Effect of Surface Modified Fullerene C₇₀ on the ROS Production and Cellular Integrity Using Chinese Hamster Ovarian Cells

S. Anju,[‡] J. Ashtami,[‡] and P. V. Mohanan*

Toxicology Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram 695 012, Kerala, India

Email: mohanpv10@gmail.com (P. V. M.). [‡] The authors contributed equally.

Abstract Fullerene, a distinct buckyball structured carbon allotrope, has immense popularity among various scientific disciplines. Comparing various fullerene allotropes, recent attention is focused on C₇₀ fullerene owing to its potent applications in various interdisciplinary arenas including optoelectronics, photovoltaics as well as in biomedical technology. Although C₇₀ suggests as a tunable material in biomedicine, convincing results about its toxic effects are still under controversy, which recommends the necessity for proper toxicity evaluation. Dextran polysaccharide was effectively coated on C₇₀ to overcome the poor dispersion in water and for achieving proper stabilization. The as-prepared material was further characterized using various sophisticated techniques such as DLS, TEM, FTIR and zeta potential. Chinese Hamster Ovarian cell lines (CHO) served as the subject for major experiments. Various cytotoxicity assays and DCFH-DA probe ROS scavenging analysis were done. Morphological examinations of major sub-cellular organelles were carried out with the aid of fluorescent microscopy for nuclear condensation, mitochondrial membrane potential, lysosomal integrity, cytoskeletal integrity, *etc.* Flow cytometric FACS analysis for possible apoptosis-necrosis mediated cell death assessment and DNA ladder experiment for genotoxicity was done. Cell viability assays show up to 80% live CHO cells after dextran-coated C₇₀ treatment for 24 h. The fluorescent staining results that confirm intact organelles further prove the non-toxic nature of dextran-coated C₇₀. DNA laddering assay results exclude chances for genotoxic potential of dextran-coated C₇₀. Experimental results of the present study indicate that dextran stabilized C₇₀ fullerene is a potent candidate material for futuristic healthcare applications.

Keywords ROS, fullerene-C₇₀, cellular integrity, mitochondrial membrane potential, flow cytometry, apoptosis, cytotoxicity

Introduction

The advent of nanotechnology has gained popularity and acceptance across various scientific and engineering disciplines owing to its potential for various application-level strategies. Nanoparticles (NPs) are proved to be successful candidates for various biomedical applications such as fluorescent labeling of target analytes, tissue engineering, drug delivery, regenerative medicine, *etc.*^[1] Nanoparticles exhibit their unique physicochemical properties in the particular nanosize distribution only, and the small size will lead to increased surface reactivity which will subsequently lead to toxicity.^[2] The outbreak of nanotechnology has resulted in an abrupt exposure of nano environment to humans, which make safety aspects of nanoparticles an important query to solve. The study on the effect of nanomaterials on cell structure, intracellular localization and the possible routes of NPs entering into cells has great value for comprehending the demonstrated biological characteristics and the potential biomedical applications. It was reported that NPs can cause lipid peroxidation, oxidative stress, DNA denaturation, protein misfolding as well as severe health problems like neurodegenerative disorders, foetal abnormalities, teratogenicity, chronic inflammation, *etc.*, once it enters the system. All these dilemmas eventually paved the whole way towards in-depth toxicological assessments of NPs at the biochemical and molecular level.

Fullerenes, a distinct allotrope in the family of carbon NPs, composed entirely of carbon atoms, were discovered in 1985 in carbon soot resulting from laser-induced graphite ablation. On

to these spherically arranged carbon atoms, side chains can be added to modify the compound for various biomedical applications. The unique soccer ball-like structure and electron-deficient nature tends to impart excellent properties, which make them an outstanding candidate in the field of nanomaterial science.^[3-5] Fullerenes behave as superconductors to semiconductors and possess exceptional free radical scavenging activity also.^[3,5-7] One specific feature of fullerene is that they show extreme durability.^[8] Fullerenes are experimented as highly potent candidates for lithium-ion cells, solar cells, fuel cells as well as for various biomedical applications like treatment of cancer, AIDS, gene therapy, *etc.* Among various fullerene allotropes, recent attention has focused on C₇₀ fullerene. C₇₀ fullerenes are closed hollow cage-like structure consisting of 70 carbon atoms containing 37 faces interconnected in 12 pentagonal and 25 hexagonal rings. Each carbon is bonded to three carbon neighbouring carbon atoms and its bonds are in sp² hybridization. C₇₀ can readily accept and donate electrons, which contribute to its outstanding properties in nanotechnology and material science. C₇₀ displays an ellipsoidal geometry in contrast to C₆₀'s spherical structure as a result of the insertion of 10 carbon atoms equatorial into the fullerene sphere. C₇₀ is usually extracted from fullerene carbon soot and the solubility of bare C₇₀ is typically lower than that of C₆₀ for any given solvents.^[7] Owing to its unique properties, fullerene C₇₀ is of particular interest in biomedical field as nanocarriers for drug/gene delivery, tissue engineering scaffolds, biosensing, bioimaging and wound healing. Wudl and coworkers^[9] demonstrated that

fullerene derivatives have the potential to specifically inhibit HIV protease (HIV-P) by perfectly fitting into its catalytic site. Further anti-HIV property of cationic fullerene derivatives was studied by Marchesan *et al.*^[10] by synthesizing a series of regioisomeric bis-fulleropyrrolidines, which are potent anti-HIV agents. Their effect against HIV-1 and HIV-2 have been evaluated confirming the importance of relative positions of the substituent on fullerene cage structure. Previous researches have shown that fullerene derivatives cause cytotoxicity in some cell lines mainly by inhibiting cell growth and promoting apoptosis. Whereas at the same time, these are reported to be potent antioxidant scavengers. Yet then, in contrast, fullerenes were found to protect some cells from induced toxicity by various other factors. Makarova *et al.* studied the effect of fullerene in the prevention of neurotoxicity induced by intrahippocampal microinjection of amyloid-beta peptide. The evidence from the study gives promise that functionalized fullerenes can be used to develop anti-amyloid drugs combining antioxidant and anti-aggregative properties.^[11]

