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RESEARCH ARTICLE

A comparative study on the *in vitro* cytotoxic responses of two mammalian cell types to fullerenes, carbon nanotubes and iron oxide nanoparticles

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Abstract

The present study was designed to evaluate and compare the time- and dose-dependent cellular response of human periodontal ligament fibroblasts (hPDLFs), and mouse dermal fibroblasts (mDFs) to three different types of nanoparticles (NPs); fullerenes (C₆₀), single walled carbon nanotubes (SWCNTs) and iron (II,III) oxide (Fe₃O₄) nanoparticles via *in vitro* toxicity methods, and impedance based biosensor system. NPs were characterized according to their morphology, structure, surface area, particle size distribution and zeta potential by using transmission electron microscopy, X-ray diffraction, Brunauer–Emmett–Teller, dynamic light scattering and zeta sizer analyses. The Mössbauer spectroscopy was used in order to magnetically characterize the Fe₃O₄ NPs. The hPDLFs and mDFs were exposed to different concentrations of the NPs (0.1, 1, 10, 50 and 100 µg/mL) for predetermined time intervals (6, 24 and 48 h) under controlled conditions. Subsequently, NP exposed cells were tested for viability, membrane leakage and generation of intracellular reactive oxygen species. Additional to *in vitro* cytotoxicity assays, the cellular responses to selected NPs were determined in real time using an impedance based biosensor system. Taken together, information obtained from all experiments suggests that toxicity of the selected NPs is cell type, concentration and time dependent.

Keywords

Nanoparticles, cytotoxicity, iron (II,III) oxide, carbon nanotube, fullerene, *in vitro* toxicity, impedance based assay

History

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Introduction

Nanoparticles (NPs) which are popular building blocks of new engineered materials are in the range of 1–100 nm (Auffan et al., 2009). Among these, Fe₃O₄ NPs and carbon based NPs – fullerenes (C₆₀) and single walled carbon nanotubes (SWCNTs) – both have attracted much attention due to their advantageous characteristics making them candidates for use in a wide range of applications, most of which are biomedical applications with a high probability of human exposure. The commercial production and use of fullerenes in consumer products, including cosmetics, tissue engineering and wound healing (Benn et al., 2011; Inui et al., 2014; Lens, 2009, 2011; Pulicharla et al., 2014; Sitharaman et al., 2007; Takada et al., 2006), create the need for a detailed investigation of toxicity. Additionally, future applications of fullerenes as building blocks of nanorobots for being used in biomedical fields, including dentistry is proposed by several groups (Bhardwaj et al., 2014; Kanaparthi & Kanaparthi, 2011; Kukreja et al., 2012). SWCNTs are graphitic nano-scale

materials synthesized for their extraordinary properties like ultra-high surface area and characteristic structure with a unique surface chemistry. Either alone or in modified form, they are used in applications including biosensors (Zelada-Guillen et al., 2013), tissue engineering (Gupta et al., 2014; Hernández-Ferrer et al., 2014; Sitharaman et al., 2007), drug delivery (Madani et al., 2011; Meng et al., 2012), cancer diagnosis-treatment (Andreoli et al., 2014), antimicrobial agents in wound bandages (Simmons et al., 2009) and dentistry (Akasaka et al., 2009; Zhang et al., 2008). Fe₃O₄ NPs have been used in various applications not only because of their size-dependent superparamagnetism (Buyukhatipoglu & Clyne, 2011), but also for their inexpensive production, especially in the biomedical field as contrast agent for magnetic resonance imaging (MRI) (Jun et al., 2005; Stephen et al., 2011; Tiefenauer et al., 1996), targeted drug delivery (Sadighian et al., 2014), gene delivery as vectors (Fouriki & Dobson, 2013), tissue engineering (Ito et al., 2004; Shimizu et al., 2007) and hyperthermia treatment (Xiao et al., 2015). Additionally, functionalized Fe₃O₄ NPs are introduced as antifungal and antimicrobial agents in wound textile dressings (Anghel et al., 2012; Grumezescu et al., 2014) and are also used in dentistry for the treatment of periodontitis (Chifiriuc et al., 2013; Di Turi et al., 2011).

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Numerous studies on the toxicity of these NPs have been published so far. Sayes et al. (2004) investigated the toxic effects of C₆₀ to human dermal fibroblasts, and HepG2 human liver carcinoma cells, and found toxic effects at the 20 ppb level. Another study conducted on mouse midbrain cells showed that C₆₀ dose-dependently inhibited cell differentiation and proliferation (Tsuchiya et al., 1996). According to a recent *in vivo* study (Shipelin et al., 2015), C₆₀ was administered to rats at doses of 0.1, 1.0 and 10 mg/kg body weight over 92 days and found to be toxic to the hepatic tissue only at the highest given dose of 10 mg/kg. As some of the studies on C₆₀ conclude in low or no toxicity (Gharbi et al., 2005; Kyzyma et al., 2015; Levi et al., 2006) whereas some find it toxic depending on the cell type and concentration (Sayes et al., 2004; Shipelin et al., 2015). According to the study of Meng et al. (2012) conducted on HeLa cells, SWCNTs can enter the cells and exert a cytotoxic effect. A more recent study (Kim & Yu, 2014) demonstrated that, SWCNTs triggered the formation of reactive oxygen species (ROS) in human peripheral blood lymphocytes. In two other studies, SWCNTs induced cytotoxicity and generation of ROS in neural PC12 cells and human gingival fibroblast cell line (Cicchetti et al., 2011; Zhang et al., 2010). The cytotoxicity and biocompatibility of Fe₃O₄ NPs were investigated with normal, glia and breast cancer cells (Ankamwar et al., 2010) and found to be nontoxic in the range of 0.1–10 µg/mL for all three cell types. In another study (Mahmoudi et al., 2011), again conducted with three different human cell lines; HCM (heart), BE-2-C (brain) and 293T (kidney) concluded in a concentration dependent toxicity.

There are several methods including *in vitro* cytotoxicity tests to assess the toxicity of NPs. *In vitro* cytotoxicity assays based on detection of mitochondrial dehydrogenase activity and the release of lactate dehydrogenase (LDH) enzyme, etc., play an important essential role in preliminary testing of NPs to evaluate its potential risks before *in vivo* studies. However, the *in vitro* cytotoxicity tests, which rely on detection of final biochemical products of cellular cytotoxic response via optical detection have some drawbacks. These assays can interfere with the interaction of some NPs with compounds used during detection or labeling steps (Kroll et al., 2012). They determine the cell responses to substances only at a specific time point. Also, the reproducibility of the cytotoxicity tests can be affected by parameters, such as the type of NPs, culture conditions, type of cells, as well as by the implemented test method (Patil et al., 2015). In contrast to these *in vitro* methods, impedance-based high-throughput systems have drawn interest in toxicology studies recently, since they provide long-term, label-free and dynamic monitoring of cell adhesion, spreading, proliferation, death and morphological changes (Urcan et al., 2010; Xing et al., 2006). The attached cells cause a local insulation in the biosensor surface and create a different interface between the electrode and the cell culture medium, resulting in an increase in impedance. Hence, larger impedance values mean that the number of cells growing on the electrodes is greater (Xiao & Luong, 2005).

