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Biological cellular response to carbon nanoparticle toxicity

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Abstract

Recent advances in nanotechnology have increased the development and production of many new nanomaterials with unique characteristics for industrial and biomedical uses. The size of these new nanoparticles (<100 nm) with their high surface area and unusual surface chemistry and reactivity poses unique problems for biological cells and the environment. This paper reviews the current research on the reactivity and interactions of carbon nanoparticles with biological cells *in vivo* and *in vitro*, with ultrastructural images demonstrating evidence of human cell cytotoxicity to carbon nanoparticles characteristic of lipid membrane peroxidation, gene down regulation of adhesive proteins, and increased cell death (necrosis, apoptosis), as well as images of nontoxic carbon nanoparticle interactions with human cells. Although it is imperative that nanomaterials be systematically tested for their biocompatibility and safety for industrial and biomedical use, there are now ways to develop and redesign these materials to be less cytotoxic, and even benign to cell systems. With this new opportunity to utilize the unique properties of nanoparticles for research, industry and medicine, there is a responsibility to test and optimize these new nanomaterials early during the development process, to eliminate or ameliorate identified toxic characteristics.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Recently, advances in developing new nanomaterials for use in industrial and biomedical applications have yielded new types of nanoparticle less than 100 nm in size. Both engineered nanoscale materials developed in the laboratory, and naturally occurring (biological particulates, anthropogenic particulate by-products from diesel fuel, power plants,

incinerators etc) particulates falling within the 1–100 nm size range, are included in the term nanoparticles [1]. These nanoparticles vary in composition, physico-chemical characteristics and biological toxicity, and include metallic and polymer quantum dots [2, 3], carbon nanotubes (single walled carbon nanotubes, SWCNTs; multiwalled carbon nanotubes, MWCNTs, [4–10]), fullerenes [11–14], carbon nanotube derived structures (horns, loops, peapods), ultrafine particles in commercial products such as aerosols and sunscreens (i.e. SiO_2, TiO_2) [15], or in the work environment (asbestos, beryllium etc) [16–20], and diesel and air pollution particles [20, 21]. The effect and mode of action of these nanoparticles in the environment and within living systems cannot yet be predicted with any certainty because no nanoparticles are exactly the same [22]. Each new type of ultrafine nanoparticle has unique physico-chemical properties (surface charge, surface properties in terms of biologic reactivity, shape and size, deformability, durability, tendency to aggregate, hydrophobicity), which will determine how this particle will interact with the environment and biological systems [22, 23]. Furthermore, some research studies indicate that 'when harmless bulk materials are made into ultrafine particles, they tend to become toxic'. The National Institute for Environmental and Health Sciences (NIEHS) in the US has found that 'generally the smaller the particles, the more reactive and toxic are their effects', and therefore safety precautions should be taken when making and handling these new materials[4]. Both the European Union and European Science Foundation have supported activities to explore the opportunities and risks involved in nanotechnology, as well as to identify the opportunities to exploit this technology in the life and physical sciences [25, 26]. Similarly, the National Nanotechnology Initiative (NNI) was established as a US federal research and development program to coordinate multi-agency efforts in nanoscale science, engineering and technology [1]. With the possibility that nanotechnology could produce changes in industrial productivity, international trade, environmental remediation, diagnosing and treating disease, and foster economic growth in many parts of the world, scientists must begin to question whether there might be health and environmental contamination risks following exposure to these new materials, during manufacture, internalization for medical treatment or diagnosis, or following disposal in the environment.

A number of investigators found that some forms of carbon nanoparticles had the ability to cause environmental contamination problems [11, 13], as well as inflammatory lung responses under experimental conditions (using intratracheal instillation in rodents) [5, 6, 8]. In general, the potential for bio-reactivity of a nanoparticle increases as the particle size decreases due to two inherent factors: (1) the smaller the particle the greater the surface area per unit mass [23, 26], and (2) the particle surface characteristics [27, 28]. With bio-applications of nanoparticles for drug delivery and imaging for health and disease, nanoparticles have been purposely reacted, and in some cases coated, with surfactants, monoclonal antibodies, purified protein, DNA and/or organic molecules for targeting specific desired organs or cells, or to make them biocompatible. These newly engineered nanoparticles with attached surface complexes create physiologically new materials that may be more reactive with living cells, reacting differently with each cell type, hormone and immune factor encountered after entry into a living organism. There is also the potential for each organ system to respond differently to these bioengineered probes, with a benign effect in one organ or tissue, only to elicit an inflammatory response in another tissue such as the blood, or glomerulus of the kidney during excretion.