Although C₇₀ fullerenes have potent applications in various interdisciplinary arenas, there are relatively very few studies available regarding its toxicity. Convincing results about the potent cytotoxicity of C₇₀ fullerenes are still under controversy, which makes the necessity for the toxicity evaluation of the same. Based on the literature survey, it was evident that C₇₀ fullerenes are found to have excellent application strategies in various interdisciplinary arenas. Mostly in the field of biomedicine, fullerenes possess outstanding applications. However, relevant information regarding the toxicity concerns for C₇₀ fullerene NPs is lacking. Therefore, the present study has aimed at evaluation of cytotoxicity of dextran stabilized C₇₀ fullerene NPs on Chinese Hamster Ovarian (CHO) cell lines as an *in vitro* system to understand the effect of dextran-coated C₇₀ nanoparticles in the living system.

Materials and Methods

Chemicals and equipments

Fullerene C₇₀ NPs were purchased from Sigma Aldrich, and the coating material Dextran from *Leuconostoc* spp. M.W. 6000 was also from Sigma Aldrich. High glucose (HG)—Dulbecco's modified eagles medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (Ca²⁺, Mg²⁺ free PBS), trypsin EDTA, antibiotic and antimycotic solution (10,000 g/mL of streptomycin, 10,000 units/mL of penicillin and 25 µg/mL of Fungizone® Antimycotic) were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue and neutral red dye, Lipopolysaccharide (LPS) were purchased from Sigma Chemicals Co., Ltd. (St. Louis, MO, USA). Rhodamine phalloidin, JC1 probe, Annexin/PI, calcein AM and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular probes, Invitrogen (Carlsbad, CA, USA). Giemsa stain, 4,6-diamidino-2-phenylindole (DAPI) and acridine orange (AO) were purchased from Hi-media Pvt., Ltd. (Mumbai, India). FCCP mitochondrial oxidative phosphorylation uncoupler was purchased from abcam (US). Agarose for gel electrophoresis was purchased from Sisco Research Laboratory (SRL) (Mumbai, India). DNA isolation kit from Sigma (GenElute Mammalian Genomic DNA Miniprep Kit). Ultrapure deionized water was used in the experiments.

Surface functionalization and characterization

Effective surface functionalization can improve the solubility and dispersibility of fullerene NPs in water, which can make use for its applications in the field of biomedicine. Dextran polysaccharide functionalization is used for making water soluble C₇₀ dispersion. For that 1 mg C₇₀ fullerene and 2 mg dextran,

Leuconostoc spp were mixed in 1 mL deionized water and sonicated for 30 min followed by heat treatment at 65 °C for 1 h and dialysed overnight in deionized water. The whole suspension was again sonicated for 30 min and stored at 4 °C for further experimental analysis. The dry weight concentration was 1.8 mg/mL. Size and morphology of dextran-coated C₇₀ fullerene was determined by Dynamic Light scattering (DLS), Zeta potential, Transmission Electron Microscopy (TEM) and Fourier Transform Infrared Spectroscopy (FTIR). TEM images were procured using JEOL JEM 2100 TEM (China). Dynamic DLS and Zeta potential were performed by Malvern Zetasizer Nano ZS (UK). FTIR spectra were taken using IR Tracer 100 Shimadzu.

In vitro Cytotoxicity Studies

CHO cell culture

CHO cells for *in vitro* studies were purchased from National Centre for Cell Science (NCCS), Pune. Cells were cultured in High glucose (HG)—Dulbecco's modified eagles medium (DMEM) provided with 10% FBS and 1% AB/AM and incubated at 37 °C with 5% CO₂ incubator.

Cytotoxicity assessment using MTT assay

MTT assay was used for cell viability assessment by evaluating mitochondrial function.^[12] In brief 1 × 10⁴ cells were seeded on to each well of a 96 well plate and after 24 h incubation dextran-coated C₇₀ fullerene NPs prepared in DMEM media were added according to different concentrations (10, 20, 40, 80 µg/mL), phenol (0.02 µg/mL) was used as the positive control and cell without any treatment was the negative control. After 24 h incubation in a CO₂ incubator at 37 °C, the media was discarded and 100 µL MTT working standard was added. The formazan crystals formed by the intracellular reduction of tetrazolium salt was dissolved with the addition of 100 µL of DMSO to each well and incubated under dark for 15 min. Absorbance was measured using a multiwell plate reader at 540 nm (Bio-Tek instruments Inc). Viability rate (%) was calculated from the relative absorbance at 540 nm with respect to the percentage of control. Percentage cell viability was calculated as MTT cell viability% = $\frac{A_{\text{exp}} - A_{\text{neg}}}{A_{\text{con}} - A_{\text{neg}}}$, where A_{exp} is the amount of experimental group absorbance, A_{neg} is the amount of blank group absorbance, and A_{con} is the amount of control group absorbance.

Cell viability assessment using neutral red uptake assay

Neutral red uptake assay is based on the ability of viable cells to take up and bind the supravital dye neutral red in the lysosomes.^[13] For this experiment, cells were seeded in a 96 well plate with a seeding density 1 × 10⁴ cells/well. After 24 h incubation in a CO₂ incubator at 37 °C, the cells were treated with different concentration of dextran-coated C₇₀ fullerene NPs (10, 20, 40, 80 µg/mL), phenol (0.02 µg/mL) was used as the positive control, cells without NP treatment as the negative control and incubated for 24 h in a CO₂ incubator at 37 °C. After 24 h post-treatment incubation, 10 µL of 1% neutral red reagent was added to each well and incubated for 3 h in a 5% CO₂ incubator at 37 °C. After the incubation period, the dye was removed and the cells were washed with PBS twice. Finally, the dye entrapped inside the cell was solubilized using neutral red desorbing reagent (1% glacial acetic acid and 50% ethanol) and the plate was kept under dark for 30 min. Absorbance was read spectrophotometrically using a multi-plate reader at 540 nm.