The objectives of this study were to compare the *in vitro* cytotoxic responses of human periodontal ligament fibroblasts (hPDLFs) and mouse dermal fibroblasts (mDFs) to three

different commercially available NPs (C₆₀, SWCNTs and Fe₃O₄) using MTT, LDH, ROS and impedance-based assay, and thereby determine the cell type dependency of NP cytotoxicity.

Materials and methods

Chemicals and reagents

DMEM (Dulbecco's modified Eagle's medium) high glucose with L-glutamine medium was purchased from Lonza (Biowhittaker, Verviers, Belgium), 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) from Invitrogen (Molecular Probes, Inc., Eugene, OR), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and LDH detection kits from Sigma-Aldrich (St. Louis, MO). Fe₃O₄ NPs and C₆₀ were purchased from Sigma-Aldrich (St. Louis, MO). The Elicarb[®] SWCNTs were purchased from Thomas Swan (Consett, UK), as a dry powder.

Characterization of NPs

BET analysis

The Brunauer–Emmett–Teller (BET) surface area of the NPs was determined using a NOVA 2200e volumetric gas adsorption instrument (Quantachrome Instruments, Boynton Beach, FL), according to the procedures described elsewhere (Yagmur et al., 2008).

XRD analysis of NPs

NPs were characterized by XRD using a RadB-DMAX II model X-ray powder diffractometer (Rigaku, Tokyo, Japan) in the range of 20–80° by using Cu-Kα radiation as incident beam in 2θ (30 kV, 15 mA, λ = 1.5405 Å) at room temperature (RT). Scan speed was selected as 2°/min in the range of 2θ = 5–80°.

TEM analysis of nanoparticles

The shape, size and morphology of selected NPs were investigated using a transmission electron microscope (TEM) (JEOL-100CX, Tokyo, Japan). NPs were suspended in appropriate media and sonicated with probe sonicator at 70 W for 3 min and dropped over carbon coated grids. Grids were air-dried in a dust-protected environment and examined under the TEM. Suspension media for C₆₀, SWCNTs and Fe₃O₄ are toluene, distilled water and ethanol, respectively.

Mössbauer spectroscopy of Fe₃O₄ NPs

The magnetic phases in the Fe₃O₄ NPs were identified using a Wissel Mössbauer measurement system working in constant acceleration mode. The velocity calibration was performed by using a Wissel MVC 450 model laser calibrator (Wissenschaftliche Elektronik GmbH, Starnberg, Germany). The measured velocity was also validated by measuring a ⁵⁷Fe Mössbauer spectrum of α-iron foil at RT. Approximately 40 mg of Fe₃O₄ NP sample was located in a nylon sample holder of 1 cm² cross-sectional area for measurement. No external magnetic fields were applied. The spectrum was fitted to Lorentzian functions by least-square fitting using the

Win Normos-for-Igor software package (ver. 6.36) and the hyperfine parameters isomer shift (IS), quadrupole splitting (QS) and full line width at half-maximum (W), expressed in mms^{-1} , were obtained. Isomer shifts are given relative to $\alpha\text{-Fe}$ at RT.

DLS and zeta potential analysis

Photon correlation spectroscopy was used to measure the zeta potential and the size of the particles via Malvern's NanoZetasizer-ZS (Worcestershire, UK). For this purpose, NPs were prepared as $100\text{ }\mu\text{g/mL}$ suspensions in cell culture medium and sonicated. Measurements were performed in disposable cuvettes at an ambient temperature of 25°C . A total of 13 sequential measurements were performed for each NP.

Cell culture

hPDLF cell isolation and culture

The root surfaces of premolar teeth without caries or restorations were used as a source for hPDLF cell isolation. The teeth were extracted for orthodontic reasons only. For the extraction of hPDLF cells; ethics committee approval and informed consent from the donors were obtained. According to a previously described method (Albandar, 2005; Inanc et al., 2007), periodontal ligament fibroblast tissue was minced with a sterile scalpel and seeded in culture flasks containing DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, $100\text{ }\mu\text{g/mL}$ streptomycin, 1% nonessential amino acid stock solution and 2 mM L-glutamine (all from Lonza). The explants were cultured in a 95% humidified incubator at 37°C , 5% CO_2 until confluence and cells between passages 8 and 10 were used in all experiments.

mDF cell isolation and culture

Protocols with animals were carried out according to the standards of international regulations. mDF cells were isolated from 8 to 10 weeks adult mouse according to the method described elsewhere (Lichti et al., 2008). Tail skin was used as a source, which was obtained from intraperitoneally anesthetized mice with avertin. Seventy percent ethanol was used to disinfect the tails prior to cutting. Ten to 15 tails were cut at the base and the skin was sectioned and removed. Small square pieces were cut with antisepsis from the sectioned skin into a petri dish ($60 \times 15\text{ mm}$) containing 20 mL of trypsin solution. Epidermis was separated from the dermis by storing the petri dishes overnight at 4°C . The separated dermal explants were placed in DMEM high-glucose medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, $100\text{ }\mu\text{g/mL}$ streptomycin and 1% non-essential amino acids. Cells were kept at 37°C in a humidified atmosphere with 5% CO_2 for 48 h and non-adherent cells were removed. Adherent cells were cultured and grown as described by changing the culture medium twice a week.

Preparation of NP stock solutions and exposure samples

NPs suspended in sterile cell culture medium ($100\text{ }\mu\text{g/mL}$) were used as stock solutions. Before dilution, NP stock

solutions were dispersed by using an ultrasonic bath (Fisher, FS60H) for 30 min in order to minimize the degree of aggregation. After sonication, prepared stock solutions were immediately diluted to predetermined exposure concentrations in cell culture medium by serial dilution with approximately 10 min resonication periods between each dilution. The stock and diluted solutions were kept at $+4^\circ\text{C}$ for a week. The suspensions were freshly prepared for every experiment.

Nanoparticle exposure

The cells were cultured with various concentrations (0.1, 1, 10, 50 and $100\text{ }\mu\text{g/mL}$) of C_{60} , SWCNTs and Fe_3O_4 for predetermined time intervals. For this purpose; cells were seeded onto 96-well plates at a density of 1.0×10^4 cells per well after being trypsinized with 0.25% trypsin-EDTA solution (Sigma). Cell culture was performed in a humidified incubator at 37°C and 5% CO_2 atmosphere overnight. NP exposure was continued for 6 h, 24 h and 48 h by replacing cell medium with the NP-dispersed culture medium. Cells seeded under the same conditions and densities without NPs were used as control.

Cell morphology

Morphological signs of cellular toxicity were investigated by using an inverted microscope (Primo Vert Zeiss, Germany). hPDLF and mDF cells were treated with NPs and phase contrast images were taken after 48 h of exposure.

Subcellular localization of NPs

In order to visualize the cellular uptake and localization of NPs, cells were seeded into six-well tissue culture-treated plates at a density of 3.0×10^5 cells per well in 5 mL culture medium. After three days of incubation, $\sim 70\%$ confluent cells were treated with C_{60} , SWCNTs and Fe_3O_4 at concentrations equal to their IC_{50} values for 48 h. The untreated cells were used as a control. The cells were washed three times with media at the end of the exposure and trypsinized. Then, the cells were centrifuged for five minutes at 282 g (Beckman Coulter Allegra X-15R) and collected for investigation. Cells were prepared for TEM according to a previously described method (Elcin et al., 2003). Accordingly, cells were collected after centrifugation, prefixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in epoxy resin. Samples were sectioned using a Leica ultramicrotome (Ultracut UCT, Vienna, Austria) and examined under a Jeol 100 CX TEM (Tokyo, Japan) after staining with 1% toluidine blue and post-staining with uranyl acetate and lead hydroxide.

