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Research Article

Effect of Size, Shape, and Composition on the Interaction of Different Nanomaterials with HeLa Cells

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The application of nanomaterials in the fields of medicine and biotechnology is of enormous interest, particularly in the areas where traditional solutions have failed. Unfortunately, there is very little information on how to optimize the preparation of nanomaterials for their use in cell culture and on the effects that these can trigger on standard cellular systems. These data are pivotal in nanobiotechnology for the development of different applications and to evaluate/compare the cytotoxicity among the different nanomaterials or studies. The lack of information drives many laboratories to waste resources performing redundant comparative tests that often lead to partial answers due to differences in (i) the nature of the start-up material, (ii) the preparation, (iii) functionalization, (iv) resuspension, (v) the stability/dose of the nanomaterial, etc. These variations in addition to the different analytical systems contribute to the artefactual interpretation of the effects of nanomaterials and to inconsistent conclusions between different laboratories. Here, we present a brief review of a wide range of nanomaterials (nanotubes, various nanoparticles, graphene oxide, and liposomes) with HeLa cells as a reference cellular system. These human cells, widely used as cellular models for many studies, represent a reference system for comparative studies between different nanomaterials or conditions and, in the last term, between different laboratories.

1. Introduction

Nanomaterials offer revolutionary solutions to traditional problems, and thus, they have been incorporated into many different consumer goods including many for human consumption such as cosmetics, biotechnological and pharmacological products, medicines, or food additives. Unfortunately, major developments are never exempt of associated problems. Nanomaterials have been connected with all types of toxicological, cumulative, or environmental problems [1–6]. However, the reality is that during evolution, vegetable and animal species have been exposed to environmentally generated nanomaterials, and this has resulted in the appearance of natural resistances. The issue to be faced now is to understand and control the effect of the many different anthropogenic nanomaterials currently in use. Society needs to know the implications of the use of the different nanomaterials in everyday products. But to find out the possible side effects of the exposure for each nanomaterial, it is necessary to establish a series of *in vitro* and *in vivo* objective tests.

Cellular models are very convenient because they do not require complex laboratory facilities and can provide pivotal information on the toxicological effects of nanomaterials; furthermore, nowadays, there are many cellular models to investigate these interactions with nanomaterials. In fact, many studies have been carried out using cells of different origins and diverse natures. However, this poorly protocolised research has resulted in the production of confusing

and incoherent data that result in chaos when it comes to understanding and comparing the effect of a particular nanomaterial at the same dose in a unique cellular system.

In our laboratories, we have been studying the *in vitro* effects of several types of nanomaterials for almost a decade. This has offered us a broad critical view on the effects produced by many of these compounds in different cellular systems. We now know that the same nanomaterial can produce different cytotoxic effects in different cells, depending on the nature and origin of the cell [7]. For example, macrophages generally suffer more cumulative or degradative effects-i.e., reactive oxygen species (ROS) accumulation-than other cells for the same material at the same dose due to their high avidity for capturing nanomaterials [8–13]. On the contrary, malignant melanoma or glioblastoma multiforme cells have great resistance to cytotoxicity for most nanomaterials [7, 14, 15]. Also, some cells have a special idiosyncrasy that makes them tolerant to certain types of nanomaterials, such as neurons. These cells can survive multiwalled carbon nanotube exposure better than macrophages but are much more sensitive to any other nanomaterials [8, 16–18].

There are many examples in the literature where cells exposed to the same nanomaterial respond differently. This is the case of carbon nanotubes where the reported effects range from innocuity to very acute toxicological or long-term accumulative effects [19–24]. Moreover, in the case of carbon nanotubes, there are different cellular phenotypes, after exposure, depending on the type of the used nanotubes. For example, single-walled carbon nanotubes (SWCNTs) seem to interact more with DNA [18, 25, 26] than multiwalled carbon nanotubes (MWCNTs) that display biomimetic properties with the cytoskeleton [9, 15, 19, 27]. This fact reveals the unique features of each nanomaterial, for example in this case, carbon nanotubes, the importance of the diameter of the tube in the interaction with different biological filaments such as DNA (2 nm), intermediate filaments (10-15 nm), microtubules (25 nm), or actin (4-8 nm).

For all these reasons, it seems necessary to carry out standard tests where a single cell type is exposed to the same concentration of different nanomaterials, functionalized, and processed identically, following the same protocol. This test allows the direct comparison of the effect of the different nanomaterials on a unique system *in vitro*. For this study, we have chosen HeLa cells as a reference cellular system. This is a human epithelial cell line originally obtained from a cervical carcinoma that is universally employed as a reference cellular model to test numerous toxic products, among them, nanomaterials. One of the great advantages of using this cell line is that its genome and proteome are known in great detail [28–30], and there are numerous studies performed in many laboratories that provide extraordinary experimental support for these analyses.

On the other hand, this cell line has been used in many laboratories on the assumption that it comprises putatively homogeneous clonal cell population. However, recent studies demonstrate that HeLa cells are heterogeneous [31]. This fact means that the results between different laboratories are not always directly comparable or reproducible, reinforcing the idea that a standard protocol must be carried out in the same cell line-from the same laboratory-to test different nanomaterials processed in identical conditions. In this study, we investigate the effect of identically prepared (functionalization/dose) nanomaterials. These were incubated for equal periods of time and were analysed following the same protocol for direct comparison of their effect on these cells, thus to establish a comparison of their exposure phenotypes on this unique cell model.

