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Application of the adverse outcome pathway (AOP) framework for investigating skin sensitization potential of nanomaterials using new approach methods

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Conflicts of interests

The authors declare no conflict of interest.

Abstract

Background

Currently, there are considerable efforts to standardize methods for accurate assessment of properties and safety aspects of nanomaterial. However, immunomodulation effects upon skin exposure to nanomaterial have been negligibly explored.

Objective

To investigate the immunotoxicity of single-wall carbon nanotubes, titanium dioxide and fullerene using the current mechanistic understanding of skin sensitization by applying the concept of adverse outcome pathway (AOP).

Methods

Investigation of nanomaterials ability to interact with skin proteins using micro-direct peptide reactivity assay (mDPRA); the expression of CD86 cell surface marker using U937 cell activation Test (OECD No 442E/2018); and the effects of nanomaterials on modulating inflammatory response through inflammatory cytokine release by U937 cells.

Results

The nanomaterials easily internalized into keratinocytes cells, interacted with skin proteins like and triggered activation of U937 cells by increasing CD86 expression and modulating inflammatory cytokine production. Consequently, these nanomaterials were classified as skin sensitizers *in vitro*.

Conclusions

Our study suggests the potential immunotoxicity of nanomaterials and highlights the importance of studying the immunotoxicity and skin sensitization potential of

nanomaterials to anticipate possible human health risks using standardized mechanistic non-animal methods with high predictive accuracy. Therefore, it contributes towards the applicability of existent OECD TG for accurate assessment of nanomaterial skin sensitization potential.

Keywords: nanomaterials, immunotoxicity, skin sensitization, single-wall carbon nanotubes, titanium dioxide, fullerene

1. Introduction

The advance of nanotechnology has spread the use and applications of nanomaterials in diverse fields, including industry, agriculture, engineering, cosmetics and medicine. As a consequence, environment and human exposures have raised concerns concerning toxic potential and exposure risk to engineered nanomaterials.¹⁻³ Global efforts have been made to establish regulatory risk assessment of nanomaterials to ensure the correct management and that potential safety issues are being addressed concomitantly with their development and applications.⁴⁻⁷ The Organization for Economic Co-operation and Development (OECD) has systematically worked to address human health and environmental safety implications associated with manufactured nanomaterials. The OECD project on Safety of Manufactured Nanomaterials comprises a series of publications, conferences and applicability evaluation of existing OECD Testing Guidelines (TG) as well as validation of new OECD TG for accurate assessment of nanomaterial properties.^{8,9} However, the toxicological profile of these materials are still limited and remains incompletely understood, in particular on the immune system.⁶

Although skin represents one of the main routes of exposure to nanomaterials, immunomodulation effects and inflammatory response upon skin exposure to such materials have not been deeply investigated. In general, the induced immunomodulation has involved activation of cells that play key role in the immune system, such as macrophages, with subsequent cellular inflammatory responses, mainly through IL-1 expression, and cellular damage. The interaction of nanomaterials with skin proteins and immune cells of immune system can potentially lead to skin sensitization.¹⁰ Skin sensitization consists of a type IV hypersensitivity response triggered in susceptible individuals after repeated dermal exposure to a potential allergenic material.

New approach methodologies (NAMs) are in continuous development to provide information for preliminary determination of chemicals hazard to the public health, moving from animal data to mechanistic human data for human risk assessment. Adverse outcome pathway (AOP), a relatively new toxicological concept, which connects, in a formalized and quality-controlled manner, toxicological mechanistic information to regulatory sciences.¹¹ The AOP is a framework that describes the initial event of a toxicant with a biomolecule and continuing with dependent series of intermediate key events (KEs) at different levels of biological organization, while finally ending in an adverse outcome pathway (AOP). As described by the OECD, the AOP for skin sensitization is defined in four KEs: interaction of material with skin proteins, culminating with formation of hapten-protein complexes (KE-1 or molecular initiating event, MIE), inflammatory keratinocytes response (KE-2), activation of dendritic cells (KE-3), and activation and proliferation of T-lymphocytes (KE-4).¹²