Intracellular ROS generation assessment using DCFH-DA assay

DCFH-DA (Dichloro dihydro fluorescein diacetate) assay was used to determine the amount of ROS generation and free radical scavenging activity.^[14] DCFH-DA is a fluorimetric probe,

which passively enters the cell forming dichlorofluorescein (DCF) in the presence of reactive oxygen species. For this assay, the cells were seeded at a density of 1×10^4 cells/well and cultured for 24 h in a 5% CO₂ incubator maintained at 37 °C. After this incubation period 100 µL of 0.1 µg/mL, DCFH-DA was added to each well and incubated in dark for 45 min. This was followed by NP treatment at the selected concentrations of 10, 20, 40, 80 µg/mL, respectively. Hydrogen peroxide (0.09%) was used as the positive control and cells without NP treatment as the negative control. This was further incubated for 24 h in a CO₂ incubator at 37 °C. Replaced media with 100 µL PBS and absorbance was read using a fluorescent microplate reader (Plate Chameleon TMV, Hidex, Finland) with excitation and emission at wavelength 450 and 535 nm, respectively.

DNA ladder assay

DNA laddering assay was done to evaluate the genotoxic effect and possible DNA fragmentation that could happen as a result of C₇₀ fullerene NP exposure. To analyze this, CHO cells were seeded at a density of 1×10^6 cells per well in a 6 well plate and incubated at 37 °C for 24 h in a CO₂ incubator. Dextran-coated C₇₀ fullerene NPs were treated at a concentration of 10, 40 and 80 µg/mL and incubated for another 24 h. Trypsinised the cells and centrifuged at 300 g for 5 min, then followed the procedure of DNA isolation as per GenElute Mammalian Genomic DNA Miniprep Kit (Sigma). For agarose gel electrophoresis, 0.8 g agarose was mixed with 40 mL 1 × TAE buffer and boiled till agarose dissolves completely. Then the solution was allowed to cool and added 2 µL Ethidium Bromide (EtBr). Poured on to a gel casting tray and placed comb for wells. After adding sufficient amount of isolated DNA mixed with loading dye to each well, electrophoresis setup was allowed to run at a voltage of 80 V for 1 h. Bands were visualized under Gel Doc imaging system (Alpha Digi Doc (RT), Alpha Innotech, USA).

Assessment of mitochondrial membrane potential using JC1 dye

JC-1 probe (5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-benzimidazolylcarbocyanine iodide) staining was used to evaluate mitochondrial membrane potential.^[15] JC-1 is a cationic and lipophilic dye that accumulates in the mitochondria in a potential-dependent manner.^[16] CHO cells were exposed to 10, 40 and 80 µg/mL concentrations of dextran-coated C₇₀ fullerene NPs and 10 µM of FCCP mitochondrial oxidative phosphorylation uncoupler. Untreated cells were taken as negative control. After 24 h exposure, 10 µL of 200 µM JC1 probe was added and incubated for 30 min at 37 °C, 5% CO₂ incubator. After incubation time, the cells were washed with PBS thrice and observed under a fluorescent microscope (Axio Scope.A1, Carl Zeiss, Germany).

Cytoskeleton integrity assessment using Rhodamine-phalloidin dye

The effect of C₇₀ fullerene NPs on cytoskeletal integrity of CHO cells was assessed using F-actin staining with the aid of fluorescent dye Rhodamine-phalloidin. A six-well plate was seeded with an initial density of 1×10^5 cells/well and incubated for 24 h in a CO₂ incubator at 37 °C. Cells were then treated with a range of concentration of NPs (10, 40, and 80 µg/mL) and incubated for 24 h. Post-treatment incubation, media was removed and cells were washed with PBS and fixed using 1.2% paraformaldehyde and kept for 15 min at room temperature. Excess aldehyde was quenched using 0.1 M glycine with 5 min incubation. Fixed cells were then permeabilized using 0.1% Triton X-100. The actin filaments were then stained using Rhodamine-phalloidin stain (1 : 50) and the nuclei were counterstained using DAPI (5 µg/mL) for 1 min. Cells were washed with PBS thrice and observed under fluorescent microscope using red and blue filters (Carl Zeiss, Germany).

Lysosomal integrity assessment using acridine orange staining

Acridine orange is a lipophilic amine that readily diffuses into the cells and inside the cell. It enters into the acidic lysosomal compartment, where it is protonated and sequestered shifting its emission spectrum towards a longer wavelength. Hence, lysosomal integrity can be measured as a "loss of red dots" or as a quantitative rise in green fluorescence inside the cells.^[17] Cells seeded on a six-well plate at a density of 1×10^5 were cultured for 24 h at 37 °C in a CO₂ incubator. After incubation, the medium was discarded and the cells were treated with different concentrations of dextran-coated C₇₀ fullerene NPs (10, 40 and 80 µg/mL). 10 µg/mL lipopolysaccharide (LPS) was used as positive control. After 24 h incubation, lysosomes were stained using acridine orange fluorescent dye (6 µg/mL) for 5 min. Excess stain was removed by washing the cells with PBS and observed under fluorescence microscope using red and green filters (Axio Scope.A1, Carl Zeiss, Germany).

Nuclear condensation using DAPI

DAPI (4,6-Diamido-2-phenylindole dihydrochloride) staining is widely used for the determination of nuclear condensation. After incubation of different concentration (10, 40 and 80 µg/mL) of dextran-coated C₇₀ fullerene NPs for 24 h, the cells were washed with PBS and fixed using 1.2% paraformaldehyde for 15 min followed by permeabilisation with Triton X 100 (0.1%). Then the cells were incubated with 5 µg/mL of DAPI for 1 min in dark. Excess DAPI was removed by washing cells with PBS. Nuclear condensation if present was then observed using fluorescence microscope (Axio Scope.A1, Carl Zeiss) under blue filter.