Of even greater concern is how these coated nanoparticles and naked nanoparticles will react following biodegradation in the environment and within cells, and whether these degraded

[4] National Institute of Environmental Health Sciences (NIEHS) OPPE fact sheet #03 July 2003 Nanotechnology Safety Assessment.

nanoparticles will be altered sufficiently to present problems in terms of bioaccumulation within cells or organs, produce intracellular changes in organelles, or inflammatory cellular responses leading to acute or chronic pathologies and gene alterations [5, 6, 9, 29]. This review examines carbon nanoparticle bio-toxicity in the light of current data, and with additional new data from our laboratory testing of carbon nanotube materials in an *in vitro* system using early passage confluent human lung and colon cell monolayers (polarized cells joined by tight junctions) to study the cellular responses during carbon nanoparticle exposure by light microscopy, transmission (TEM) and field emission scanning electron (FESEM) microscopy.

2. Biological nanoparticle toxicity

2.1. In vivo response to carbon nanoparticles

It should be noted that carbon nanotubes occur in varying lengths, from nanotube fragments ($\sim$10 nm) to SWCNTs $>$20 μm long [5, 11, 20, 23]. Because *in vivo* studies require inhalation or injection of these nanotubes, carbon nanotubes >1 μm in length were typically used. Early studies reported that carbon nanotubes produced toxic responses in rodent lung following inhalation, in comparison to quartz or graphite particles similarly tested *in vivo* using intratracheal instillation [5, 30]. With SWCNTs (measuring $\sim$1.4 nm in diameter and >1 μm in length), both groups reported some form of lung inflammation and granulomas. However, Warheit *et al* [5] reported that some of these findings may not have physiological significance because of the instillation of 'agglomerated' nanotubes, and that the stress experienced by individual animals during the surgical installation procedure also may have altered the mortality and inflammatory responses. Lam *et al* [6, 30] concluded that in mice, carbon nanotubes were found to be more toxic in the lung than carbon black or ultrafine quartz particles. They also found, using mice exposed to 'raw and purified' carbon nanotubes produced under different conditions (some nanotubes having iron, or nickel–yttrium present), that the lungs of the animals that died following the 0.5 mg (high) dose showed large aggregates of particles in alveolar macrophages; some nanotube aggregates were found between lung cells, forming granulomas; and there were signs of inflammation in the lung tissue. Of even greater interest biologically is that carbon nanotubes were also observed to pass rapidly through the walls of the air sacs, suggesting that these insoluble, nonbiodegradable fibres had the ability to be translocated within living tissue, indicating that more work needed to be done to assess the health risks of these new nanomaterials [31, 32]. Exposure to uncoated C_{60} fullerenes induced oxidative stress in juvenile largemouth bass, causing lipid peroxidation in the brain, with nanoparticle translocation in the central nervous system via axons and dendrites from the olfactory nerve into the olfactory bulb [33]. A primary question today is how the persistence of these non-degradable nanoparticles incorporated into biological organisms or the environment may pose health risks over time through inflammatory processes, bioaccumulation, and translocation of nanoparticles within living systems from initial entry sites to new areas; and whether these incorporated nanoparticles alter biological cell cycles, change gene expression, or cause tumour induction.

Some investigators feel that some aspects of these animal experiments, such as the intratracheal mode of administration and the high doses given, may make it more difficult to come to definitive conclusions [5]. Schvedova *et al* [8] found that pharyngeal aspiration of SWCNTs in mice caused robust acute inflammation with early onset of fibrosis and granulomas, but more importantly they found neutrophil (day 1) and lymphocyte (day 3) accumulation followed by macrophage infiltration (day 7), indicating a progressive inflammatory response. Equal doses of ultrafine carbon black particles or crystalline SiO_2 particles did not produce the

granulomas or alveolar wall thickening seen with SWCNTs. Biochemically this study found that exposure to SWCNTs was also accompanied by release of TNF alpha and IL-1beta as well as transforming the fibrogenic growth factor. Similarly, lung exposure to MWCNTs in rats produced pulmonary inflammation and fibrosis [34]. All of these *in vivo* studies clearly show that multiple factors interact together following carbon nanoparticle exposure, producing acute and chronic changes within individual cells and the organism itself.