Accepted Article

The characterization of skin sensitization AOP allowed the development of non-animal based innovative methods with higher predictive capacity for allergenicity assessment of chemical, when compared to the traditional animal-based methods,¹³ e.g. local lymph node assay (LLNA).¹⁴ To this end, this study investigated the potential mechanisms of immunotoxicity of widely used nanomaterials such as single-wall carbon nanotubes (SWNTs), titanium dioxide (TiO₂) and fullerene (C₆₀) using the framework of skin sensitization AOP. The nanomaterials were characterized by particle size, charge distribution and homogeneity of suspensions, as well as their ability to interact with the most abundant cell in the skin, the keratinocytes. First, the participation in KE-1 in the AOP was evaluated by quantifying the ability of nanomaterials to interact with skin proteins using micro-direct peptide reactivity assay (mDPRA).¹⁵ Participation of these nanomaterials in KE-3 was performed by measuring the expression of CD86 cell surface marker using U937 cell activation test (OECD No 442E/2018 - U-SENSTM).¹⁶ Moreover, the effects of the nanomaterials in modulate an inflammatory response was measured by analysis of inflammatory cytokine release by U937 cells.

2. Material and Methods

2.1 Preparation and characterization of nanomaterials

SWNTs (CAS no. 308068-56-6) , TiO₂ (CAS no. 1218841-9) and C₆₀ (CAS no. 99685-96-8) (Sigma-Aldrich, San Louis, Missouri) were dispersed in Dulbeccos's Modified Eagle Medium (DMEM) (Sigma-Aldrich) at 200 µg/mL. Samples were sonicated for 30 s with 1 min intervals during total of 5 min. Images from nanomaterials

Accepted Article

were obtained using CytoViva Hyperspectral Imaging System (CytoViva, Auburn, Alabama). Hydrodynamic diameter was determined by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) and zeta potential was determined by nanoparticle tracking analysis (NTA) using NanoSight NS500 (NanoSight, Amesbury, UK). Analysis of samples were conducted after appropriately diluting with PBS at 25 °C.

2.2 Cell culture

HaCaT (human immortalized keratinocyte) and U937 (human histiocytic lymphoma) cell lines were obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). HaCaT and U937 cells were cultured in DMEM and Roswell Park Memorial Institute-1640, respectively, supplemented with 10% v/v fetal bovine serum (FBS) (Sigma-Aldrich) 1% v/v penicillin/streptomycin (Sigma-Aldrich,) in humidified atmosphere of 5% CO₂ at 37 °C.

2.3 Cellular uptake

HaCaT (1x10⁵ cells/well) were seeded in round cover slips, which were placed in 6-well plate, for 24 h. Cells were exposed to SWNTs, TiO₂ and C₆₀ at 200 µg/ml for 2 h. Thereafter, the cover slips were washed with PBS, the slides prepared and observed using CytoViva Hyperspectral Imaging System (CytoViva).

2.4 mDPRA

The mDPRA¹⁵ is an analogous method to direct peptide reactive assay (DPRA) adopted by OECD as Test Guideline 442C¹⁷ and DPRA DB-ALM protocol No 154. Lysine (Ac-RFAAKAA-COOH) or cysteine (Ac-RFAACAACOOH) (>95% purity) (Genome Biotechnologies, Rio de Janeiro, RJ, Brazil) - containing peptide solutions were freshly prepared before use at 0.667 mM in pH 10.2 ammonium acetate buffer and pH 7.5 phosphate buffer, respectively. Cysteine or lysine-containing peptide solutions were incubated with SWNTs, TiO₂ and C₆₀ at 1:10 and 1:50 ratio, respectively, in 250 µl glass inserts coupled into autosampler vials for a total volume of 100 µL (5 µL nanomaterial sample, 20 µL acetonitrile and 75 µL cysteine solution or 25 µL nanomaterial sample and 75 µL lysine solution). Reference (peptide samples not incubated with nanomaterials), positive (*p*-phenylenediamine, CAS no. 106-50-3) and negative (pyridine, CAS no. 110-86-1) (Sigma-Aldrich) controls were also performed. All samples were incubated for 24 h in the dark at 25 °C. Samples and standard curve of cysteine and lysine peptides (0.0167 – 0.534 mM) were analyzed in triplicate using Agilent 1260 series chromatography system (Agilent, Palo Alto, California). Analysis was performed using C-18 column (5 µm, 150 x 3.0 mm, Agilent Technologies, Santa Clara, California) at a wavelength of 220 nm. The mobile phase consisting of ultrapure water containing 0.1% v/v trifluoroacetic acid (A) and ultrapure water containing 0.085% v/v acetonitrile (B) at flow rate of 0.35 mL/min with a gradient of B from 10% to 25% over a period of 10 min followed by an increase to 90% within 3.5 min and decrease to 10 % during 20 min of running. Peptide depletion (%) promoted by each sample was determined following the equation: depletion (%) = [1-(peptide peak area of the test material/peak area of reference)] x 100. Negative

depletion was considered as zero. The mean peptide depletion was used to classify the nanomaterials as negative or positive and to determine their reactivity class according to OECD TG 442C¹⁷ (Table 1).