Live/Dead assay using Calcein-AM/PI FACS

Live/dead cell assessment was done using Calcein-AM/PI staining using FACS analysis. Calcein AM is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In live cells, the non-fluorescent Calcein AM is converted to a green-fluorescent Calcein after acetoxymethyl ester hydrolysis by intracellular esterases.^[18] Loss of this Calcein retention can be easily analyzed using FACS analysis. Cells were seeded on a six-well plate at a density 3×10^5 cells/well and incubated for 24 h at 37 °C in a 5% CO₂ incubator. This was followed by dextran-coated C₇₀ treatment at different concentrations of 10, 40 and 80 µg/mL and again incubated for 24 h in a CO₂ incubator. After post treatment, the cells were trypsinised and resuspended in PBS containing 1 µg/mL Calcein. After 30 min of incubation before FACS analysis 0.5 µL (stock concentration 1 mg/mL) of PI was added and the samples were incubated in dark for 15 min before FACS analysis (Guava EasyCyte, Millipore).

Annexin V/Propidium Iodide FACS for apoptosis/necrosis analysis

Apoptotic cells were identified using Fluorescein-Isothiocyanate Conjugated (FITC)-annexin V/Propidium iodide staining. Annexin V binds to the phosphatidyl serine residues exposed on the surface of the cells undergoing apoptosis, whereas propidium iodide stains necrotic cells only.^[19] Briefly, cells were seeded on a six-well plate at a density 3×10^5 cells/well and allowed to grow and divide for 24 h. This was then exposed to different concentrations of dextran-coated C₇₀ fullerene (10, 40 and 80 µg/mL) for 24 h. Apoptosis/necrosis assessment was done using FACS analysis. After treatment, the cells were trypsinised, incubated with annexin binding buffer, resuspended in PBS and stained with Annexin V and propidium iodide using Alexa Flour 488 Annexin V/Dead cell apoptosis kit (Invitrogen, USA). 5 µL Annexin V and 1 µL propidium iodide were added and the samples were incubated in dark for 15 min before FACS analysis (BD FACS Aria II 14 color system, USA).

Statistical analysis

The results were statistically analyzed by Student *t* test. All experiments were repeated thrice and data presented as mean with standard deviation (mean $\pm$ SD). In all cases, $p < 0.05$ (*) and $P < 0.005$ (**) were considered as significant compared to control.

Results

Dextran-coated C₇₀ fullerene characterization

In this study, C₇₀ surface modified by dextran was characterized using DLS, TEM, FTIR and Zeta potential. DLS analysis gives the hydrodynamic particle size. In case of C₇₀, the hydrodynamic size was found to be 92 nm with Poly Dispersity Index (PDI) being 0.333 (Figure 1A). Zeta potential suggests the charge of dextran-coated C₇₀ fullerene to be around -20.6 mV (Figure 1B). The size and shape of dextran-coated C₇₀ NPs were determined using TEM analysis. The size was found to be in the range of 20–40 nm and the shape was analysed to be spherical (Figure 1C). FTIR spectra of dextran, bare C₇₀ fullerene and dextran-coated C₇₀ fullerene were analyzed (Figures 2A–2C) to confirm the effective surface modification and conjugation of dextran on C₇₀ fullerene NPs. FTIR spectral values showed the effective surface coating of dextran on C₇₀ fullerene clearly. Characteristic peaks of dextran at 3294 (OH), 2918, 2337 (CH) and 1001 (C–O) were visible in the FTIR spectra of dextran-coated C₇₀ fullerene. FT-IR shows clear cut evidence for effective dextran coating over C₇₀ fullerene. All characteristic FTIR peaks of bare C₇₀ fullerene were found in dextran-coated C₇₀ NP, which includes characteristic peak values of 1427.32, 1132.21, 792, 532.35, 457.13 and 418. FTIR spectra thus clearly mentioned effective surface modification of C₇₀ fullerene with dextran.

Figure 1 (A) Hydrodynamic size distribution analysis of dextran-coated C₇₀ fullerene by DLS, (B) Zeta potential for charge, and (C) TEM image showing size in the range of 20–40 nm.

In vitro Cytotoxicity Studies

Cytotoxicity assessment using MTT assay

Percentage of cell viability with respect to mitochondrial membrane integrity was analyzed using MTT assay (Figure 3A) and shows the percentage of mitochondrial activity at the selected concentrations of 10, 20, 40 and 80 $\mu\text{g/mL}$, which were almost similar to that of control 24 h post-treatment. Although a dose-dependant cell viability reduction was seen, more than 80% cells were viable at the maximum concentration of 80 $\mu\text{g/mL}$. More than 80% of cells were viable at all the se-

Figure 2 (A) Characteristic FTIR spectra of dextran, (B) bare C₇₀ fullerene, and (C) dextran-coated C₇₀ showing characteristic peaks.

lected concentrations of 10, 20, 40 and 80 $\mu\text{g/mL}$. There was no significant reduction in mitochondrial activity at these concentrations. From the results, it is clearly observed that mitochondrial activity was not compromised to any extent following dextran-coated C₇₀ NP treatment. Hence, it is evident that this nanoformulation is non toxic to Chinese hamster ovarian (CHO) cell lines in a time dependant and concentration dependant manner.

Cell viability assessment via neutral red uptake assay

CHO cell lines were exposed to dextran-coated C₇₀ NPs (10, 20, 40, 80 $\mu\text{g/mL}$) concentrations for 24 h lysosomal activity analysis. Results of neutral red uptake assay reveal no evident cytotoxicity related loss of lysosomal integrity (Figure 3B). The percentage of lysosomal activity observed for CHO cell lines after 24 h exposure to dextran-coated C₇₀ NPs was found to be 99.8, 99.51, 85.9, 82.25 for 10, 20, 40 and 80 $\mu\text{g/mL}$ concentrations, respectively. After 24 h treatment, more than 80% of lysosomal integrity was observed for CHO cells at all the selected concentrations, which remarkably strengthen the reduced toxicity of this sample.