2.2. In vitro testing

To simplify studying biological cellular responses to specific nanoparticles, *in vitro* models were developed which eliminated many variables found in animal studies, and allowed the investigator to have greater control over experimental conditions. In order to collect biochemical, molecular biology, and cellular (light microscope and ultrastructural) data following nanoparticle exposure to specific cell types (i.e. lung, liver, macrophage, skin. . .), many investigators [9, 14, 23, 29, 34, 35], including those in our own laboratory, have developed *in vitro* model systems using both human and animal cells. If immortalized cells are to be used, they must be used in early passage so that they retain the characteristics of their original tissue line, and when possible, primary cell lines should be substituted if the cells can be cultured to grow normally and are morphologically and physiologically identical to their *in vivo* counterpart. The ability to examine the response of specific types of living cells to different preparations of nanomaterials under very controlled (time, concentration, dispersion, functionalization etc) conditions offers immediate information about nanoparticle attachment, incorporation, intracellular and intercellular localization, alteration of organelles, biochemical and electrolyte changes, gene expression, protein expression, viability (necrosis and apoptosis) and cell cycle changes [10, 14, 29]. By combining molecular biology techniques, immuno-markers with light, fluorescence and confocal microscopy, electron diffraction and higher resolution microscopies (transmission electron microscopy, field emission scanning electron microscopy), information about the response of each cell type to specific nanoparticles can be obtained rapidly with ample statistics. For developing safe nanoparticles for use in industry and the biomedical fields, *in vitro* testing is an inexpensive and immediate tool for screening and fine-tuning nanoparticle design to maximize safety and target specificity. Using animals for *in vivo* studies involves maintenance costs, the use of genetically similar animals for comparisons between control and experimental trials, and adequate personnel to maintain the animals, equipment and process the tissue. *In vitro* testing on the other hand can be done with tissue culture cells that have been thoroughly characterized in terms of genetics, morphology, and growth requirements for optimum healthy cells, and the cells are used only as needed (stored cells are frozen). In an *in vitro* system the investigator is able to eliminate such variables as antibody responses, hormonal and electrolyte affects, and animal excitatory responses to procedures. Conversely, *in vitro* testing provides a limited view of the responses of only the cell types being tested, and ultimately it is essential to have new nanomaterials tested *in vivo*, where the combined reactions of many cell types and tissues, as well as the blood, immune and hormonal factors, are all taken into account to assess biocompatibility, and assure safety.

2.3. Fullerenes

Fullerenes are carbon nanoparticles that have a characteristic cage structure and offer unique promise for use in newly developing electronic, optical and biomedical materials and applications [36]. Sayes *et al* [37] demonstrated the differential cytotoxicity of some water-soluble fullerene species, and concluded that changes in the fullerene cage structure directly

affected the *in vitro* cytotoxicity. Water-soluble nano-C_{60} (C_{60}) demonstrated significant cytotoxicity to tissue culture cells *in vitro*, but a highly hydroxylated, water-soluble fullerene, $C_{60}(OH)_{24}$, produced no evidence of cytotoxicity under the same conditions. More recently Sayes *et al* [14] reported that the cytotoxicity caused by C_{60} was due to lipid peroxidation of the cell membranes (not mitochondrial membranes) in human dermal fibroblasts, human liver carcinoma cells (HepG2), and neuronal astrocytes after 48 h exposure. Oberdorster [33] similarly reported oxidative stress and lipid peroxidation of brain tissue in juvenile largemouth bass following exposure to fullerenes, as well as translocation of nanoparticles along central nervous system neurons. Sayes *et al* [14] also reported that lipid peroxidation during C_{60} exposure was prevented by the addition of L-ascorbic acid during C_{60} incubation with the human dermal fibroblast cells, resulting in normal cell viability, compared to controls. It would be most interesting to see ultrastructurally if these ascorbic acid protected fibroblasts had normal morphology intracellularly and at the cell nucleus, and if the antioxidant L-ascorbic acid worked effectively well on the other cell types (liver and astrocytes) in the study, protecting them sufficiently to grow and function normally.