Table 1: Cysteine and lysine prediction model for *in chemico* mDPRA

Mean cysteine and lysine depletion (%)	Reactivity class	Prediction model*
0 < depletion ≤ 6.38	Minimal	Negative
6.38 < depletion ≤ 22.62	Low	Positive
22.62 < depletion ≤ 42.47	Moderate	Positive
42.47 < depletion ≤ 100	High	Positive

*Prediction model based on OECD TG 442C¹⁷.

2.5 U-SENS™

CD86 expression in U937 cells was evaluated after exposure to SWNTs, TiO₂ and C₆₀ using U-SENS, according to OECD 442E¹⁶. Initially, a proficiency testing was performed with skin sensitizers [resorcinol (CAS no. 108-46-3), *p*-phenylenediamine, diethyl maleate (CAS no. 141-05-9) and cinnamic alcohol (CAS no. 104-54-1)] and non-skin sensitizers [salicylic acid (CAS no. 69-72-7) and lactic acid (CAS no. 50-21-5)]. Subsequently, we determined the concentration of nanomaterials that cause cytotoxicity threshold of 70% (CV₇₀) in U937 cells. U937 cells were exposed to SWNTs, TiO₂ and C₆₀ for 45 h at 37 °C. Cell viability was determined using propidium iodide (3 µg/ml) staining. U937 cells were exposed to five concentrations of nanomaterials <CV₇₀ or ≤200 µg/mL (the highest concentration allowed) for 45 h at 37 °C. Thereafter, cells were

Accepted Article

washed twice with ice cold buffer (PBS with 5% v/v FBS) and centrifuged at 300xg 4 °C for 5 min. Cells were incubated with FITC-conjugated anti-human CD86 (FUN-1) monoclonal antibody (5 µL) or FITC-isotype control anti-IgG1 (MOPC-21) (5 µL) for 30 min at 4 °C. Cells were washed twice with ice-cold buffer, stained with propidium iodide (3 µg/mL) and analyzed using flow cytometer (BD FACS Canto II, BD Biosciences, San Jose, California). Stimulation index (SI) was calculated following the equation: $SI = (\% \text{ CD86 treated cells} - \% \text{ isotype IgG1 treated cells}) / (\% \text{ CD86 control} - \% \text{ isotype G1 control}) \times 100$. Positive response for skin sensitization was considered when $SI \geq 150\%$. Concentration at which the test material induced SI of 150% (EC_{150}) was obtained through linear regression.

2.6 Inflammatory cytokines quantification

About 5×10^5 cells/well of U-937 cells were seeded in 24-well plates 24 h prior to the assay. Cells were pre-treated with SWNTs, TiO_2 and C_{60} at 100, 150 and 200 µg/mL for 24 h. Subsequently, supernatant was removed and protease inhibitor cocktail (Sigma-Aldrich) was added before storage at -80 °C. Cytokine levels (TNF, IL-1 β , IL-6, IL-8, IL-10 and IL-12p70) were quantified using BD CBA Human Inflammatory Cytokines Kit (BD Biosciences, San Jose, California) in accordance with manufacturer's instructions.

2.7 Data analysis

Data are expressed as mean $\pm$ standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using Graph Pad Prism version 5.0

(GraphPad, San Diego, California). The intergroup variation was measured with one-way ANOVA followed by Tukey multi comparison post-hoc test. Differences were considered statistically significant at $P < .05$.