Intracellular ROS generation assessment using DCFH-DA

ROS generation is one of the initial responses of a cell prior to NP exposure. DCFH-DA fluorescent probe is commonly employed for ROS generation assessment and it will react with several ROS including H₂O₂, hydroxyl radicals and peroxy nitrate. Increase of fluorescence intensity marks increased free radical production. In this experiment, ROS generation was measured after 24 h exposure of CHO cells with dextran-coated C₇₀ NPs at all the selected concentration (Figure 3C). Results clearly state that at the initial concentration of 10 $\mu\text{g/mL}$ ROS production was evident to a significant level when compared to control untreated cells. But for 20 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ concentrations, ROS scavenging activity was found to be more prominent than ROS production. At higher concentrations, ROS

Figure 3 (A) MTT assay of dextran-coated C_{70} fullerene in CHO cell lines to determine percentage cell viability. (B) Neutral Red uptake assay showing percentage lysosomal activity in CHO cell lines. (C) ROS production and free radical scavenging property assessment using DCFHDA probe ($SD \pm$ mean). (D) DNA ladder assay Gel Doc image showing DNA fragmentation analysis data.

production was found to be increased significantly when compared to control.

DNA ladder assay

DNA ladder assay was done to analyse the possible DNA fragmentation and genotoxicity of dextran-coated C_{70} NPs. From the results, it was evident that no DNA fragmentation was caused to the genetic material of CHO cell lines post 24 h NP exposure (Figure 3D). All the lanes corresponding to negative control and the selected concentrations of 10 µg/mL, 40 µg/mL and 80 µg/mL showed the presence of intact DNA in the Gel Doc image. All the results were similar to that of control.

Assessment of mitochondrial membrane potential using JC1 dye

The loss of mitochondrial membrane potential, a peculiar element of apoptotic cell, was analyzed by a cationic carbocyanine JC-1 dye. This dye exists as green fluorescent monomeric form in cytosol of normal cells and accumulates in mitochondria as red fluorescent aggregates (spots). But in apoptotic cells, disintegrated mitochondrial membrane retains the monomer form of JC-1 dye and emits more green fluorescence. In this experiment, particle treated CHO cells maintained mitochondrial membrane potential as that of control cells, which reveals potent non toxic property of dextran-coated C_{70} fullerene NPs on mitochondria of CHO cell lines. At all the selected concentrations of 10, 40 and 80 µg/mL, presence of red fluorescence was found as similar to that of control (Figure 4A).

Cytoskeleton integrity assessment using Rhodamine-Phalloidin dye

After 24 h exposure of dextran-coated C_{70} NPs, CHO cells did not show any evident cell damage or loss of cytoskeletal integrity. For the selected concentrations of 10, 40 and 80 µg/mL, cells were found to be morphologically similar to control (Figure 4B). It can also be seen that similar to control, the actin filaments were intact and healthy in all the selected concentrations.

Lysosomal integrity assessment using acridine orange staining

Autophagy, the type 2 programmed cell death was studied using acridine orange vital staining.^[20] Autophagy is characterized by the presence of acidic vesicular organelles (AVOs) in cytoplasm. AVO formation after NP treatment was detected using this staining. Intact healthy lysosomes will stain orange

Figure 4 Fluorescent microscopic images of CHO cells using JC-1 probe for assessment of mitochondrial membrane potential (A) negative control without NP treatment and the selected concentrations of (B) 10 µg/mL, (C) 40 µg/mL and (D) 80 µg/mL (E) CHO cells treated with 10 µM FCCP-mitochondrial oxidative phosphorylation uncoupler (positive control), respectively. Fluorescent microscopic images of Rhodamine-Phalloidin staining for cytoskeletal actin filament integrity assessment for (F) negative control and selected concentrations of (G) 10 µg/mL, (H) 40 µg/mL and (I) 80 µg/mL, respectively (magnification: 20x; scale represents 20 µm).

Figure 5 Fluorescent microscopic images of CHO cells using acridine orange staining for lysosomal integrity analysis (A) negative control without dextran-coated C_{70} NP treatment and selected concentrations of (B) 10 µg/mL, (C) 40 µg/mL, (D) 80 µg/mL (E,) CHO cells treated with LPS (positive control), respectively. (F, G, H, I) shows nuclear staining fluorescent microscopic images for nuclear condensation analysis in CHO cells for negative control and selected concentrations of 10, 40 and 80 µg/mL, respectively (magnification: 20x; scale represents 20 µm).

spots whereas damaged lysosomes emit green fluorescence. Therefore, loss of red spots indicates loss of lysosomal integrity. Acridine orange positive (orange spots) cells were more in all the selected concentrations of 10, 40 and 80 µg/mL and results were comparable to that of control cells (Figure 5A).

Nuclear condensation using DAPI

No visible change in the morphology of nucleus was found in CHO cells after treatment with dextran-coated C_{70} fullerene NPs (Figure 5B). Moreover, no evident chromatin condensation was visible at all the selected concentrations, which confirm the potent non toxic effect of this material.

Live/Dead assay using Calcein-AM/PI FACS

Live/dead cells were identified using Calcein-AM/PI using FACS analysis. Red spots represent live cells and black spots represent dead cells in the FACS data (Figure 6A). At all the selected concentrations dead cells were not prominent when compared to control. At 10 µg/mL concentration, the cell viability was around 94% cells. At 40 µg/mL concentration 89%, cell viability was seen and at the maximum concentration of 80

Figure 6 (A) Calcein AM/Propidium Iodide staining for live/dead assay of CHO cells after 24 h exposure of dextran-coated C₇₀ fullerene for negative control and selected concentrations of 10 µg/mL, 40 µg/mL and 80 µg/mL, respectively. (B) Characteristic graphical representation of the FACS data showing percentage viable cells with respect to different concentrations of dextran-coated C₇₀ treatment (Red bar shows live cells and black bar shows dead cells).

µg/mL the cell viability was found to be around 85% (Figure 6B). Therefore, at all the selected concentrations, more than 80% cell viability was evident from FACS data analysis.