2.4. SWCNTs

Many studies have been done using *in vitro* models to study SWCNT exposure to animal and human cell lines. Mattson *et al* [38] found that carbon nanotubes inhibited the growth of embryonic rat brain neurons, and similar cytotoxicity was reported in keratinocytes exposed to SWCNTs in culture [7, 39]. Cui *et al* [29] found evidence in human embryo kidney cells (HEK293) of alterations of gene regulation induced by SWCNT exposure. Cells showed inhibited cell proliferation and adhesive ability in a dose–time-dependent relationship, with the up regulation of cell cycle associated genes, the down regulation expression of signal transduction associated genes and adhesion associated proteins such as laminin, fibronectin, cadherin, FAK and collagen IV, as well as G1 arrest of cells and apoptosis (programmed cell death). They observed that HEK293 cells actively responded to SWCNTs by secreting proteins to aggregate and wrap the nanotubes. After incubation for several hours with these cells, the SWCNTs aggregated together forming bundles in the medium, and similarly HEK293 cells with attached SWCNTs formed cell aggregations that became apoptotic. However, those HEK293 cells far from the SWCNTs 'grew quite well', suggesting that direct contact with the nanoparticles may be necessary to initiate cytotoxicity [29].

If carbon nanoparticles are to be used for drug delivery or bio-imaging in health and disease, an understanding of the response of blood cells to nanomaterials is essential for parenteral administration. Both phagocytic (macrophages, monocytes, neutrophils) and non-phagocytic (lymphocytes, erythrocytes) blood cells and platelets participate in the recognition of foreign material in the blood, and could pose a major obstacle to nanoparticle transport via the circulatory system. Bottini *et al* [40] analysed the toxicity of three different preparations of SWCNTs with normal human lymphocytes from blood donors, and commercially available human T cell leukemia Jurkat cells. SWCNTs preparations of (1) air oxidized; (2) nitric acid treated and further processed to reduce oxygen functionality; and (3) carboxylated nanotubes (1.4 nm diam. $\times$ 50–500 nm length), were resuspended to make concentrations of 1 nM, 10 nM, 100 nM and 1 μM. All of the nanotubes ranged in length from 50–500 nm, with a mean diameter of 1.4 nm. Cell growth and viability following 48 h exposure (using Trypan blue to measure cell death) and Annexin V staining to identify apoptosis, revealed that both peripheral lymphocytes and Jurkat cells only showed increased cell death or apoptosis when exposed to SWCNT concentrations above 10 nM, regardless of modifications of the nanotube surface. Kam *et al* [41] reported internalization of SWCNT–protein conjugates into various

tissue culture cells (human leukemia (HL60), Chinese hamster ovary cells, 3T3 fibroblasts and Jurkat T cells), and concluded that the functionalized nanotubes, which had entered the cells via the endocytic pathway, were not toxic. However, these cells were only followed for 48 h, and no information was collected on subsequent ultrastructural intracellular changes with time, nor changes in gene expression or normal function. Many cells that seem normal after exposure may actually be damaged and function abnormally at the molecular level, leading to future apoptosis or tumour induction. The bioaccumulation of SWCNTs within mammalian cell endocytic vacuoles is not normal for a cell; and with time, the release of these nanotubes into the cytoplasm has the potential for producing serious damage.

2.5. MWCNTs

Monteiro-Riviere *et al* [9] reported that proinflammatory cytokines were released from human keratinocytes incubated with MWCNTs, similar to findings of others using the same cells exposed to SWCNTs [7]. MWCNTs were sonicated in keratinocyte growth medium (0.4 mg ml^{-1}), and incubated with the cell cultures. When the cells were embedded and thin sectioned for TEM, MWCNTs were observed predominantly within cytoplasmic vacuoles, although occasionally MWCNTs were seen free in the cytoplasm and lying close to the cell nucleus. MWCNTs were more numerous in the cells incubated at higher concentrations for longer exposure times, and release of interleukin (IL-8) increased at the highest MWNT concentrations, suggesting cell irritation. The sonication of the nanotubes in growth medium for nanotube dispersal may have coated or wrapped the MWCNTs with protein. If protein had attached to the nanotube surface, the protein could facilitate recognition and phagocytic uptake of the MWCNTs by the fibroblasts. IL-8 release could also have been augmented by the damage within the cell associated with the internalized MWCNTs, and lysosomal or phagosomal processing.