3. Results

3.1 Characterization of nanomaterials

Nanomaterials were dispersed to obtain homogeneous and stable particles. The CytoViva photomicrographs shows hyperspectral images of SWNTs (Figure 1 A1), TiO₂ (Figure 1 A 2) and C60 (Figure 1 A3). All three nanomaterials exhibited some degree of aggregation, even after sonication in the dispersion media. Resulting nanoparticles for SWNTs showed mean average diameter of 220 nm (Figure 1 A2), while TiO₂ (Figure 1 B2) and C60 (Figure 1 C2) had mean particle sizes of approximately 295 nm and 342 nm, respectively. Additionally, SWNTs and C60 had small size distribution (PdI<0.2), whereas higher PdI of TiO₂ (PDI>0.5) suggested a non-homogeneous aggregation. We observed negative zeta potential for all nanomaterials (SWNTs, -25.9 mV; TiO₂, -27.9 mV; and C60, -29.8 mV), as depicted in Figure 1 (A3, B3 and C3).

3.2 Cellular uptake

We evaluated the internalization of nanomaterials (200 µg/mL) into keratinocyte cell line HaCaT after 2 h of incubation. Dark field microscopy showed the difference in brightness of SWNTs (Figure 2 B), TiO₂ (Figure 2 C) and C₆₀ (Figure 2 D) into HaCaT cells and the non-specific background illumination of non-treated cells (Figure 2 A).

3.3 mDPRA

Potential of SWNTs, TiO₂ and C₆₀ to induce skin sensitization was investigated using mDPRA. The skin-sensitizer *p*-phenylenediamine was used as positive control, the non-sensitizer pyridine was used as negative control. The results found for these controls were in accordance with historical data from the Lab. SWNTs, TiO₂, and C₆₀ were predicted to be moderate skin sensitizers with mean depletion of lysine and cysteine of 32.5%, 28.1% and 28.6%, respectively (Table 2). Furthermore, these nanomaterials induced higher cysteine depletion % as compared to lysine depletion %. Considering that mean depletion was in the range of 22.62% - 42.47%, these nanomaterials were predicted to be skin sensitizers with moderate reactivity class.

Table 2: Peptide reactivity assessment and skin sensitization prediction of SWNTs, TiO₂ and C₆₀ using mDPRA

Test material	Depletion (%)		Mean depletion	Reactivity class	Prediction
	LYS	CYS			
Positive control	24.1 ± 0.5	100 ± 0	62.0	High	Positive
Negative control	8.9 ± 5.2	0.3 ± 0.5	4.6	No or minimal	Negative
SWNTs	7.6 ± 0.3	57.6 ± 0.9	32.6	Moderate	Positive
TiO ₂	5.5 ± 0.2	50.5 ± 0.7	28.1	Moderate	Positive
C ₆₀	12.3 ± 0.2	38.9 ± 0.5	28.6	Moderate	Positive

Data are expressed as mean ± SD from three independent experiments

LYS: Lysine. CYS: Cysteine. Positive control: *p*-phenylenediamine. Negative control: pyridine. SWNTs: single-wall carbon nanotubes; TiO₂: titanium dioxide. C₆₀: fullerene.

3.4 U-SENS

Before the assessment of third KE of skin sensitization through U937 cell line activation test (U-SENS), we performed the proficiency testing using five chemicals known as skin sensitizers (resorcinol, *p*-phenylenediamine and cinnamic alcohol) and non-skin sensitizers (salicylic acid and lactic acid). This assay provided correct identification of skin sensitizer potential of tested reference controls, as shown in Table 3. Similarly to resorcinol and negative controls (salicylic acid and lactic acid), CV₇₀ of SWNTs, TiO₂ and C₆₀ were above 200 µg/mL. Based on calculated values of stimulation index and EC₁₅₀, the tested nanomaterials were classified as skin sensitizers, as depicted in Table 3.

Table 3: Quantification of CD86 expression in U937 cells after exposure to skin sensitizer chemicals, non-skin sensitizer chemicals and nanomaterials and prediction of skin sensitizer potential using U937 cell line activation test (U-SENS)

Test Material <u>Reference chemicals</u>	Solvent	U-SENS results		
		CV ₇₀ (µg/mL)	EC ₁₅₀ (µg/mL)	Prediction
Resorcinol	Medium	>200	23.5	S
<i>p</i> -Phenylenediamine	Medium	>30	12.6	S
Diethyl maleate	DMSO	>20	11.5	S
Cinnamic alcohol	DMSO	>100	59.1	S
Salicylic acid	DMSO	>200	-	NS

Lactic acid	Medium	>200	-	NS
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Nanomaterials

SWNTs	Medium	>200	91.7	S
TiO ₂	Medium	>200	183.1	S
C ₆₀	Medium	>200	189.5	S

Data are expressed as mean $\pm$ SD of three independent experiments
 CV₇₀: concentration of tested material that promotes 70% cell viability. EC₁₅₀: concentration of tested material that induces stimulation index of 150%. S: sensitizer; NS: non-sensitizer. DMSO: dimethylsulfoxide. SWNTs: single-wall carbon nanotubes; TiO₂: titanium dioxide. C₆₀: fullerene.