Annexin V/Propidium Iodide FACS for apoptosis/necrosis analysis

Apoptotic/necrotic cells were identified using Annexin V /Propidium Iodide FACS analysis. Negligible level of dose-dependant apoptotic cells were found at the higher concentrations (Figure 7A). 10 µg/mL showed strictly similar level of both apoptosis and necrosis as that of control, whereas 40 µg/mL and 80 µg/mL concentrations showed a slightly more number of apoptotic cells than necrotic cells when compared to that of control (Figure 7B). Therefore, at higher concentrations more than necrosis, apoptosis was more evidently seen. With particle treatment at higher concentrations, more possibility for cells entering apoptosis mediated cell death was prominent. However, at low concentration, normal necrosis mediated cell death was seen. Even then the cell viability was around 80% for all selected concentrations.

Discussion

Fullerenes are a major class of carbon compounds possessing excellent applications especially in the fields of cosmetology, therapeutics and various biomedical technology related areas. Fullerenes are found to be excellent free radical scavengers, too. Although fullerenes are found to be useful candidates for these purposes, a well established toxicological examination should be done in order to use it on an application level strategy. A long biological half-life raises concern about

Figure 7 (A) Annexin V/Propidium Iodide staining for apoptosis/necrosis mediated cell death assessment using flow cytometry FACS after 24 h exposure with dextran-coated C₇₀ fullerene for negative control and selected concentrations of 10 µg/mL, 40 µg/mL and 80 µg/mL, respectively. (B) Characteristic graphical representation of the FACS data with index data showing corresponding data (blue: % viable cells, red: % early apoptotic cells, green: % late apoptotic cells and violet: % dead cells by necrosis).

bioaccumulation and long term effects. In general, the acute oral, dermal and airway toxicity of fullerene is low. However, a few relevant experimental studies of repeated-dose toxicity, reproductive toxicity and carcinogenic effect of C₆₀ fullerene are available till now.^[21] To date toxicity concerned researches were mainly focused on C₆₀ fullerene (Buckminster fullerene), adequate information regarding the toxicity and related aspects of its C₇₀ derivative is still unknown.

There are relatively very few studies available specific to the toxicity of C₇₀ fullerene. Convincing results about the potent cytotoxicity of these materials are still under controversy. Also, the potent effect of this particular compound in reproductive organs and the possible developmental toxicity hazards are very much less studied to date. Toxicity concerns about this NP in ovary and associated organs are very rarely considered. Chinese Hamster Ovarian cell lines (CHO) are excellent source for these related research fields and with this taken into consideration, CHO cell lines has been chosen as a subject for this entire study. The present study reveals the possible effects of dextran-coated C₇₀ fullerene NPs on CHO cell lines and provides a significant understanding of the possible mechanism, through which these NPs exert their impact on the biological system. The characterizations of dextran-coated C₇₀ NPs were done by DLS, TEM, FTIR and Zeta potential. From the results, the size of dextran-coated C₇₀ NPs was confirmed to be in the range of 20–40 nm and shape was spherical. Zeta potential suggests the charge to be -20.6 mV. Effective surface functionalization of dextran over C₇₀ was found to be evident from the FTIR spectra of dextran, bare C₇₀ fullerene and dextran-coated C₇₀ fullerene. In the FTIR spectra of dextran-coated C₇₀ fullerene, characteristic peaks of dextran at 3294 (OH), 2918, 2337 (CH) and 1001 (C—O) were visible, which shows a clear

cut evidence for effective dextran coating over C₇₀ fullerene. All FTIR peaks of bare C₇₀ fullerene were found in dextran-coated C₇₀ fullerene NPs also, which include characteristic peak values of 1427.32, 1132.21, 792, 532.35, 457.13 and 418. This clearly mentioned the effective surface modification of C₇₀ fullerene with dextran polysaccharide.

In the present study, two independent assays were done to evaluate the cytotoxicity of dextran-coated C₇₀ NPs via MTT assay and Neutral red uptake assay at the selected concentrations of 10, 20, 40 and 80 µg/mL. From MTT assay, it was evident that dextran-coated C₇₀ fullerene NPs didn't exert any potent cytotoxic effect at the selected concentrations. At the maximum concentration, more than 80% cell viability was seen itself. Neutral red uptake assay is based on the ability of viable cells to take up and bind supravital dye Neutral red in the lysosomes. Uptake of Neutral red depends on the cell's capacity to maintain pH gradient through ATP production.^[22] When the cells die, the pH gradient cannot be maintained.^[23] In this study, at all the selected concentrations, cells retained lysosomal integrity. More than 80% cell viability was seen at the maximum selected concentration also, which confirms the potent nontoxic effect of dextran-coated C₇₀ *in vitro* in CHO cell lines. Thus, it can be inferred that dextran-coated C₇₀ NPs possess very low toxicity in CHO cell lines. It was proposed that C₆₀ fullerene has an ability to acquire positive charge by absorbing several protons inside the cell and this complex could penetrate into mitochondria and such a process allows for mild uncoupling of respiration and phosphorylation, which in turn leads to the decrease in ROS production.^[24] To understand whether C₇₀ also possess such a kind of ROS scavenging property, DCFHDA assay was done. The results clearly showed that at the medium concentrations of 20 and 40 µg/mL in a 80%–90% confluent six well plate free radical scavenging property was evident. But at maximum concentration of 80 µg/mL, more than free radical scavenging property ROS production was evident. These results validate the studies done by Liu *et al.*^[25] that significantly low concentrations of carboxyfullerenes can elucidate good antioxidant property. The different mechanisms, through which this particular response happens, need to get explored.