2. Materials and Methods

2.1. Nanomaterials. Different nanomaterials were used in this work. High-purity MWCNTs were obtained from Nanocyl NC3100™. These nanotubes have been fully characterized in previous publications [15]. Monodisperse silica spheres (500 nm) were prepared using the Stöber method as described in our previous work [32]. These silica particles were coated with carbon nanotubes as detailed elsewhere [33]. Cationic liposomes (CLPs) were commercially obtained from Nanovex Biotechnologies SL and prepared from basic components by a hydration process. All liposomes showed a narrow size distribution with mean particle sizes of 150-200 nm and polydispersity indexes of less than 0.4. TiO_2 and ZnO nanoparticles (Z-COTE®) were commercial (BASF Chemical Company). ZnO:Co^{2+} nanowires were synthesised in-house [34–36]. Chemically exfoliated graphene oxide (GO) was purchased commercially (Graphenea, Spain). Morphological characterization of the nanomaterials was performed using a JEOL JEM 1011 transmission electron microscope (TEM) operating at 100 kV. Nanomaterials were suspended in ethanol and adsorbed onto 400 mesh carbon-coated copper grids.

2.2. Functionalization of Nanomaterials. Nanomaterials were resuspended and functionalized in a saline solution containing 30% fetal bovine serum (FBS, Gibco) by mild probe sonication (3-5 cycles, 2-5″ at a frequency of 20 kHz) in a SONICS Vibra-Cell VCX130 Ultrasonic Processor (Sonics & Materials Inc.), before resuspension in cell culture medium and addition to the cell cultures.

2.3. Cell Culture, Cycle and Viability Tests, Staining, Immunofluorescence, and Imaging. HeLa cells (from the European Molecular Biology Laboratory Cell Bank, passage 10) were cultured under standard conditions in Minimum Essential Medium (MEM) containing 10% FBS and antibiotics (Gibco, Thermo Fisher Scientific). Phase contrast micrographs were taken at different time points using a Progress CT5 (Jenoptik) digital camera coupled to a Nikon Eclipse TS100-F. Cell viability assessments were performed using a standard trypan blue assay. The cell cycle distribution was analysed by flow cytometry using a Muse® Cell Analyzer (Merck KGaA) following the manufacturer's instructions. Immunostaining was performed on cells fixed in 4% paraformaldehyde. Phalloidin-tetramethylrhodamine B isothiocyanate, Hoechst dye (bisbenzimide), and Acridine Orange hemi (zinc chloride) salt (all from Sigma-Aldrich) were used to stain actin, DNA, and cytoplasm, respectively. Microtubules were immunostained with the B512

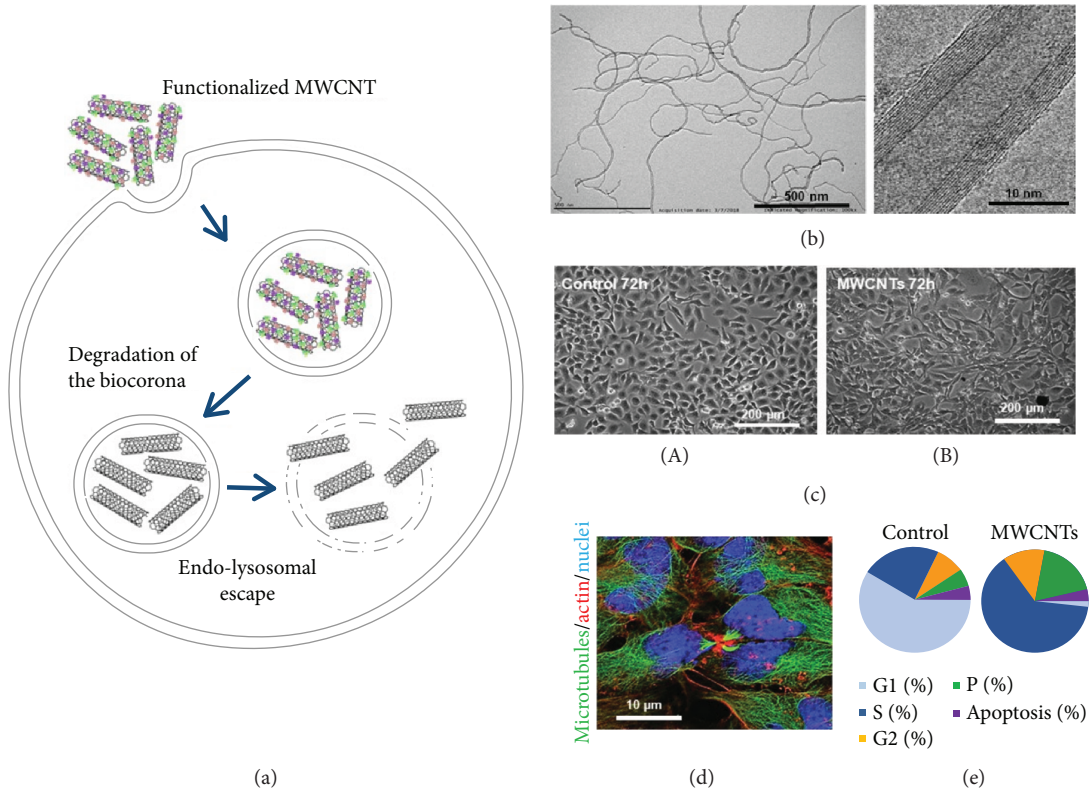

Figure 1: Multiwalled carbon nanotube (MWCNT) interaction with HeLa cells. (a) Diagram of their internalization and endo-lysosomal escape. (b) Low- and high-resolution TEM images of MWCNTs. (c) Phase contrast images of the control and 72 h MWCNT-exposed HeLa cells. (d) Asymmetric triple mitosis representative of the biomechanical defects triggered by MWCNTs in the microtubule cytoskeletal machinery. (e) MWCNTs treatment resulted in a drop in the number of cells at the G1 stage (nondividing cells, represented in light blue) and a rise of cells at S (DNA synthesis), G2 stage (mitotic), polyploidy (P, aberrant genomic load), and apoptosis. Changes in the cell cycle after 72 h incubation with MWCNTs are indicative of cell cycle blockage.

anti-α-tubulin antibody (Sigma-Aldrich) and a secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen). Confocal microscopy images were performed with a Nikon A1R confocal microscope and were processed with the NIS-Elements Advanced Research software. All confocal cell images are pseudocoloured.