3.5 Quantification of inflammatory cytokines

The levels of TNF, IL-1 β , IL-6, IL-8, IL-10 and IL-12p70 were quantified in U937 cells after exposure to SWNTs, TiO₂ and C₆₀ at different concentrations (50, 100, 150 and 200 μ g/mL) for 24 h. We observed that exposure of U937 cells to C₆₀ at 50 and 100 μ g/ml significantly ($P<.05$ and $P<.001$) increased IL-8 expression (Figure 3). Although exposure of cells to different concentrations of SWNTs and TiO₂ also increased IL-8 expression, the levels were not significantly different as compared to IL-8 levels of control (untreated cells). We did not observe significant differences in the TNF, IL-1 β , IL-6, IL-10 and IL-12p70 levels of control as compared to the levels expressed by exposed cells (data not shown).

4. Discussion

The wide applications of nanomaterials have followed the nano-revolution in the early 1980's.³ The expanding presence in the environment of such materials has raised

the concerns with the potential health and safety risks following exposure.^{5,6} OECD Testing Programme of Manufacture Nanomaterials comprises safety assessment of specific manufactured nanomaterials along with the applicability of existing OECD TG and validation of new OECD TG.^{7,8} Despite OECD work to address human health and environment safety concerns associated with nanomaterials, the current knowledge of their related adverse effects is still limited. Additionally, characterization of immunomodulatory effects after skin exposure to nanomaterials have been neglected once it was thought that skin penetration of such materials would be minimal.² In the present study, the investigation of immunotoxicity of widely used nanomaterials (SWNTs, TiO₂ and C₆₀) showed the ability of these nanomaterials to internalize HaCaT keratinocytes, potential to interact with skin proteins and trigger activation of U937 cells through CD86 expression and modulation of inflammatory cytokine production.

Recently, there is an increasing number of studies investigating skin penetration by nanomaterials using *in vitro* and *in vivo* models,⁵⁻⁹ but only a few reports dedicated to investigate penetration of nanomaterials into human skin.^{10,11} However, the ability of nanomaterials to overcome the skin barriers and reach deep layers of skin remains controversial. Most of studies reported that skin layers preclude the penetration of nanomaterials deeply into epidermis and dermis.^{6,8,12,13} While dermal permeation of small amounts of nanomaterials were detected by Gulson et al.^{10,11,14} The latter indicated that nanomaterial composition and size, repeated applications, methods for analyzing the samples and integrity of skin are included as critical factors that may influence nanoparticle penetration. Considering that the scientific evidence of human skin

permeation by nanomaterials are still poor, health concerns should be only raised when detailed investigations confirm dermal absorption of nanoparticles.

Determination of nanomaterial characteristics such as particle size, surface charge, morphology, are of major importance since they influence nanomaterial properties and biological activities.¹⁸ SWNTs, TiO₂ and C₆₀ displayed uniform particle size and negative surface charge, which were in good correlation with findings from reports studying the same nanomaterials.^{19–21} Negatively charged nanoparticles has shown particular role in immunomodulating effects as well as inflammatory response after systemic administration.^{22–24} To understand further the role of nanomaterials in skin sensitization, initially we observed the uptake of SWNTs, TiO₂ and C₆₀ into HaCaT keratinocytes. Hyperspectral images confirmed the presence of nanomaterials in the cytoplasm of the cells.