Various fluorescent microscopic analysis also suggests the potent nontoxic effect of dextran-coated C₇₀. Actin filaments are the major components of cell division machinery and they are ubiquitous protein, which is continuously undergoing polymerisation and depolymerisation reactions. It is the main protein involved in microtubule dynamics. Therefore, any tampering to this during cell division produces abnormal spindle leading to apoptosis or unequal chromosomal distribution. Rhodamine phalloidin staining reveals that CHO cell lines exposed to dextran-coated C₇₀ NPs showed no obvious change in cytoskeletal integrity (actin filament integrity) at all the selected concentrations and treated cells remained morphologically closer to the control also. Mitochondrial membrane potential was examined using JC-1 probe with the aid of fluorescent microscopic analysis. Being the powerhouse and energy repository of cell, mitochondria are the major target organelle for oxidative stress-induced cytotoxicity and cell death. NPs can generate mitochondrial reactive oxygen species once they reach the cell and thereby impair electron transport chain machinery. JC-1 probe staining is particularly used in this study to evaluate mitochondrial membrane potential. Characterized red spots represent rod shaped mitochondria in the green background of cell cytoplasm. More red spots represent good mitochondrial membrane potential. At all selected concentrations, cells were found to retain good mitochondrial membrane potential to a greater extent again suggesting the potential nontoxic effect of dextran-coated C₇₀. The red spots diminished in positive control depicting the loss in mitochondrial membrane

potential when exposed to FCCP-mitochondrial oxidative phosphorylation uncoupler. These results contradict the findings of Dellinger *et al.*^[26] that mitochondrial membrane integrity was not lost to any extent with fullerene NP treatment.

Lysosomal integrity assessment was done using acridine orange (AO) straining. AO is a lipophilic amine dye, which readily diffuses into the cell cytoplasm. Inside the cell, it enters into the acidic lysosomal compartment, where it is protonated and sequestered shifting its emission spectrum towards a longer wavelength (red). Loss of red spots suggests the loss of lysosomal membrane potential. From the fluorescent microscopic analysis, it was evident that no obvious loss of lysosomal integrity was found in the treated cells even at the maximum selected concentration also. The lysosomal activity was found to be evidently compromised when exposed to LPS (positive control). Depending on the size and composition some NPs may enter nucleus and exert its genotoxic effect also. Here DAPI is the nuclear stain used for staining nucleus to analyse the possible nuclear condensation dextran-coated C₇₀ NPs can cause. Fluorescent microscopic analysis suggests nontoxic effect of dextran-coated C₇₀ in nucleus and no observed nuclear condensation was found even at maximum selected concentration. Intact nuclear morphology and absence of nuclear condensation suggests the possible nontoxic effect of dextran-coated C₇₀ NP at nuclear level. Genotoxic effect of dextran-coated C₇₀ in CHO cell lines was evaluated using an agarose gel DNA ladder assay. On the gel run, intact DNA was found as such, which suggests the absence of denatured DNA fragments giving strength to the possibility of non genotoxic efficacy of dextran-coated C₇₀. This result contradicts the findings of Dhawan *et al.*^[27] on C₆₀ fullerene NPs. They have reported that stable colloidal suspension of C₆₀ fullerene showed a genotoxic response. In this study, from the DAPI nuclear condensation analysis as well as from the DNA ladder experiment, it was clear that dextran-coated C₇₀ fullerene NPs showed no obvious genotoxic effect as well as nuclear condensation at any of the selected concentrations.

Flow Cytometry using Fluorescence Activated Cell Sorting (FACS) was done to analyse the possible live/dead cell count after dextran-coated C₇₀ treatment. Calcein-AM/PI is the fluorescent dye combination chosen as marker. Calcein is a fluorescent dye with excitation and emission wavelengths of 495/515 nm, respectively. It has the appearance of orange crystals commonly used as an indicator of lipid vesicle leakage, which is a characteristic feature of necrotic cell death. From the FACS data analysis, it was clear that at all the selected concentrations more than 85% cell viability was possessed by CHO cells treated with dextran-coated C₇₀ fullerene. Less than 15% cells were analysed to be dead. These results validate the findings of Levi *et al.* reports about C₆₀ fullerene.^[28] Apoptosis is generally accepted as a non-inflammatory process, the lytic nature of necrosis leads to the release of intracellular damage-associated molecular patterns (DAMPs) and triggers inflammation. In order to better understand the possible mechanism of cell death, whether apoptosis or necrosis mediated cell death happened after dextran-coated C₇₀ fullerene NP treatment. Annexin V/Propidium Iodide flow cytometric analysis was done using FACS. 10 µg/mL concentration showed strictly similar level of both apoptosis and necrosis as that of control, whereas 40 µg/mL and 80 µg/mL concentrations showed a slight more number of apoptotic cells than necrotic cells when compared to that of control. At the highest concentrations, apoptosis mediated cell death was found to be more prominent. To date, more experimental toxicological researches were focused on C₆₀ fullerene derivatives. But more application level potential of C₇₀ fullerenes above C₆₀ marks the need for its toxicity assessments. Before validating any nanomaterial for

biomedical applications, it is mandatory to assess their toxicological evaluation. The results of this study, which focused on C₇₀ fullerene biocompatibility in CHO cell lines, suggest dextran-coated C₇₀ fullerene as a good nanomaterial for future biomedical applications.

Conclusion

The present study aimed at evaluation of cytotoxicity of dextran-coated C₇₀ fullerene NPs on CHO cell lines concluded the fact that no obvious toxicity was induced by dextran-coated C₇₀ fullerene. Dextran coating improves the solubility and stability of hardly soluble fullerene C₇₀ to a substantial extent. The results clearly indicate that mitochondrial membrane potential, lysosomal integrity, cytoskeletal integrity, nuclear integrity, etc., were intact as compared to untreated control cells and no clear evidence of any nuclear condensation observed. Absence of DNA fragmentation post dextran-coated C₇₀ treatment was found with DNA ladder assay. Effective free radical scavenging was found at significantly low concentration of dextran-coated C₇₀ treatment suggesting dose dependant ROS scavenging property. Calcein AM/PI FACS data reveals that more than 85% cells were viable at the maximum selected concentration. Annexin/PI FACS results make it evident that more than necrosis, apoptosis mediated cell death was found in cells treated with maximum concentration. Even then, more than 80% cell viability was found at all the selected concentrations. The results from the present study make it evident that dextran-coated C₇₀ fullerene is a safe candidate material for futuristic healthcare applications.