3. Results and Discussion

3.1. Carbon Nanotubes. Carbon nanotubes (CNTs) represent a class of highly versatile materials that display very interesting mechanical, thermal, electronic, and biological properties [37]. These nanomaterials have been broadly used in numerous *in vitro* and *in vivo* toxicity studies, and there is a lot of documentation regarding their effects. However, there is some confusion in the literature that we think could be due to the purity, the surface treatment, or the morphological properties of the nanotubes, among others. Here, we investigate the effect of MWCNTs on HeLa cells incubated with 50 μg/ml. Figure 1(b) shows the characterization of these nanomaterials by TEM and the different steps of the internalization in these cells. For their use, MWCNTs are functionalized with serum proteins to trigger receptor-mediated endocytosis (Figure 1(a)) [38–40]. Figure 1(c) (B) shows HeLa cells incubated with 50 μg/ml of MWCNTs for 72 h. Previous studies report how MWCNTs have a high affinity for the cellular cytoskeleton [9, 21, 41], causing detectable morphological changes and alterations in the biomechanics of HeLa cells which results in slower migration rates [15, 19], proliferative blockage, and, depending on the dose/exposure time, genomic instability and cytotoxic effects (Figure 1(d)) [7, 42–44]. In this study, HeLa cells exposed to nanotubes display an elongated morphology (Figure 1(c)), abnormal mitotic figures (Figure 1(d)), and aberrant cell cycles (Figure 1(e)) where we can see an increased S and/or G2 phases, depending on the incubation dose/times. Confocal microscopy examination of MWCNT-treated cell cultures confirmed indicative signs of the biomechanical and disruptive effects of these nanotubes on HeLa cells, including (i) cell retraction, (ii) membrane blebbing, (iii) nuclear DNA compaction, and (iv) the presence of micronuclei, as well as other previously described cytoskeletal changes including disorganized microtubular patterns or a reactive cortical actin [14]. These morphological changes and cytotoxic effects were corroborated by flow cytometry (Figure 1(e)), showing a patent blockage in the S phase of the cell cycle, suggesting MWCNT interference with DNA replication.