Bottini *et al* [42] examined the toxicity of pristine, hydrophobic, and oxidized MWCNTs with human T cells and Jurkat T leukemia cells. Although these blood cells are highly responsive to foreign material, the more hydrophobic pristine MWCNTs were less toxic than the oxidized MWCNTs. These oxidized MWCNTs induced significant loss of cell viability (apoptosis) at doses of 400 μg ml^{-1}, with less toxicity at a tenfold lower concentration. Following MWCNT exposure at 40 μg ml^{-1}, lymphocyte function was monitored by Western blot and revealed no detrimental effects on receptor-induced T-cell activation, suggesting that these cells could function in the presence of lower doses of MWCNTs. From these MWCNTs studies, it appears that toxicity of these nanotubes not only depends on exposure time and concentration, but also on the cell type being tested, as well as the nanotube surface characteristics.

3. Not all carbon nanoparticles seem to be toxic

As mentioned in most of the previously described studies, using shorter exposure times and lower concentrations of carbon nanoparticle preparations reduced cytotoxicity [40, 42]. Sayes *et al* [14] also reported that fibroblasts incubated with C_{60} fullerenes showed no cytotoxic responses when a potent antioxidant (L-ascorbic acid) was added to the culture medium along with the fullerenes during cell incubation. Scientists at Rice University in Texas [43] had reported that even minor surface modifications of buckyball preparations could dramatically reduce the cytotoxicity. With higher degrees of surface modification, buckyballs and SWCNTs produced less toxic responses when incubated with tissue culture cells [44, 45].

Similarly, in our laboratory, a 'carbon nanotube-derived nanoloop', used as an immuno-carrier, produced no recognizable cytotoxic effects in colon or lung cell growth, or

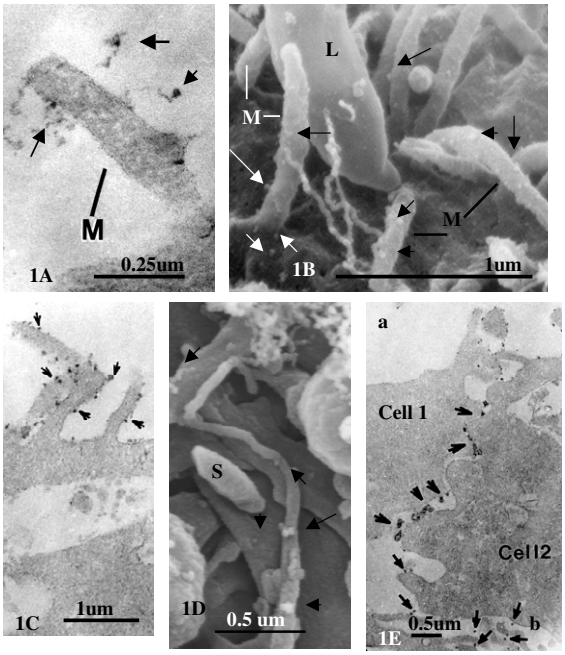

Figure 1. Antibody functionalized carbon nanoloops used to study gClq-R expression during bacterial attack of human cells. (A) Clusters of monoclonal antibody-functionalized carbon nanoloops (arrows) indicated sites of gClq-R expression on colon microvillus (M) during *Listeria monocytogenes* attack. (B) Antibody-functionalized nanoparticles (arrows) seen on colon microvilli (M) adjacent to attached Listeria (L). (C) Lung cells incubated with bacterial spores showed clusters of antibody functionalized nanoparticles (arrows) attached to apical cytoskeletal extensions, identifying sites of gClq-R expression. (D) Functionalized groups of nanoparticles (arrows) were attached directly to membrane extensions adjacent to a *B. cereus* spore (S) protoplast. (E) Following bacterial attack, apical (a) membrane bound nanoparticles (arrows) were transported via the intercellular space (between neighbouring cells 1 and cell 2) to the basal cell surface (b), with no accumulation of carbon nanoparticles seen within cells or organelles. Cells remained intact during, and following, nanoloop exposure, showing no signs of toxicity.

ultrastructural morphology [35, 46, 47]. The carbon nanotube-derived nanoloops (22–44 nm diameter) were made by cutting and cleaning Carbolex nanotubes in 60 °C sonicated sulfuric acid and hydrogen peroxide. The cut nanotubes were differentially centrifuged in phosphate buffered saline (PBS) to isolate and clean the SWNTs, followed by sonication with carbodiimide and functionalization with either antibodies (nonimmune IgG or monoclonal antibodies to gClq-R invasion protein) or recombinant purified invasion receptor protein (gClq-R) [35, 46, 47].