MTT assay

mDF and hPDLF cells were tested for their metabolic activity using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma) after exposing to different concentrations of NPs for 6, 24 and 48 h. Briefly, cells were incubated with different amounts of C_{60} , SWCNTs and Fe_3O_4 , and at the end of the exposure, cell medium was aspirated and cells were washed without

disrupting, three times with serum-free medium in order to remove NPs. Twenty microliters of fresh MTT solution (5 mg/mL) and 180 μ L of DMEM (without serum) were added into each well, and the cells were incubated at 37 °C and 5% CO₂ for 4 h. After incubation, the medium was aspirated cautiously and 200 μ L MTT solvent (0.1 N HCl in anhydrous isopropanol) was added to each well and mixed to dissolve the blue formazan crystals. The 96-well plates were centrifuged for 5 min at 250 $\times$ g at 25 °C, supernatants were transferred to new 96-well plates to remove remaining NPs. Formation of formazan was measured at 570 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) (Emin et al., 2008). The increasing concentration of formazan product as measured by the amount of absorbance [A] is directly proportional to the number of living cells in culture. The relative cell viability (%) was calculated from the formula;

$$[A]_{\text{sample}}/[A]_{\text{control}} \times 100$$

where [A]_{sample} is the absorbance of the test sample and [A]_{control} is the absorbance of the control sample.

LDH assay

Cell membrane integrity was measured by determining the LDH released from the cytoplasm using a commercial enzymatic LDH detection kit. mDF and hPDLF cells in 96 well plates were treated with different concentrations of C₆₀, SWCNTs or Fe₃O₄ NPs for 6, 24 and 48 h. At the end of exposure time, the NPs were removed from the test media via centrifugation of the plates at 250 g for five minutes at RT, in order to avoid any interference with the test material. The collected supernatants were then added to freshly prepared LDH assay mixture (substrate/dye/cofactor) in another clean 96-well plate. Fifty microliters of supernatant was added to 100 μ L of LDH assay mixture and incubated at RT for 30 min. Ten microliters of lysis solution was added to each control well containing cells without NPs, 45 min prior to the centrifugation, as a positive control. Fifteen microliters of 1 N HCl per well was used to terminate the reaction. Finally, the absorbance was recorded at 490 nm using a microplate reader. The enzymatic analysis steps were protected from light and all samples were read in triplicate. The 690 nm background readings were subtracted from the primary 490 nm readings in order to find the actual sample and control absorbance values. Calculation of LDH activity (% of positive control) was made using the formula;

$$([A]_{\text{sample}} - [A]_{\text{negative}}) / ([A]_{\text{positive}} - [A]_{\text{negative}}) \times 100$$

where [A]_{positive} is the measured average absorbance for the lysed control cells; [A]_{negative} is the measured average absorbance for the untreated control cells and [A]_{sample} is the measured average absorbance for the NP-treated cells.

Detection of intracellular ROS

The detection of intracellular ROS mediated cell death was performed using carboxy-H₂DCFDA. Prior to incubation, cells were exposed to a series of different concentrations of the NPs for 6 h. Since ROS formation is a quite rapid process,

NP incubation was performed as short-term exposure to better analyze the oxidative potential of the NPs. In order to minimize the interaction of NPs with carboxy-H₂DCFDA, they were removed from the test media by washing the cells with PBS three times consecutively. The cells were then loaded with 20 μ M carboxy-H₂DCFDA (Invitrogen, Carlsbad, CA) for 30 min at 37 °C and the fluorescence intensity was measured at 485-nm excitation and 520-nm emission using the microplate reader. Cells incubated in culture medium without NPs were used as negative control and cells incubated in culture medium containing 400 mM H₂O₂ were used as the positive control.

Impedance-based assay

In order to measure the cytotoxic response of the hPDLFs and mDF cells in real-time; cells were seeded on gold microelectrodes embedded at the bottom of the 16 well microplates (E-plates, Roche Diagnostics, Basel, Switzerland) at a density of 6.25 $\times$ 10³ and 12.50 $\times$ 10³ cells/well, respectively. The microplates were placed in the incubator and cells were grown to the log phase at 37 °C and 5% CO₂ (Şeker et al., 2014). At this point, cells were exposed to different concentrations of NPs and the change in the impedance was measured simultaneously at intervals of 15 min with the xCELLigence Real-Time Cell Analyzer (RTCA) DP-system (Roche Applied Science, Penzberg, Germany). Cells with cell culture medium only were used as the negative control. Highest concentration of NPs with cell culture medium without cells were also tested for any potential interference. As a positive control, we performed additional experiments with ZnO (100 μ g/mL) that leads up to a known toxic effect. IC50 values were calculated by the RTCA-DP Software (Roche) using the sigmoidal dose response curves.

Statistical analysis

Data from all experiments were obtained from three independent experiments, and were expressed as the mean $\pm$ standard deviation. A value of $p < 0.05$ was considered to be statistically significant.

Results

BET surface area measurement

The specific surface areas measured by BET analysis are presented in Table 1. According to the data obtained, SWCNTs have larger surface area compared to C₆₀ and Fe₃O₄.

Table 1. Characterization of nanoparticles.

	C ₆₀	SWCNT	Fe ₃ O ₄
BET surface area (m ² /g)	10.58	808.3	40.89
Average diameter in DLS (nm)	142.0	112.9	316.6
Zeta potential (mV)	-37.4 $\pm$ 6.45	12.9 $\pm$ 3.19	-18.4 $\pm$ 4.49
Polydispersity index	0.548	0.573	0.644
Sizes in TEM (nm)	34.64	9.00*	28.33

*Diameter of SWCNT.

XRD analysis of NPs

The powder XRD patterns of C_{60} , SWCNTs and Fe_3O_4 NPs are shown in Figure 1(A)–(C), respectively. XRD analysis indicated that C_{60} showed eight distinct diffraction peaks: (111), (220), (311), (222), (331), (420), (422) and (511) (Figure 1A). SWCNTs showed two distinct diffraction peaks: (002) and (101) (Figure 1B). Fe_3O_4 NPs showed (111), (220), (311), (222), (400), (422), (511), (440), (620) and (622) reflections (Figure 1C).

TEM characterization of NPs

TEM micrographs of the NPs are shown in Figure 1(D)–(F). TEM micrographs of NPs reveal that the C_{60} NPs (Figure 1D) tend to form circular shaped and larger agglomerates in

solution. SWCNTs were naturally aggregated and did not exist as individual tubes; but as bundles and clumps of aggregates (Figure 1E) due to the Van der Waals forces between the molecules. Fe_3O_4 NPs (Figure 1F) have a relatively uniform size distribution. Agglomerate formation is observed from the TEM micrograph, but relatively in smaller sized clusters when compared to C_{60} and SWCNTs. The average particle sizes are given in Table 1.