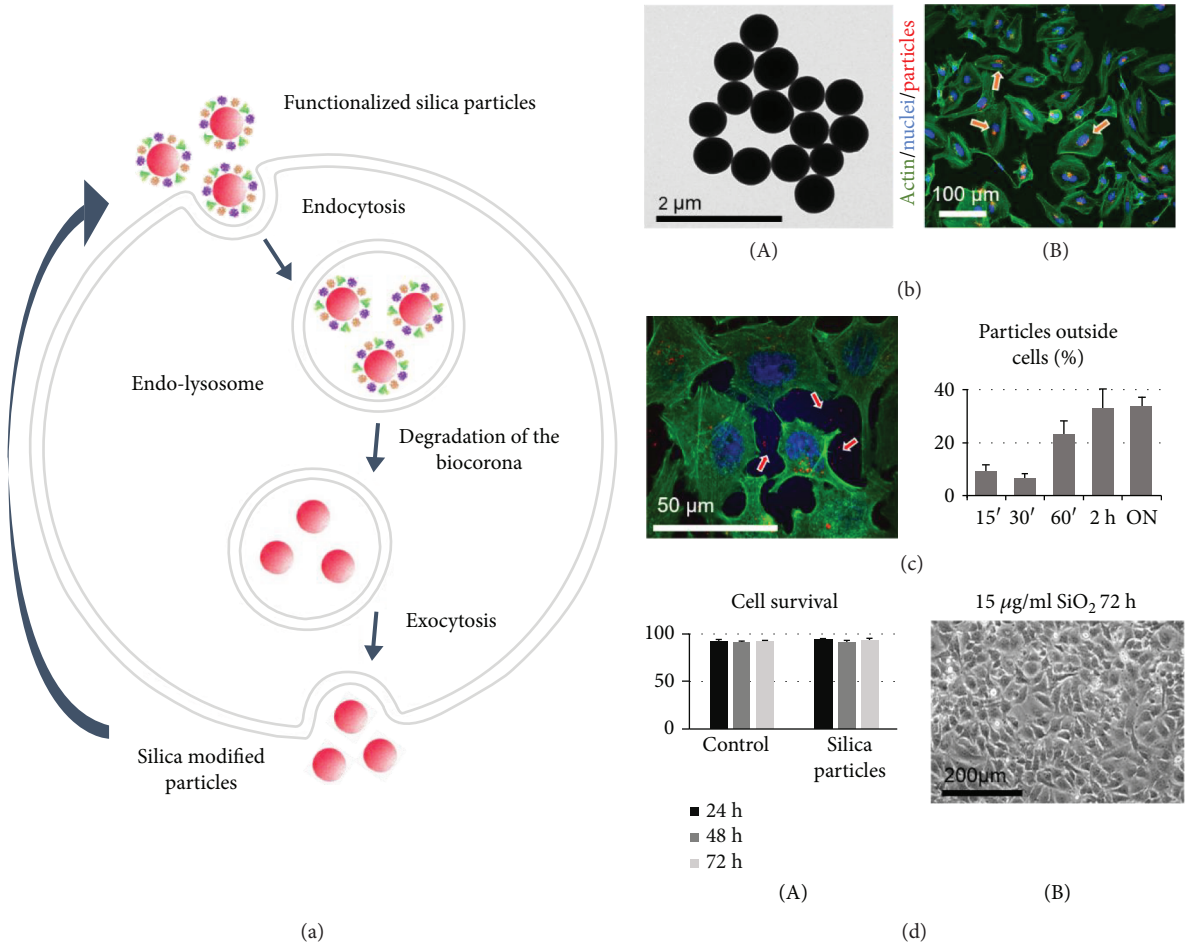

Figure 2: Silica particle interaction with HeLa cells. (a) Schematic diagram of silica particles processing in HeLa cells. Particles are internalized in cells via endocytosis. In endo-lysosomes, their biocorona is degraded, and stripped particles are finally exocytosed. ((b), A) TEM characterization of the as-prepared silica particles. ((b), B) Confocal image demonstrating silica particles inside HeLa cells. (c) Confocal image where the exocytosed silica particles are detected (red arrows). The accompanying histogram shows the percentage of extracellular particles after 15′, 30′, 1 h, 2 h, and overnight. ((d), A) Percentage of live cells at different exposure times. ((d), B) Phase contrast image of HeLa cells presenting no detectable cytotoxicity.

3.2. Silica Particles. Silica particles are traditionally considered to be quite biocompatible and have been used as a therapy delivery system due to their interesting physico-chemical properties [45]. Figure 2 compilates the characteristics of these particles and several aspects of their relationship with the HeLa cells used in this study. These particles of ca. 500 nm diameter size, when functionalized with serum proteins, are receptor-mediated endocytosed. Figure 2(b) (B) shows the trajectory of these particles inside HeLa cells: particles are rapidly internalized by HeLa cells after overnight exposure. In the endo-lysosomal route, the silica particle biocorona proteins are degraded by the local lysosomal proteases. Protein-stripped silica particles are exocytosed from these cells a few hours after engulfment (see Figure 2(c)) [33]. These exocytosed silica particles can be reendocytosed after adsorbing other proteins from the surrounding culture medium on their surfaces as part of the biocorona. Previous studies demonstrate a constant concentration of approximately 30% of the total particles in the culture outside the cells [32]. As shown in Figure 2(d), silica particles are highly biocompatible at 50 μg/ml, and no significant changes in the morphology of the cells after 72 h of exposure were appreciated.

3.3. CNT-Coated Silica Particles. Carbon nanotubes can be used to coat silica particles to trigger the lysosomal exit imitating viral escape mechanisms [33, 46]. Figure 3 shows a summary of the results obtained after the exposure of HeLa cells to silica particles coated with MWCNTs. Since the carbon nanotubes of the coating are functionalized with serum proteins, nanotube-coated silica particles also enter cells via endocytosis (Figure 3(c), A). Once inside the endo-lysosome, the local proteases degrade the coating proteins, stripping the carbon nanotubes that then interact with the lysosomal membrane (Figure 3(c), B), tearing the vesicle apart and escaping into the cytosol [33]. These CNT-coated silica particles do not trigger detectable cytotoxicity either (Figure 3(d)) at a final concentration of 50 μg/ml [33, 46].