Once functionalized the nanoloops were resuspended in PBS and added to *in vitro* cultures of growing lung or colon epithelial monolayers (human lung, NCI-292 or colon, Caco-2 cells), grown to 90–95% confluency on 12 mm coverslips in dram vials, and incubated at 36 °C for 1–7 h. The presence of the functionalized nanoloops in the culture media produced no changes in the apical plasma membranes of the cells, or in their intracellular contents (cytoplasm, organelles, tight junctions, no increased vacuolization or endosomal activity), when examined by TEM and FESEM as seen in figure 1 [46, 47]. The control functionalized nanoloops (IgG and purified protein functionalized nanoparticles) did not attach to the human cells. Only the monoclonal antibody-functionalized nanoloops attached to the surface of the epithelial cells,

and that only happened when the target invasion protein was expressed on the apical surface of the cells during bacterial attack (figures 1(A)–(D)). Under all other conditions, the carbon nanoloops did not attach nor accumulate on, or within, the human cells.

Following incubation, the culture medium was removed and the cells washed two times with PBS, and the media and buffer washes screened for the presence of nanoloops, dead cells and contaminants. Analysis of the combined solutions from each dram vial showed almost complete retrieval of nanoloops functionalized with recombinant purified protein, or nonimmune IgG, verifying their lack of attachment to the cells. Cell monolayers incubated with nanoloops functionalized with monoclonal antibody to gClq-R invasion protein showed similar high retrieval rates of functionalized nanoloops in the media and buffer washes when the cells were bacteria-free. However, when bacteria or bacterial spores were introduced into the media, the monoclonal antibody-functionalized nanoloops rapidly and irreversibly attached to sites of invasion protein (gClq-R) expression on the microvilli (M) of bacterially attacked cells (figures 1(A)–(D) arrows) [35, 46, 47]. There was no indication of membrane damage, lipid peroxidation or apoptosis, even after several hours of exposure to the nanoparticles. As the cells recycled their apical cell membranes, the segments of plasma membrane with attached nanoloops were also processed with the carbon nanoloops transported between the colon or lung cells via the intercellular space (figure 1(E)). Ultimately the nanoparticles were seen at the basal infoldings of the cells (lower right, figure 1(E)). At 4–7 h following bacterial attack, the apical membrane surfaces of the lung and colon cells were clear of nanoparticles, with intact, normal apical membranes showing no holes, tears or damaged cells.

4. Ultrastructural human cellular cytotoxicity response

4.1. In vitro Carbolex exposure

Manufactured carbon nanotube preparations are handled every day by technicians, investigators and students. To better understand the first response of human cells to exposure (2–4 h) to this type of raw nanotube preparation (containing predominately SWNTs, nanoropes, graphene, some nickel and trace yttrium), we used an *in vitro* model with monolayers of Caco-2 colon cells (ingestion) and lung NCI-292 epithelial cells (inhalation route). In this way we not only could measure viability, but were also able to visualize the first contact of the nanoparticles with the cells, and follow this process ultrastructurally to reveal how these epithelial cells processed this type of nanomaterial; and most importantly, what happened to the cells that did not immediately die. Many nanoparticle exposure studies have shown cell death and altered regulation of cellular proteins, cell cycle and gene regulation, but questions remain about the first point of contact of nanoparticles with biological cells, and the fate and normalcy of the surviving cells with time.

To examine what the response might be of two types of human cell (colon Caco-2 and lung NCI-292) that represent two possible portals of entry (ingestion and inhalation) to carbon nanoparticle exposure, we used an *in vitro* system where we could examine not only cell viability, but also the ultrastructural response of individual cells intracellularly, and at the first point of contact of the nanoparticle to the cell.