Mössbauer spectroscopy of Fe_3O_4 NPs

The RT Mössbauer spectrum of the Fe_3O_4 NPs which is fitted with four sextets is presented in Figure 2. The parameters suggest the existence of $Fe^{2.5+}$ on the octahedral sites (Hf: 43.904 T) and Fe^{3+} on the tetrahedral sites (Hf: 47.741 T) of

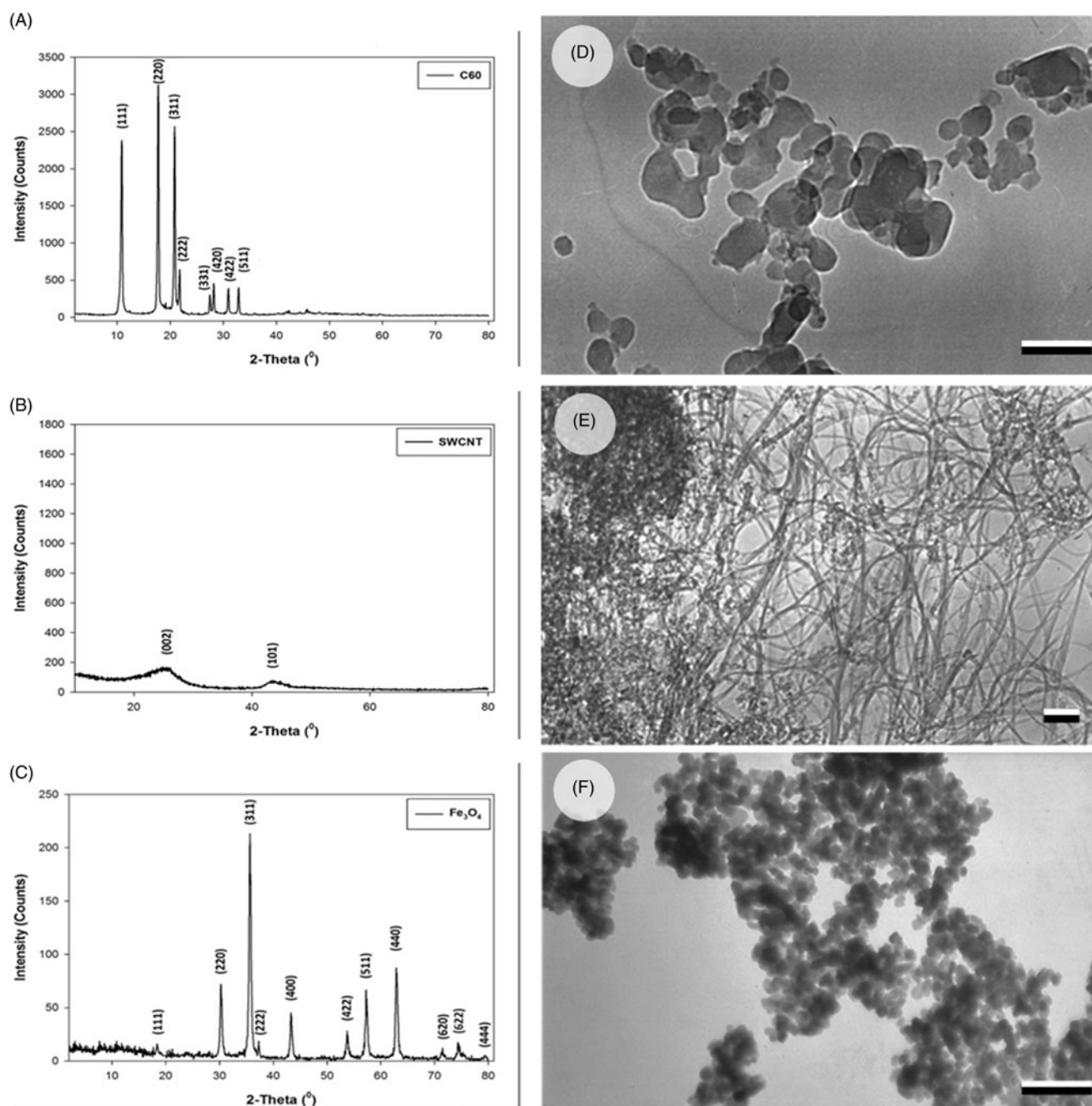
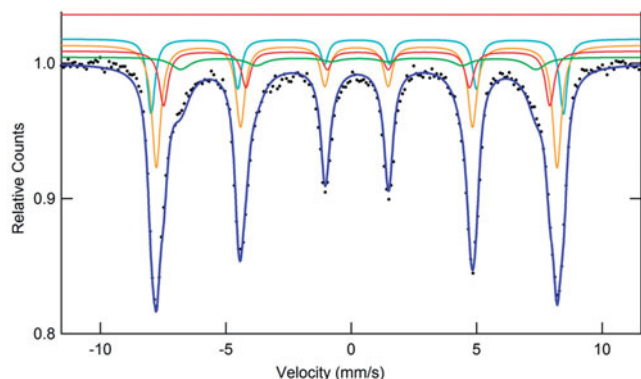


Figure 1. XRD patterns of (A) C_{60} , (B) SWCNTs and (C) Fe_3O_4 NPs analyzed for size and crystalline structure. Respective Miller indices of each peak are represented in parenthesis. TEM micrographs of C_{60} (D), SWCNT (E) and (F) Fe_3O_4 . Scale bars: 100 nm.

Figure 2. Mössbauer spectrum of Fe₃O₄ NPs.Table 2. Hyperfine parameters of the fitted Mössbauer spectra of Fe₃O₄ recorded at RT.

Component	IS (mms ⁻¹)	QS (mms ⁻¹)	Hf (Tesla)	W (mms ⁻¹)	Area (%)
Fe ^{2.5+} (Mt)	0.4071	−0.033	43.904	0.9	18.815
Fe ³⁺ (Mt)	0.3445	−0.04	47.741	0.4249	19.049
Fe ³⁺ (Mh)	0.3181	0.0109	49.539	0.4253	42.818
Fe ³⁺ (Hm)	0.341	0.0096	50.969	0.3643	19.318

IS: isomer shift relative to α -Fe at room temperature; QS: quadrupole shift; Hf: magnetic hyperfine field at ⁵⁷Fe nuclei; W: half maximum line width; Area: area of the spectrum component; Mt: magnetite; Mh: maghemite; Hm: hematite.

magnetite. The other two Fe³⁺ sextets on the tetrahedral sites are assigned to maghemite (Hf: 49.539 T) and hematite (Hf: 50.969 T) (Table 2).

Particle size distribution and zeta potential

The average particle size distribution and zeta potential (ζ) of NPs in cell culture medium measured by DLS method are presented in Figure S1 and Table 1. The average hydrodynamic diameters are 112.9, 142.0 and 316.6, for SWCNTs, C₆₀ and Fe₃O₄, respectively. Polydispersity index (PDI) is also determined using NanoZetasizer (Table 1). All the particles showed a relatively broad size distribution indicating polydispersity in cell culture medium.

Cell morphology

In order to visualize the changes in the morphology of hPDLF and mDF cells, inverted phase-contrast images are collected before and after exposure to NPs for 48 h. The representative micrographs of SWCNT, C₆₀ and Fe₃O₄ exposed cells are presented in Figure S2.