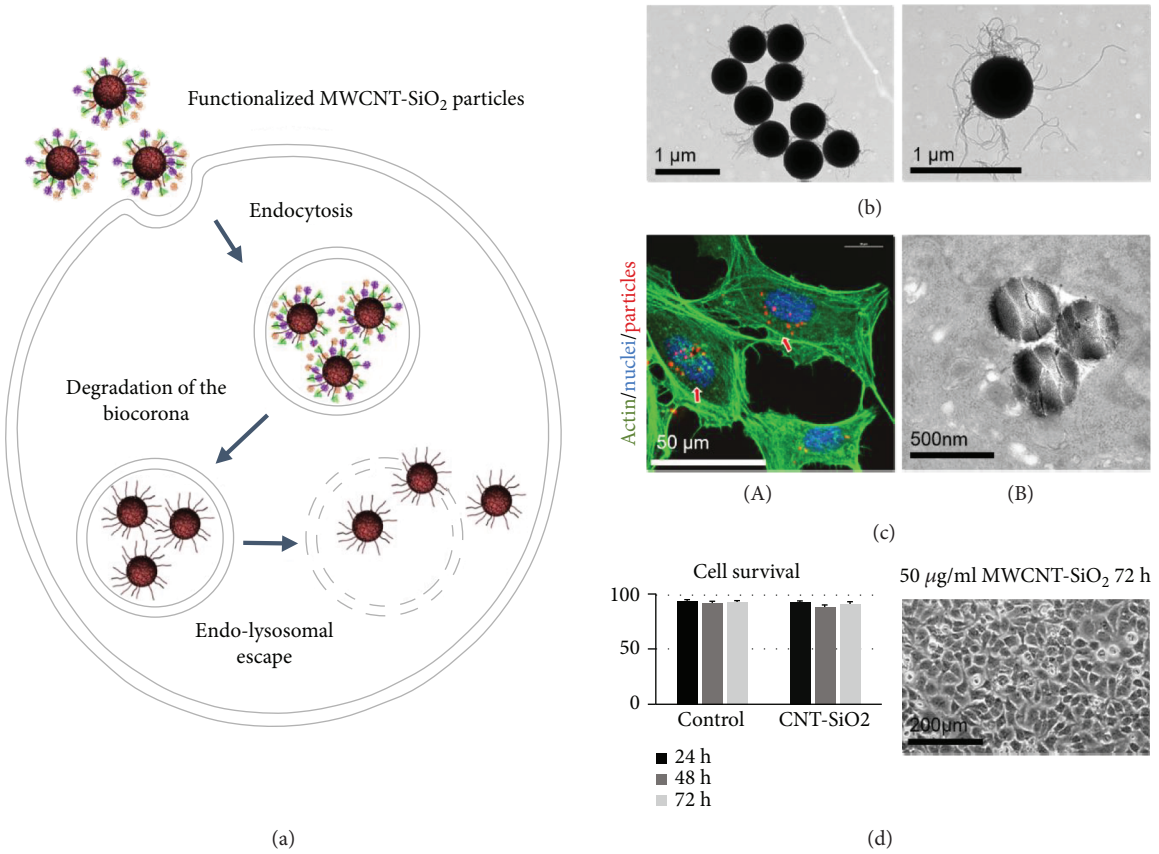

Figure 3: CNT-silica particle interaction with HeLa cells. (a) Schematic diagram of CNT-silica particle trajectory in HeLa cells. Once these particles are internalized via endocytosis, the CNT biocorona is degraded. Stripped nanotubes interact with the lysosomal membrane and escape the endo-lysosomal compartment. (b) TEM images of some representative CNT-silica particles. ((c), A) Confocal microscopy images demonstrating internalized particles in HeLa cells (red arrows). ((c), B) TEM micrograph of a section of a HeLa cell cytoplasm where the CNTs of the coating are observed piercing the endo-lysosomal membrane. ((d), A) Percentage of live cells after 24 h, 48 h, and 72 h of exposure to 50 μg/ml of the CNT-coated particles. ((d), B) Phase contrast image of HeLa cells displaying no detectable cytotoxic changes.

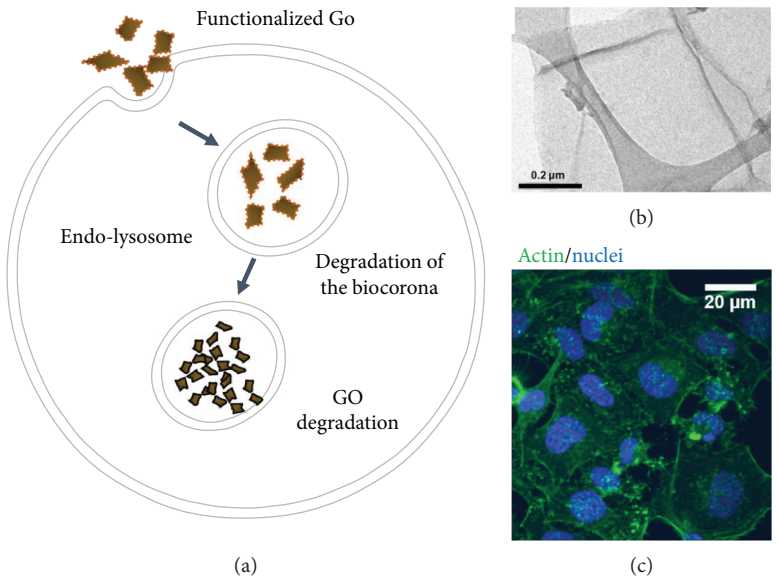

Figure 4: GO interaction with HeLa cells. (a) Schematic diagram of functionalized GO contacting with cellular surface receptors and invading the cell via endocytosis. Internalized GO flakes are progressively degraded in lysosomes. (b) TEM characterization of GO flakes. (c) Confocal microscopy image of HeLa cells exposed to 50 μg/ml functionalized GO flakes displaying a normal morphology after 72 h exposure.