Conflicts of interest

The authors declare that they have no conflict of interests.

Acknowledgement

The authors wish to express their sincere thanks to Director and Head, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram for their encouragement and support for conducting this study. Anju thanks University Grants Commission, New Delhi and Ashtami thanks SCTIMST, Trivandrum for financial support of Junior Research Fellowships.

References

- [1] Salata, O. J. *Nanobiotechnol.* **2004**, 2, 1.
- [2] Oberdorster, G.; Maynard, A.; Donaldson, K.; Castranova, V.; Fitzpatrick, J.; Ausman, K.; Carter, J.; Karn, B.; Kreyling, W.; Lai, D.; Olin, S. *Part Fibre Toxicol.* **2005**, 2, 1.
- [3] Jinno, K.; Matsui, H.; Ohta, H.; Saito, Y.; Nakagawa, K.; Nagashima, H.; Itoh, K. *Chromatographia* **1995**, 41, 353.
- [4] Ajie, H.; Alvarez, M. M.; Anz, S. J.; Beck, R. D.; Diederich, F.; Fostiropoulos, K.; Huffman, D. R.; Kraetschmer, W.; Rubin, Y. *J. Phys. Chem.* **1990**, 94, 8630.
- [5] Diederich, F.; Ettl, R.; Rubin, Y.; Whetten, R. L.; Beck, R.; Alvarez, M.; Anz, S.; Sensharma, D.; Wudl, F.; Khemani, K. C.; Koch, A. *Science* **1991**, 252, 548.
- [6] Richter, H.; Labrocca, A. J.; Grieco, W. J.; Taghizadeh, K.; Lafleur, A. L.; Howard, J. B. *J. Phys. Chem. B* **1997**, 101, 1556.
- [7] Heath, J. R. *Nature* **1998**, 393, 730.
- [8] Kroto, H. W.; Heath, J. R.; O'Brien, S. C.; Curl, R. F.; Smalley, R. E. *Nature* **1985**, 318, 162.
- [9] Sijbesma, R.; Srdanov, G.; Wudl, F.; Castoro, J. A.; Wilkins, C.; Friedman, S. H.; DeCamp, D. L.; Kenyon, G. L. *J. Am. Chem. Soc.* **1993**, 115, 6510.
- [10] Marchesan, S.; Da Ros, T.; Spalluto, G.; Balzarini, J.; Prato, M. *Bioorg. Med. Chem. Lett.* **2005**, 15, 3615.
- [11] Makarova, E. G.; Gordon, R. Y.; Podolski, I. Y. *J. Nanosci. Nanotechnol.* **2012**, 12, 119.
- [12] Mosmann, T. *J. Immunol. Methods* **1983**, 65, 55.
- [13] Repetto, G.; Del Peso, A.; Zurita, J. L. *Nat. Protoc.* **2008**, 3, 1125.
- [14] Wan, C. P.; Myung, E.; Lau, B. H. *J. Immunol. Methods* **1993**, 159, 131.
- [15] Smiley, S. T.; Reers, M.; Mottola Hartshorn, C.; Lin, M.; Chen, A.; Smith, T. W.; Steele, G. D.; Chen, L. B. *Proc. Natl. Acad. Sci.* **1991**, 88, 3671.
- [16] Mathur, A.; Hong, Y.; Kemp, B. K.; Barrientos, A. A.; Erusalimsky, J. D. *Cardiovasc. Res.* **2000**, 46, 126.
- [17] Peters, T. J.; Carol, A. S. *Clin. Sci. Mol. Med.* **1976**, 50, 75.
- [18] Bratosin, D.; Mitrofan, L.; Palii, C.; Estaquier, J.; Montreuil, J. *Cytometry A* **2005**, 66, 78.
- [19] Schutte, B.; Nuydens, R.; Geerts, H.; Ramaekers, F. *J. Neurosci. Meth.* **1998**, 86, 63.
- [20] Lin, Y. F.; Chiu, I. J.; Cheng, F. Y.; Lee, Y. H.; Wang, Y. J.; Hsu, Y. H.; Chiu, H. W. *Part Fibre Toxicol.* **2015**, 13, 52.
- [21] Nielsen, G. D.; Roursgaard, M.; Jensen, K. A.; Poulsen, S. S.; Larsen, S. T. *Basic Clin. Pharmacol.* **2008**, 103, 197.
- [22] Wolff, D. J.; Papoiu, A. D.; Mialkowski, K.; Richardson, C. F.; Schuster, D. I.; Wilson, S. R. *Arch. Biochem. Biophys.* **2000**, 378, 216.
- [23] Borenfreund, E.; Puerner, J. A. *Toxicol. Lett.* **1985**, 24, 119.
- [24] Chistyakov, V. A.; Smirnova, Y. O.; Prazdnova, E. V.; Soldatov, A. V. *Biomed. Res. Int.* **2013**, 1.
- [25] Liu, Q.; Zhang, X.; Zhang, X.; Zhang, G.; Zheng, J.; Guan, M.; Fang, X.; Wang, C.; Shu, C. *ACS Appl. Mater. Interfaces* **2013**, 5, 11101.
- [26] Dellinger, A. L.; Cunin, P.; Lee, D.; Kung, A. L.; Brooks, D. B.; Zhou, Z.; Nigrovic, P. A.; Kepley, C. L. *PLoS one* **2015**, 10, 126290.
- [27] Dhawan, A.; Taurozzi, J. S.; Pandey, A. K.; Shan, W.; Miller, S. M.; Hashsham, S. A.; Tarabara, V. V. *Environ. Sci. Technol.* **2006**, 40, 7394.
- [28] Levi, N.; Hantgan, R. R.; Lively, M. O.; Carroll, D. L.; Prasad, G. L. *J. Nanobiotechnol.* **2006**, 4, 4.

Received August 14, 2019

Accepted September 11, 2019