As shown in Figure S2, the cells of the control group exhibited a normal morphology and non-inhibited cell proliferation. The 50 μ g/mL SWCNT, C₆₀ NP-treated hPDLF and mDF cells changed into a rounded morphology from its original shape due to cytoplasmic shrinkage. We also monitored cells by video microscopy and the data obtained (not shown here) confirmed that the presence of NPs affected the morphology and attachment of cells more significantly at higher concentrations.

Cellular uptake, intracellular accumulation and morphological alterations

The detailed observation of the selected cells were made using TEM micrographs obtained from exposure studies. The results are given in Figure 3. NP-treated cells were relatively rich in cytoplasmic vesicles whereas control cells had a uniform distribution of vesicles which were free of particles and a normal cell morphology with no sign of cell injury (Figure 3A and B). No significant sign of cell injury except increased and enlarged vacuoles in the C₆₀-treated hPDLF cells (Figure 3C) could be noticed. No significant membrane blebbing could be detected.

The nucleus of SWCNT-treated hPDLF cells displayed an irregular, indented nuclear shape and margin as a result of nuclear grooving and convolutions (Figure 3E). Note the darkened (electron dense) mitochondria in the SWCNT-treated mDF cells (Figure 3F).

Fe₃O₄ NP aggregates can be visualized either in the cytoplasm or in the cytoplasmic vesicles of both hPDLF and mDF cells. A significant number of endocytotic–exocytotic vesicles were accumulated on the cell membrane. Intense membrane blebbing can be seen especially in hPDLF cells treated with Fe₃O₄ NPs (Figure 3G2). Fe₃O₄-treated mDF cells also exhibited cell blebbing with a network of microvilli (Figure 3H1).

Mitochondrial function

Cell viability was monitored via measurement of the mitochondrial dehydrogenases after exposure to NPs. As represented in Figure 4, the cell viability was higher for hPDLF cells in the first 6 h of exposure which then decreased dramatically ($\sim$ 50%) in the subsequent time points. mDF cell viability was higher for 24 and 48 h exposure times compared to hPDLF cells. For the first six hours of exposure hPDLF cell viability seemed to be concentration dependent for SWCNTs because a gradual decrease with increasing concentration is observed whereas for C₆₀ NPs, the dependency of cell viability on concentration was less, because all concentrations resulted in close cell viability values.

LDH measurements

LDH enzyme which can leak into the extracellular fluid in case of destroyed membrane integrity is investigated in hPDLF and mDF cells to find out if NP exposure caused damage on the cell membrane. According to the results of the assay, all three NPs caused loss of membrane integrity in time, in a gradually increasing manner. LDH levels measured for hPDLF cells were considerably higher compared to that of mDF cells, at all time points except for mDF cells exposed to Fe₃O₄ for 48 h. The LDH leakage for mDF cells increased time dependently for all tested NPs. Especially at 48 h of exposure, 100 μ g/mL of Fe₃O₄ NPs produced a significantly high amount of LDH ($\sim$ 84%). hPDLF cells produced a more concentration dependent LDH increase compared to mDF cells (Figure 5).

Intracellular ROS measurements

In order to control the ROS formation due to oxidative stress triggered in hPDLF and mDF cells by NPs, a fluorescence

Figure 3. TEM micrographs of control (A) hPDLF and (B) mDF cells; (C) C_{60} -treated hPDLF, (D) C_{60} -treated mDF; (E) SWCNT-treated hPDLF, (F) SWCNT-treated mDF; (G) Fe_3O_4 -treated hPDLF, (H) Fe_3O_4 -treated mDF cells. Some of the marked areas in the figures are shown in higher magnifications with arrows (arrows indicate NP agglomerates). N: nucleus; M: mitochondrion; ER: endoplasmic reticulum; V: vesicle. Scale bars = 1 μm .

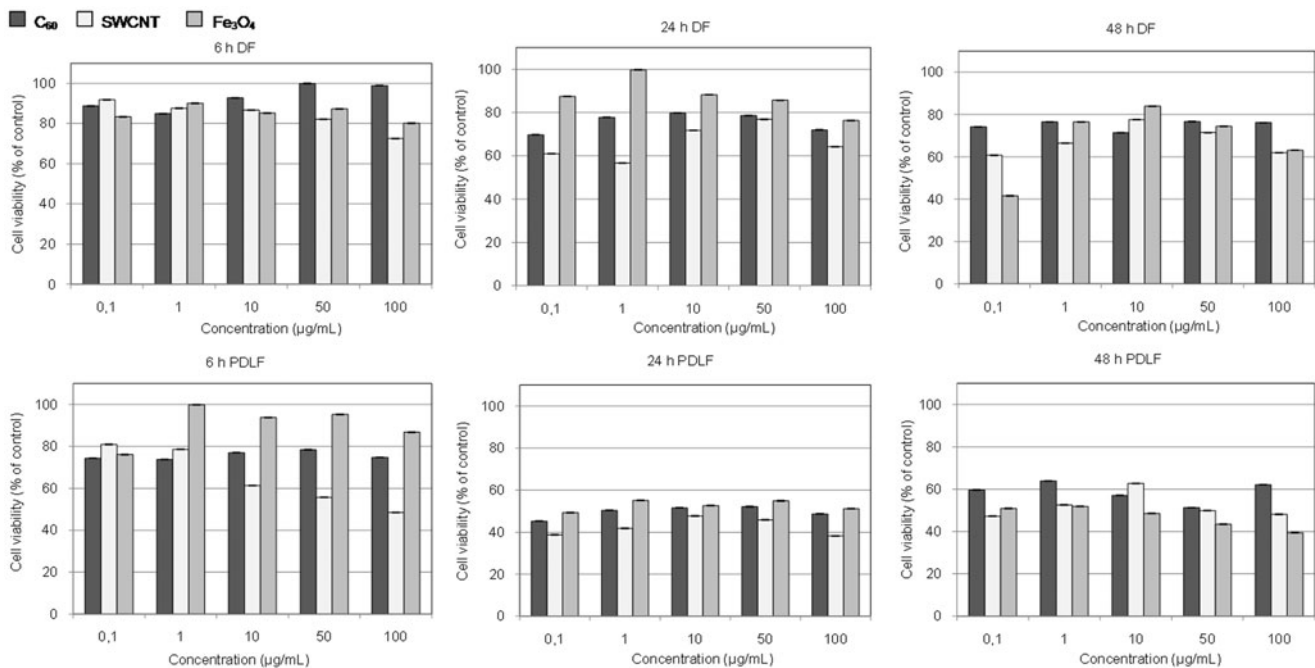
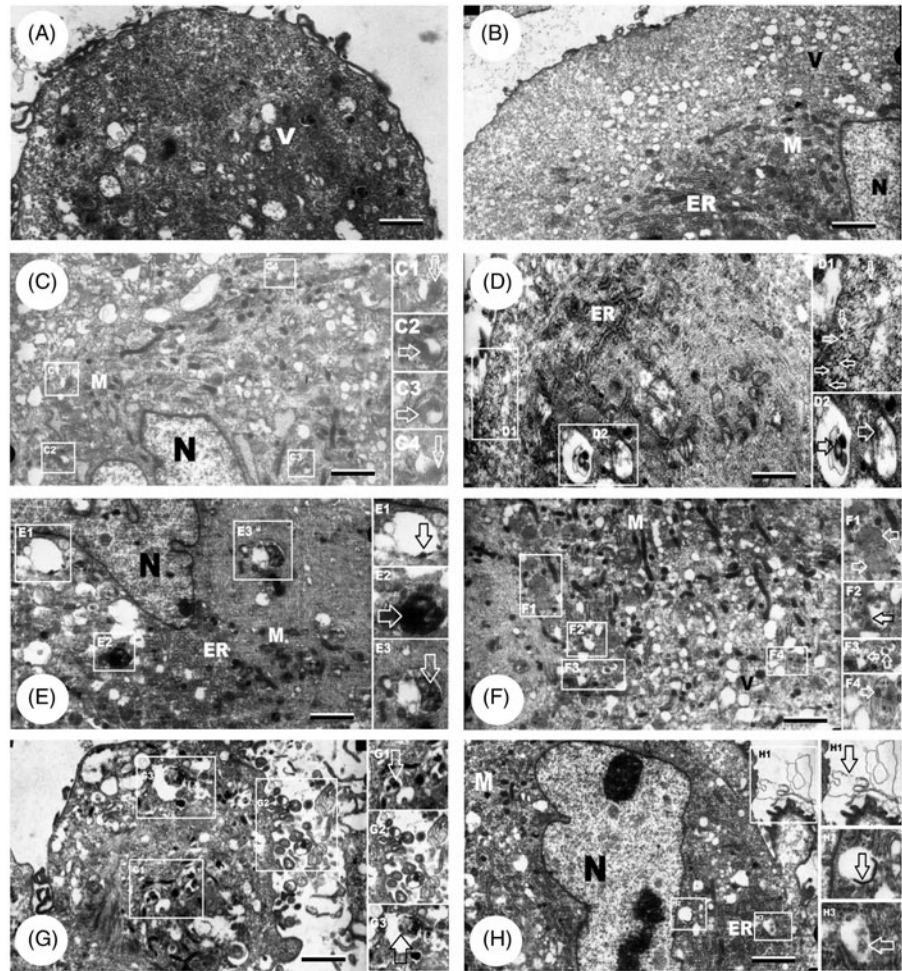