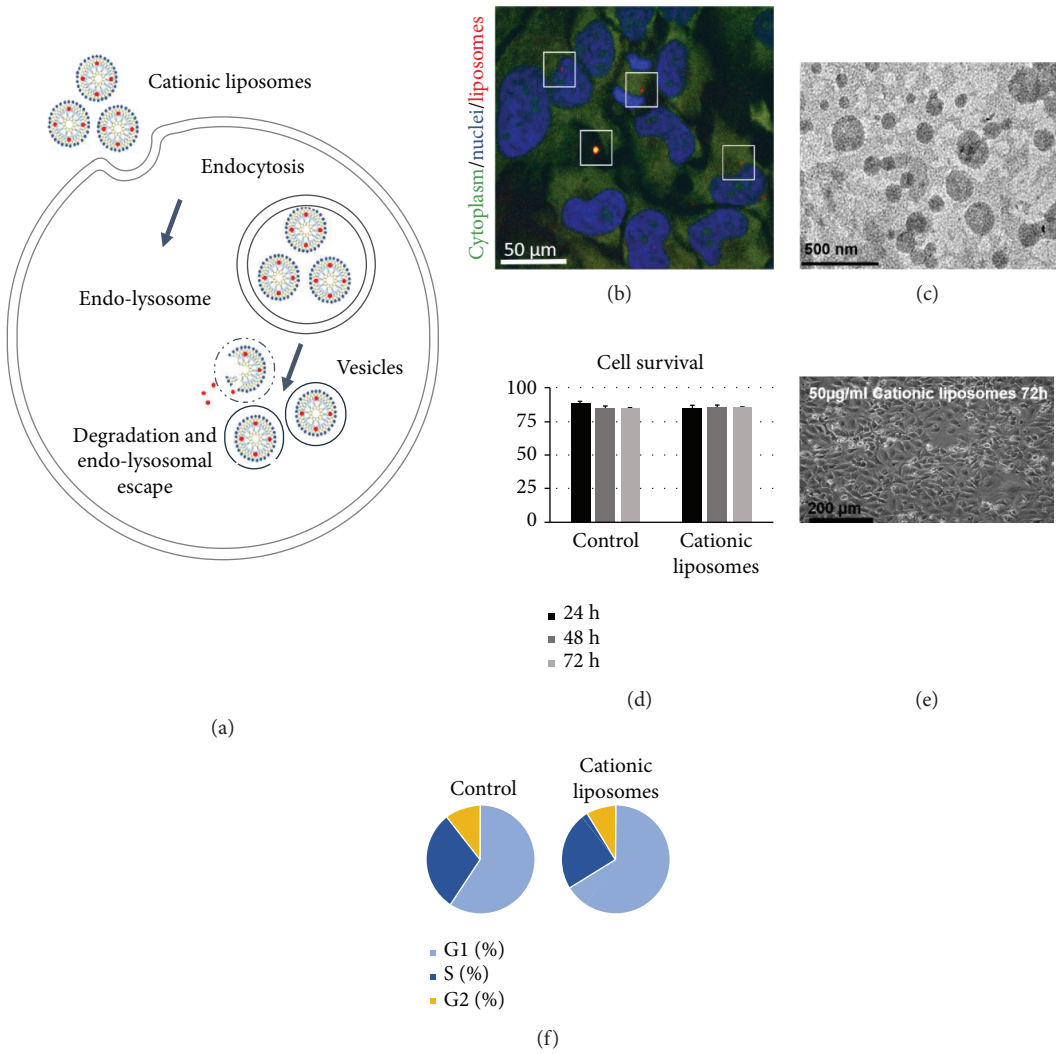

Figure 5: Cationic liposome interaction with HeLa cells. (a) Graphic illustration of the intracellular cycle of cationic liposomes in HeLa cells. (b) Confocal microscopy image of HeLa cells exposed to DiI-labelled cationic liposomes (red channel, in boxes). (c) TEM image of the as-prepared cationic liposomes. (d) HeLa cell viability after cationic liposome exposure. (e) Phase contrast image of HeLa cells exposed to the cationic liposomes for 72 h. (f) Comparative study of the cell cycle between HeLa cells with CLP by flow cytometry.

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3.4. Graphene Oxide (GO) Flakes. Graphene oxide is a nanomaterial that, up to date, has been reported to be quite biocompatible in different systems *in vitro* and *in vivo*. The GO composition, the flat morphology, and the oxidation of the structure result in a very biocompatible element. Oxidised graphene allotropes have been shown to be degradable by lysosomal enzymes, being much more biocompatible than the nonoxidised counterpart nanostructures [47–50]. Oxidation produces small enzymatic attack points on the graphene layers that favour degradation by the lysosomal enzymes. Figure 4 shows a summary of the intracellular entry and trajectory of GO in HeLa cells. GO produces little detectable morphological or biomechanical changes at the concentration of 50 μg/ml. Figure 4(b) shows a TEM micrograph of one of the GO sheets used in this study. As with carbon nanotubes, GO is functionalized by serum proteins absorbed on the surface. This protein corona triggers receptor-mediated cellular entry. Unlike for carbon nanotubes, no interaction phenomena of GO with intracellular filaments such as DNA, microtubules, or actin are observed or reported so far (Figure 4(c)).

3.5. Liposomes. Liposomes are widely used as delivery systems to transfer drugs, proteins, or nucleic acids into target cells. These nanovesicles made up of a lipid bilayer can encapsulate different types of therapies into an inner aqueous phase or lipid bilayer and are considered, in general, very biocompatible nanostructures [51–53]. To improve the carrier efficiency, it is necessary to understand the mechanism of uptake into cells and the release of the therapy in the cytoplasm of the target cell, and both depend on the composition of the lipocarrier. In our study, liposomes are functionalized in 30% serum, just like the other nanomaterials. These particular types of commercial cationic liposomes (see Materials and Methods) are captured by endocytosis, after interacting with the cell surface receptors (Figure 5(a)). As a general rule, this process is strongly influenced by the nature and density of the charge of the liposomes. Figure 5 depicts some