OECD No. 256 establishes twelve different integrated testing strategy (ITS) to identify and classify skin sensitizers using *in silico* and *in vitro* methods focused on the four AOP KEs of skin sensitization, such as “2-out-of-3” and sequential testing strategies based on KEs 1-3 and KE-1 and 3, respectively. These approaches allow a broad evaluation of exposure, skin penetration, metabolism and key events by applying the skin sensitization AOP framework.²⁵ In addition, they have recently acquired regulatory acceptance and/or applicability in the weight-of-evidence for the next-generation risk evaluation for skin sensitization¹³. This study determined the skin sensitization potential of SWNTs, TiO₂ and C₆₀ covering the first and third KEs of skin sensitization AOP. Considering that activation of skin proteins represents an initial event in skin

sensitization, the induction of first KE by nanomaterials was investigated using mDPRA, which classified the test nanomaterials as skin sensitizers with moderate reactivity class. Additionally, we observed enhanced reactivity of SWNTs, TiO₂ and C₆₀ to Cys as compared to Lys peptides. Studies focused on characterization of materials with skin sensitization potential has reported the tendency to Cys depletion by sensitizing agents.^{15,26–28}

Following the investigation of skin sensitization potential of SWNTs, TiO₂ and C₆₀, we evaluated the activation of dendritic cells (KE-3) through quantification in U937 cell line of a specific cell marker (CD86) and pro inflammatory cytokines, which are associated with the process of activation of cells after exposure to sensitizers.^{29,30} Both experiments indicated the potential of nanomaterials as skin sensitizers. Combination of our results from mDPRA, CD86 expression and released IL-8 analyses suggested the classification of nanomaterials as skin sensitizers. This finding supports earlier reports showing the skin sensitization potential of SWNTs assessed through *in vitro* (engineered skin and keratinocytes) and *in vivo* methods.^{20,23,31}

Published studies have suggested the potential of TiO₂ to modulate immune response induced by a skin sensitizer.^{32,33} Smulders et al. observed that TiO₂ could enhance skin sensitization potential of chemical sensitizers as soon as they penetrate the skin.¹⁹ However, studies with TiO₂ alone could not demonstrate their skin sensitization potential.^{32,34} Differently than our work, those studies have performed the evaluation of skin sensitization potential of TiO₂ using *in vivo* LLNA. Conversely, subchronic dermal exposure of hairless mice and porcine skin to TiO₂ nanoparticles suggested that they

represent a human health risk after long time dermal exposure.³⁵ The authors reported that these nanoparticles were able to penetrate the skin and caused damage in various tissues, mainly in skin and liver probably due to oxidative stress.

In contrast to our findings with C₆₀, studies carried out in guinea pigs following the OECD 406 suggested that fullerene nanoparticles are not skin sensitizers.³⁶ Although animal methods have been traditionally used for most risk assessment, a combination of non-animal methods has shown high predictive capacity. All available methods have limitations and substantial efforts have been made for appropriate replacement of animal methods by non-animal methods.³⁷ Therefore, it is still needed a detailed investigation of dermal toxicity, immunomodulatory effects and allergenic potential of nanomaterials. We highlight the relevance of validated non-animal methods to classify the skin sensitization potential of nanomaterials and this study is contributing towards this new scenario.

We investigated single-wall carbon nanotubes, titanium dioxide and fullerene nanomaterials with homogenous particle sizes and negative zeta potential. The nanomaterials interacted with HaCaT keratinocytes and efficiently internalized into the cells. The investigation of skin sensitization potential of nanomaterial through mDPRA, U-SENSTM assays and quantification of proinflammatory cytokines classified the tested nanomaterials as skin sensitizers. Although the present study reports the immunotoxicity and skin sensitization potential of SWNTs, TiO₂ and C₆₀, more studies are needed to improve our understanding of the health implications of exposure to nanomaterials. Additionally, it represents a contribution towards the applicability of existent OECD TG for accurate assessment of nanomaterial skin sensitization potential.

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- Accepted Article
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Figure Legends

Figure 1: Characterization of nanomaterials: CytoViva darkfield images, hydrodynamic size and zeta potential of SWNTs (A1, A2 and A3, respectively), TiO₂ (B1, B2 and B3, respectively) and C₆₀ (C1, C2 and C3, respectively). Scale bar: 10 μ m.

Figure 2: CytoViva hyperspectral imaging of (B) SWNTs, (C) TiO₂ and (D) C₆₀ internalized into human HaCaT keratinocytes after 2 h of exposure. (A) Control cells with non-specific background illumination. Scale bar: 3 μ m.

Figure 3: IL-8 expression in U937 cells after 24 h exposure to (A) SWNTs, (B) TiO₂ and (C) C₆₀ at different concentrations (50, 100, 150 and 200 μ g/ml). Data are expressed as mean $\pm$ SD from three independent experiments. Statistically significant differences shown as (*) for $P < .05$ and (**) for $P < .001$.