Figure 4. MTT measurement results of hPDLF and mDF cells at 6, 24 and 48 h of NP exposure.

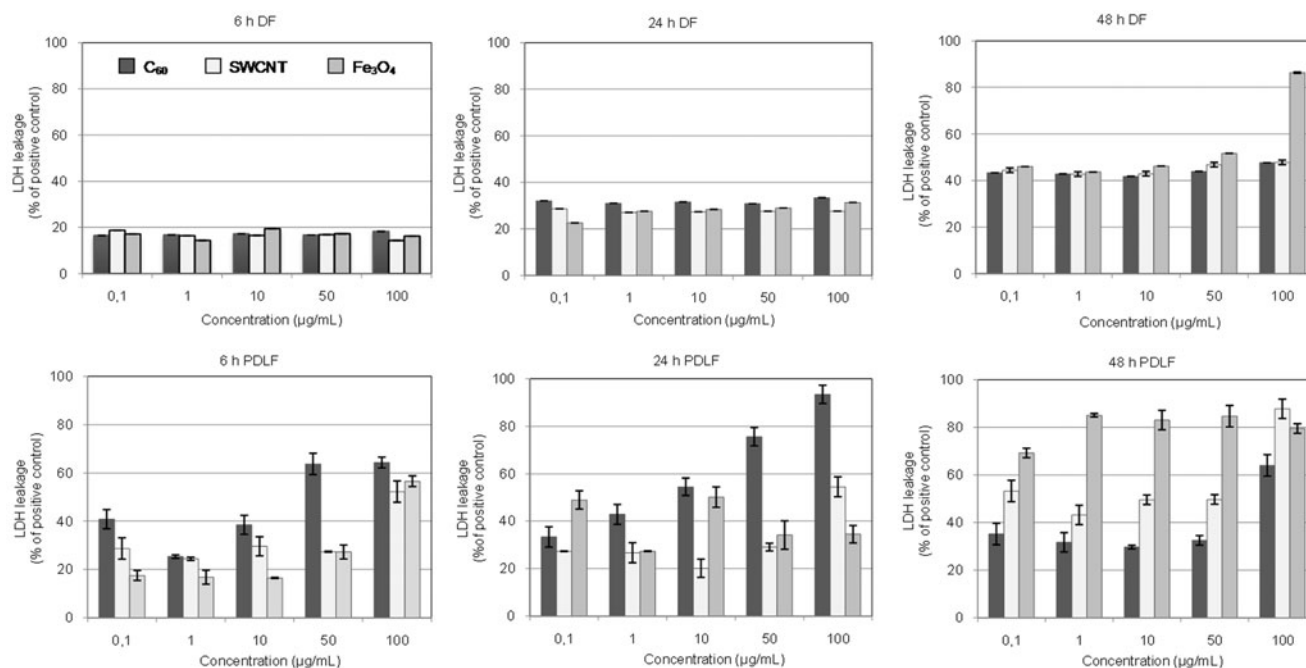


Figure 5. LDH measurement results of hPDLF and mDF cells at 6, 24 and 48 h of NP exposure.

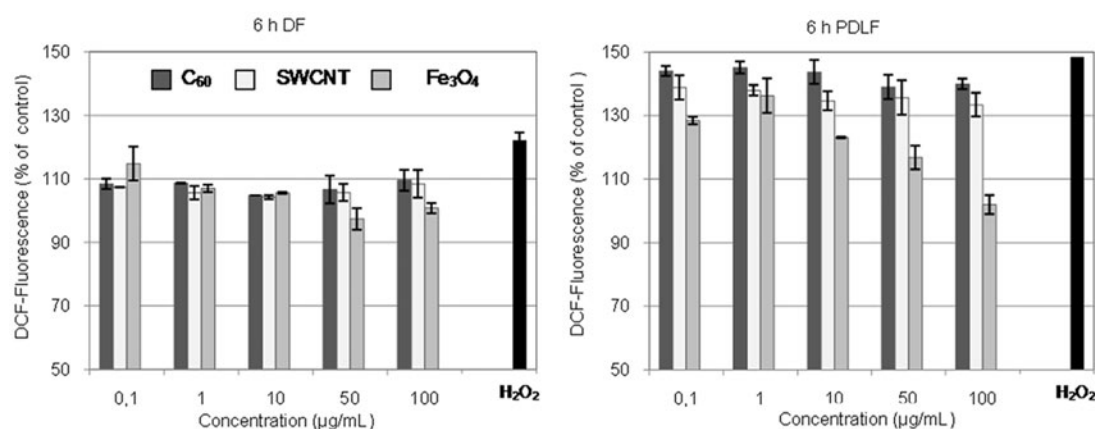


Figure 6. ROS measurement results of hPDLF and mDF cells at 6 h of NP exposure.

assay involving carboxy- H_2DCFDA as the ROS indicator was used. The results showed that, in the six hours of exposure, all three NPs triggered the formation of ROS in both hPDLF and mDF cells (Figure 6). ROS formed in the hPDLF cells in the six hours of exposure was obviously higher than that of mDF cells.