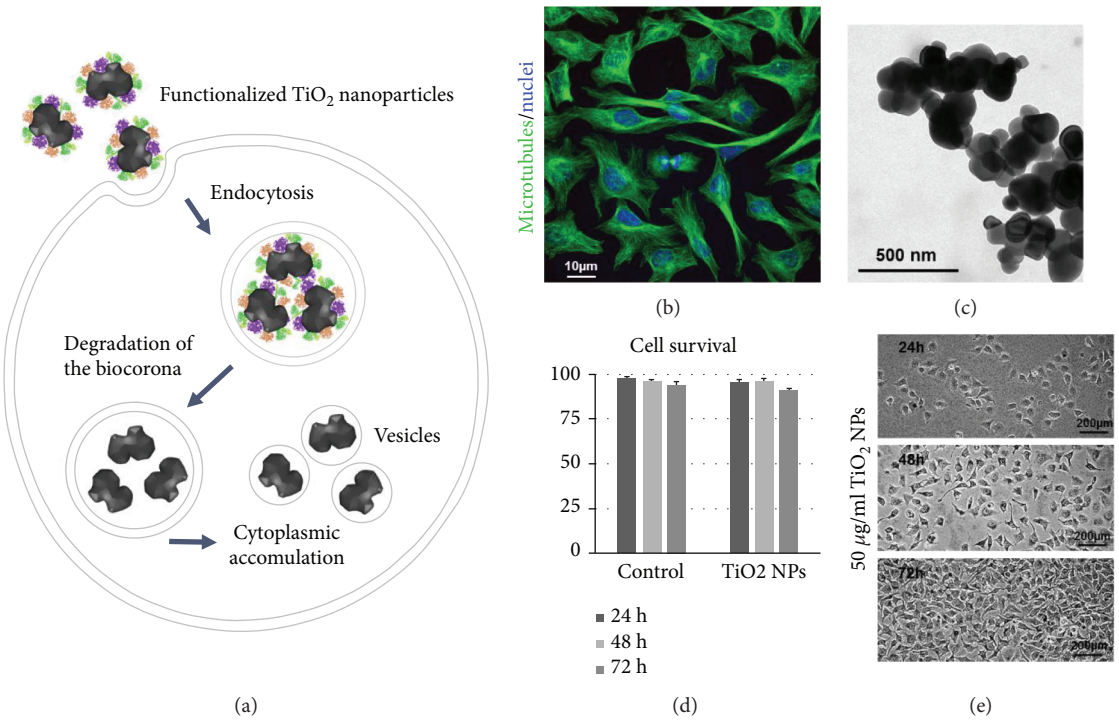

Figure 6: Titanium oxide nanoparticle interaction with HeLa cells. (a) Graphic illustration of the intracellular trajectory of TiO_2 nanoparticles in HeLa cells. (b) Confocal microscopy images of HeLa cells exposed to TiO_2 nanoparticles where no morphological changes are observed. (c) TEM images of the pristine TiO_2 nanoparticles. (d) Cell survival after exposure to 50 μg/ml of TiO_2 nanoparticles. (e) Phase contrast images of HeLa cells exposed to TiO_2 nanoparticles during 24, 48, and 72 h.

characteristic data of these liposomes and their interaction with HeLa cells. Cultures were incubated with the liposomes resuspended in MEM medium for 72 h at 37°C. Figure 5(b) (boxes) shows a few DiI-labelled cationic liposomes inside HeLa cells. This particular type of liposomes did not trigger any observable morphological changes in HeLa cells. Neither changes were observed in the cell cycle or viability of HeLa cells after 72 h incubation with these nanomaterials (Figures 5(d) and 5(f)).

3.6. TiO_2 Nanoparticles. Titanium dioxide (TiO_2) nanoparticles are some of the most commonly manufactured nanomaterials that are extensively used as components of paints, cosmetics, food, and many other consumer products [1, 54]. Due to their physical and chemical properties, traditionally, TiO_2 nanoparticles have been considered as a low-toxicity; in fact, they are often used as negative controls in several studies [55]. However, there are increasing evidences in *in vitro* studies suggesting that they can induce oxidative stress and genotoxicity upon UVA exposure [56]. In our standard assay, HeLa cells exposed to high doses (50 μg/ml) of TiO_2 nanoparticles display no observable effects [57]. Figure 6 shows a summary of the findings in HeLa cells exposed to serum-functionalized TiO_2. Confocal microscopy images of HeLa cells show a normal distribution of microtubules without any evidence of alteration in cell morphology (Figure 6(b)). Neither were there detected abnormalities in the cell cycle nor increased cell death after exposure to these particles, visible in vesicles in the cellular cytoplasm 72 h after exposure [57–60]. Furthermore, some TiO_2 nanoparticle aggregates were observed in the centrosomal region of the cells, as previously reported [61]. Interestingly, these nanoparticles can remain encapsulated in membranes for long periods of time (24-48 h) without any evidence of their exocytosis [60]. In general, most studies carried out in HeLa support the hypothesis that TiO_2 nanoparticles are highly biocompatible under standard culture conditions.

3.7. ZnO Nanoparticles and Nanowires. HeLa cells that are subjected to treatment with ZnO display a very acute phenotype of cytotoxicity due to the dissolution of the ZnO nanoparticles inside the lysosomes [57, 62–64]. Cells need very small amounts of zinc, and that is why they have exquisite membrane systems to control the entrance of films into the cytoplasm. When ZnO nanoparticles are incubated in medium containing serum proteins, they acquire a biocorona that the cell recognizes through its membrane receptors triggering receptor-mediated endocytosis. Once in the endosome, the pH of these vesicles decrease and ZnO nanoparticles dissolve, releasing massive amounts of zinc ions in the vesicle that invade the cell cytoplasm. Within the cellular cytoplasm, there is a series of proteins that captures zinc transiently functioning as an intracellular buffering system (Figure 7(a)). Two of these proteins are actin and tubulin. The microtubules, built of nanotubes of tubulin units, are transformed into sharp sheets of tubulin upon zinc incorporation in their structure [57]. This causes the hardening and thickening of the microtubules that behave like "daggers" perforating

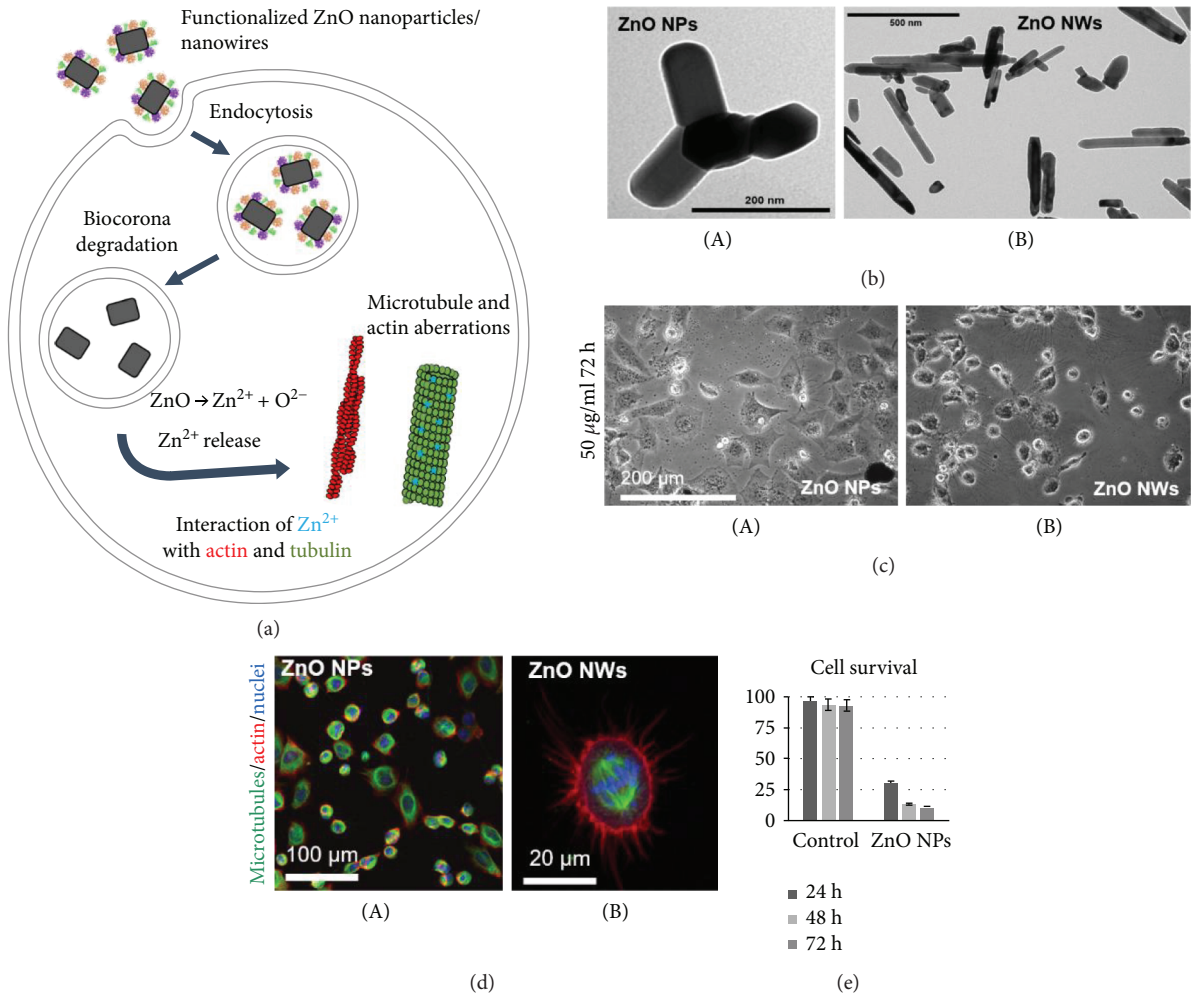

Figure 7: Interaction of ZnO nanoparticles and nanowires with HeLa cells. (a) Graphic illustration of the intracellular cycle of ZnO nanomaterials in HeLa cells. (b) TEM characterization of ZnO nanoparticles (A) and nanowires (B). (c) Phase contrast images of HeLa cells exposed to 50 μg/ml of ZnO nanoparticles (A) and nanowires (B) during 72 h. (d) Confocal microscopy images demonstrating the cytotoxic effects of ZnO nanoparticles (A) and nanowires (B) of HeLa cells. Cytoskeletal abnormalities are clearly visible. (e) HeLa cell viability after ZnO nanoparticle exposure.

the cell membrane causing immediate cell necrosis. This effect can be seen in the immunofluorescence image from Figure 7(d) (A).

ZnO:Co^{2+} nanowires caused very similar effect to ZnO nanoparticles despite the different morphology and composition. These nanomaterials functionalized with serum proteins interact with membrane receptors, trigger endocytosis, and finally, dissolve in the lysosomes virtually identical to ZnO nanoparticles [57, 65]. As observed for the ZnO nanoparticles, these nanowires produced changes in the microtubule and actin cytoskeletons (Figure 7(a)), stabilizing both cytoskeletal polymers and producing necrotic changes derived from the perforation of the cell membrane by the microtubules (Figure 7(d), B).

4. Conclusions

Our work shows how the toxicological effects of nanomaterials can result from the morphology of the nanomaterials and/or their composition. In this review, we report several cases that illustrate these behaviours. In the case of carbon nanotubes and GO, it is the morphology rather than the composition factor that triggers the cytotoxic response in the HeLa cells. However, this is not a universal dogma, for ZnO-based nanomaterials, morphology is less important than chemistry, and it is its composition and their chemical properties which trigger the cytotoxic effect in that case. Also, this work demonstrates how nanomaterials can produce unpredictable consequences in human HeLa cells; even if we can know the composition and morphology of nanomaterials, a complete cytotoxic study should be performed in each case.

Our results show that although all employed nanomaterials interact with cells by receptor-mediated endocytosis, the route that they follow once inside the cell is not the same. As we can see, our study reinforces the idea that it is necessary to develop specific tests for each nanomaterial, since it is not possible to anticipate the cytotoxic effects and/or the interaction with cells and tissues.

This exhaustive study constitutes an extraordinary tool for the modelling of more complex structures, incorporating materials endowed with magnetic, optical, or catalytic functionalities. In fact, the carbon nanotubes provide a high surface, which makes easier the adsorption of many different ligands, together with other porous or hollow materials like mesoporous SiO_2 or liposomes. Also, we can take advantage of other materials that are innocuous to these cells like TiO_2 in order to improve the biostability. This can increase the applications of these nanomaterials as drug delivery systems, therapy or diagnostic.

Nanomedicine and in particular the study of these interactions between nanomaterials and biological systems is a field in constant development and evolution that changes every day making the designs and the possibilities almost endless.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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