Cellular impedance measurements

Adhesion and proliferation dynamics under NP exposure conditions were measured and assessed as cellular impedance with the help of the xCELLigence system. Results were presented as mean $\pm$ standard error of the mean for triplicate samples. NP related changes in cell index (CI) are given in Figure 7. The CI values were normalized to the time point of NP addition. The normalized cell index (NCI) values increased continuously for control samples of both hPDLF and mDF cells in a constant rate due to cell proliferation. The xCELLigence experiments were conducted for five different

concentrations (0.1, 1, 10, 50, 100 $\mu g/mL$) of NPs to observe the effect of concentration on cellular toxic response.

The results revealed that hPDLF cells showed a similar NCI with the control cells for all NPs. For mDF cells however, exposure of NPs resulted in a concentration-dependent retardation of NCI increase for all three NPs, especially at the higher concentrations of exposure (Figure 7). The positive control results indicated that CI values for both cells had significantly decreased (near zero level) (Figure S3). None of the tested NPs caused the NCI to decrease to zero level.

IC₅₀ values ($\mu g/mL$) for the tested NPs were calculated for both cell types by using the xCELLigence RTCA software. IC₅₀ values for hPDLF and mDF cells are given in Table 3.

Discussion

C₆₀, SWCNTs and Fe₃O₄ are all NPs which have unique and extraordinary properties due to their nano-size and atomic

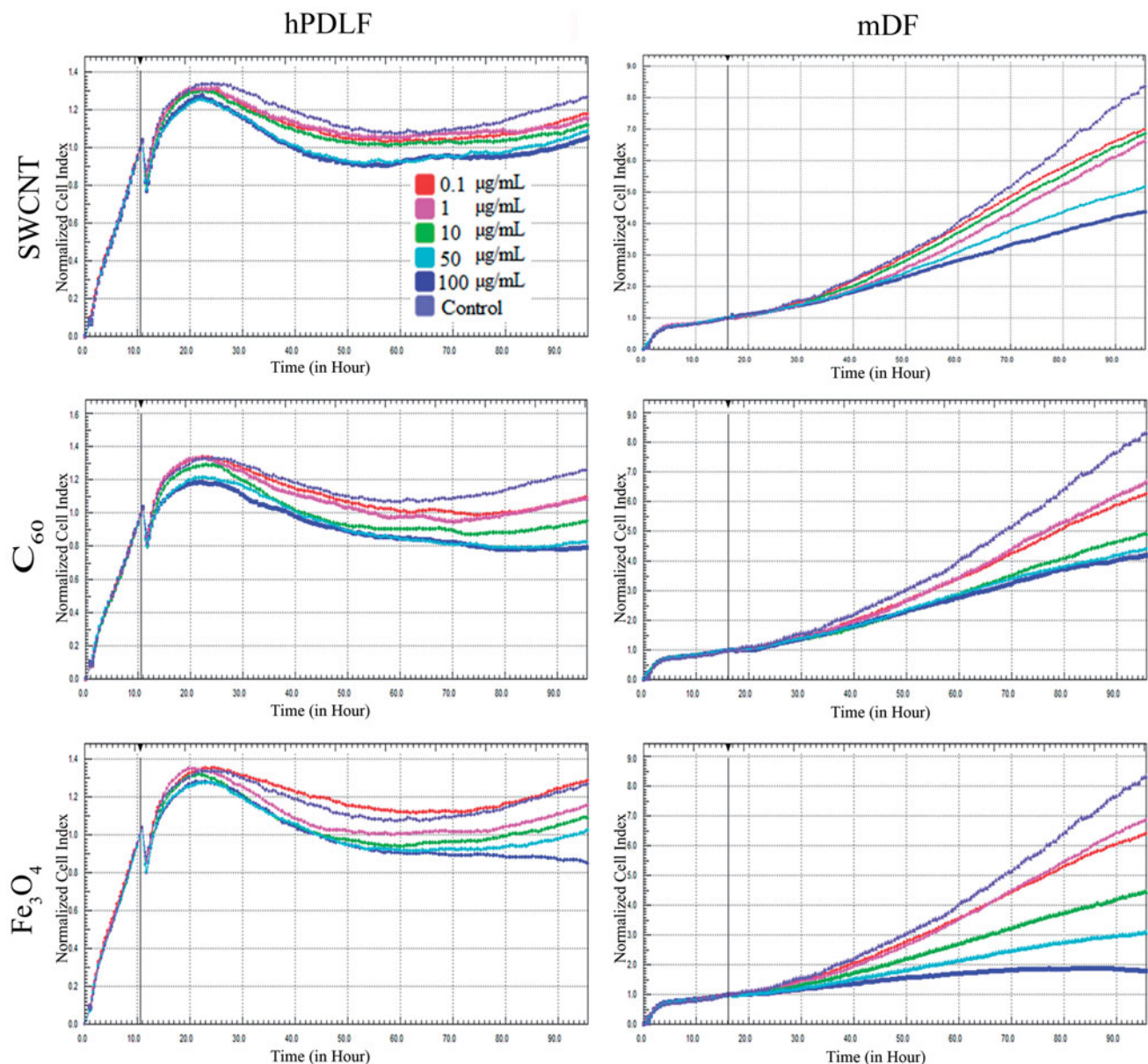


Figure 7. Impedance based (xCELLigence) cell viability results of hPDLF and mDF cells exposed to SWCNTs, C_{60} and Fe_3O_4 .

structure. For this reason, their potential use is being investigated extensively in many fields and as they find areas of application, their production increases accordingly. However, nanomaterials are of concern when they are in contact with cells and tissues because of their small size which can go beyond the barriers of the cell. For this reason, nanomaterials which are potential building blocks in a variety of applications, including the biomedical field need to be fully understood in terms of cytotoxicity.

In this study, two different cell types, hPDLFs and mDFs, were used to assess the cytotoxic effects of three selected NPs. hPDLFs are cells of the dental root lying between the alveolar bone and cementum layer of teeth. The dental implants – one of the long term applications inside patients – which pass through the periodontal ligament tissue are directly in contact with hPDLF cells. Many published or ongoing research on engineered materials for dental

applications, like periodontal tissue engineering and improvement of dental implants involve the use of NPs to benefit from their extraordinary structural properties (Kong et al., 2006; Wang et al., 2007). However, the degradation and/or wear of dental implant materials are highly possible *in vivo*, and the fate of these materials are detected as nano-scale debris in periprosthetic tissues or/and other organs (Yang & Webster, 2012).

Dermal fibroblasts are the collagen producing cells of the skin within the dermis layer which are the main participants of wound healing (Thangapazham et al., 2014). In the recent years, NPs have been involved in studies on wound healing, as antimicrobial agents (Anghel et al., 2012; Grumezescu et al., 2014; Simmons et al., 2009; Zhou et al., 2010). Besides, the incorporation of NPs in cosmetic products like skin creams and toothpastes are under investigation and their use is expanding in the industry (Benn et al., 2011; Inui et al., 2011,

Table 3. IC₅₀ values of periodontal ligament fibroblasts and dermal fibroblasts.

	hPDLFs	mDFs
C ₆₀	50.7 µg/mL	48.1 µg/mL
SWCNT	168.4 µg/mL	136.6 µg/mL
Fe ₃ O ₄	143.4 µg/mL	88.7 µg/mL

2014; Lens, 2011; Takada et al., 2006). Dermal exposure of NPs from cosmetic products is not the only way they can reach dermal cells, the commercial production of these NPs creates the risk of occupational dermal exposure as well. A study conducted on SWCNTs (Maynard et al., 2004) reported 0.2–6 mg of occupational dermal exposure per hand. For the mentioned rationale, we chose the two types of fibroblast cells, hPDLF and mDF, as prototypes in our study.

To the best of our knowledge, the cytotoxic effects of C₆₀, SWCNTs and Fe₃O₄ NPs on hPDLFs and mDF cells were evaluated for the first time in our study. Also, proliferative cytotoxic responses of hPDLF and mDF cells to the selected NPs were evaluated for the first time with the impedance-based biosensor (xCELLigence) system for real-time, label-free and long-term monitoring of cell responses to NPs.

Nanosized Fe₃O₄ is less stable than its bulk form and can be more readily oxidized to γ -Fe₂O₃ due to its high surface area (Cornell & Schwertmann, 2006). For this reason, it is important to determine if there is any formation of oxidation product in the Fe₃O₄ NPs, as it may affect the reactivity of the NPs and accordingly the state of cellular toxic response. The XRD peaks of Fe₃O₄ and γ -Fe₂O₃ (Figure 1), which both crystallize in the cubic structure, occur nearly at the same angle and accordingly, it is difficult to distinguish between these two types of iron oxides via XRD. For this reason, Mössbauer spectroscopy, which is a well-known local probe technique for its sensitivity and specificity in investigating Fe-containing compounds, was employed to characterize the fine structure and oxidation state of magnetic Fe₃O₄ NPs.

The observed Mössbauer spectra and parameters are very similar to the spectra reported by Andrade et al. (2009). According to the Mössbauer spectrum of the Fe₃O₄ NPs, it can be suspected that there are magnetite, maghemite and hematite crystal phases in the measured samples which may be the result of partial oxidation in air occurred during synthesis of the NPs and/or more likely during storage. The nanosize of the particles facilitates the oxidation process due to high surface area which produces NPs with a core of magnetite while the surface is partially oxidized. Slightly broadened resonance lines may be the result of the small particle size which reduces the Hf below that of the bulk material (Cornell & Schwertmann, 2006).

TEM was also used to examine the cellular uptake and localization of the NPs, and the effects of NP exposure on cells. According to the examined micrographs of the cells before and after NP exposure, some differences between control cells and the NP exposed cells were observed. The control cells showed a normal morphology with an intact cell membrane and endoplasmic reticulum for both hPDLF and mDF cells (Figure 3A and B). However, we observed some dissimilarities between the control cells and the NP exposed

cells, especially for Fe₃O₄-exposed cells. They had an abnormal morphology with protruding membrane blebs which may be a sign of cell injury and necrosis due to NP-triggered toxicity. In line with this finding; bleb formation has been observed in previous studies (Sadhukha et al., 2014; Yurchenko et al., 2010) on different cell types exposed to Fe₃O₄ NPs as well. Bleb formation is also linked to apoptosis as a sign of cell death. The cell to cell contact was lost accordingly and Fe₃O₄ NPs showed cellular signs resembling apoptosis which is also in line with the findings of Ramesh et al. (2012) and Shi et al. (2012). The nucleus of SWCNT-treated hPDLF cells displays an irregular, indented nuclear shape and margin as a result of nuclear grooving and convolutions (Figure 3E). This is a common characteristic morphological abnormality for nucleus of malignant cells as well (Dey, 2010). Note the darkened (electron dense) mitochondria in the SWCNT-treated mDF cells (Figure 3F).

The degree and way of internalization for the NPs may be highly dependent not only on physicochemical properties and size of the NPs, but also on type of the cells as well. The interaction of the NPs with the extracellular matrix, cell membrane and cytoplasm may also be affected by the agglomeration behavior of the NPs which alters the apparent size–shape and accordingly determines the contact surface area and degree of mobility and internalization. NPs which have a lower tendency to agglomerate may be internalized more easily which increases the exposure doses compared to the agglomerating NPs. The agglomeration is common for NPs as most of them have surface charges which interact with the cell culture medium proteins and form protein corona. Agglomeration also may prevent NPs from membrane permeation as they would not be small enough to bypass endocytotic uptake (Nazarenus et al., 2014). C₆₀ and SWCNTs are a family of synthetic carbon allotropes, where the carbon atoms are joined by strong covalent bonds. Hydrophobic surfaces of C₆₀ and SWCNTs adsorb a wide class of substances by π – π and/or van der Waals interactions. Fe₃O₄ NPs, C₆₀ and SWCNTs can adsorb serum proteins present in the cell culture medium onto their surface via hydrophobic and electrostatic interactions, leading to a change in their surface charges and agglomeration behavior (Murdock et al., 2008). Also, Fe₃O₄ NPs show superparamagnetic behavior. Magnetic attractive forces combined with native high surface energies lead to intense aggregation in fluids. DLS results showed that the size of the Fe₃O₄ NP aggregates was higher than that of the C₆₀ and SWCNTs.

The entry of NPs may induce oxidative stress in cells by disrupting the nature of the antioxidant mechanisms. Therefore, the oxidative stress of the selected cells was investigated after NP treatment. For all of the tested NPs, the measured ROS levels were elevated after six hours of exposure for both hPDLF and mDF cells compared to the control group, since nanoparticles induced oxidative stress via generation of ROS.

The obtained NCI values were significantly divergent depending upon the cell type and NP used. One thing that should be noted for hPDLF cells is that, they exhibited an obvious increase in NCIs between 60 and 70 h of exposure after a decrease up to that point which may be considered as

an adaptation of the cells to NPs. For C₆₀, mDF cells were quite sensitive whereas the toxicity was at the lowest level for hPDLF cells. These results confirmed that, the cytotoxic response is dependent upon the target cell type. The results of a previous study on SWCNT toxicity (Hu et al., 2010) is in good agreement with this finding. Three tested cell lines in the mentioned study responded differently to NP treatments. Studies conducted by different groups (Falcão et al., 2006; Patra et al., 2007; Sohaebuddin et al., 2010; Suresh et al., 2012; Treichel et al., 2004) are also highlighting similar conclusions. The results of the impedance measurements of our study as well showed that, the selected NPs exhibited dissimilar cytotoxic effects on different cell types. The reason of this different behavior may be attributed to the possible different cell membrane composition of different cell types which may alter the uptake mechanism and degree of internalization for NPs. Also, different cellular defense mechanisms the cells possess, according to their location and function in the body may cause the cytotoxic responses to differ. Another possible cause may be the different adaptation abilities of different cells to tolerate new environments (Williams & Iatropoulos, 2002).

In summary, the exposure studies of hPDLF and mDF cells to C₆₀, SWCNT and Fe₃O₄ NPs revealed that mDF cells were more sensitive to NP exposure compared to hPDLF cells, which exhibited signs of cellular adaptation at NP exposures of lower concentrations. As a result, all three investigated NPs which are potential building blocks for biomedical materials can be suitable candidates in means of biocompatibility as long as the concentrations are limited to a level that do not elicit toxic response. The different toxic responses of the two selected cell types observed during the study may give an opinion of the biomaterial's possible application sites and purposes of use. For this reason, further studies should be conducted with more different types of cells in order to identify the specific responses, safe exposure concentrations and adaptation abilities of these cells to NPs.

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Declaration of interest

The authors declare no competing financial interests in relation to this article.

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Supplementary material available online