Carbolex was resuspended in phosphate buffered saline (PBS) to make pH 6.8 solutions at 10 and 100 μM. Suspensions were sonicated and vortexed just prior to use to ensure that the nanoparticles were evenly distributed without carbon nanotube bundles or aggregations (checked by TEM on formvar carbon coated grids). Each tissue culture dram vial with human epithelial colon (Caco-2) or lung (NCI292) cell monolayers (grown to confluency on 12 mm coverslips) received 2 μl of either the 10 μM or 100 μM nanoparticle solution in buffer. Control

vials received 2 μl of PBS alone. All tissue culture vials were then mounted on a rotary tilt platform in a 36 °C incubator and the cells were incubated for 2, 3 or 4 h with constant swirling to distribute the carbon nanotubes over the cell surfaces. Following incubation the media was removed from each vial, and the cells washed twice with warm PBS to remove any unattached nanotubes, detached or dead cells, and the combined media and wash solutions screened for the presence of dead cells, possible contamination and nanoparticles. The cell monolayers were either prepared for vital staining (with 0.4% erythrosin B in PBS, diluted 1:5 PBS), and photographed for statistical necrosis determinations using an Olympus BH-2 photomicroscope; or the cells were fixed in 2.8% glutaraldehyde in 0.1 M cacodylate buffer with 10% added sucrose for TEM and FESEM imaging. For TEM, cells fixed in glutaraldehyde for 6–12 h were rinsed in 0.1 M cacodylate buffer with 10% sucrose, washed in sterile deionized water briefly and post-fixed in aqueous 1% osmium tetroxide with 10% sucrose at room temperature for 1–2 h. The fixed cell monolayers were washed in buffer to remove all osmium, dehydrated, embedded in epoxy resin, thin sectioned and imaged with a Philips 300 TEM at 80 kV. Serial sections were cut and alternate sections stained with uranyl acetate and lead citrate. In this way both stained and unstained sections could be imaged and analysed for ultrastructural cell morphology, electron diffraction and nanoparticle localization. Samples for FESEM were similarly glutaraldehyde-fixed, osmicated, dehydrated, critical point dried, mounted on brass stubs, and plasma coated with 3 nm of Pt for imaging in a JEOL 6500F at 5 and 15 kV.

For viability testing, each control and experimental vital stained coverslip was divided into six random fields and three images were recorded at 200× and 1000× in each of the six fields. Living, non-stained cells, and necrotic erythrosin B stained cells were counted, with mean counts averaged from 15 to 18 photomicrographs per coverslip (3 cell monolayers per solution tested; the total number of fields calculated $N = 45$–48). For FESEM, each monolayer was divided into four areas and 5–6 micrographs were taken in each area at low and high magnifications for qualitative comparison of cell morphology, monolayer confluency, damage, and evidence of nanoparticles, compared to controls. High-resolution imaging was done on a JEOL 3000 field emission TEM with electron diffraction and x-ray microanalytical capabilities. Carbolex suspensions placed on pyrolytic graphite planchets were analysed by FESEM x-ray microanalysis at 5 and 15 kV, and revealed nickel and trace yttrium, but no other metal content.

4.2. Human cell viability following Carbolex exposure

Table 1 shows the viability results of lung and colon cells incubated for 3 h in the presence of Carbolex, compared to control values. Both of these cell types are polar cells which form tight junctions when grown to confluency as monolayers, and when used in early passage maintain the morphology and physiology of their normal *in vivo* cell type. Therefore the only way that nanoparticles could enter these cells would be through cellular processes via the apical plasma membrane. Colon cells showed greater than a twofold increase in cell death following 3 h exposure to 10 and 100 μM Carbolex solutions, compared to the control. There was no significant difference between the lower and higher Carbolex dose solutions at 3 h, but at 2 h (100 μM), cell necrosis was slightly less (table 1). Cell growth difficulties with the lung monolayers eliminated doing multiple concentration experiments. However, the 90% confluent lung monolayers, when exposed to 100 μM Carbolex (3 h), showed higher levels of cell necrosis compared to control lung monolayers (table 1).

Increased exposure time, and increased concentration of these nanoparticles, produced increased cell necrosis in both colon and lung human cells. To understand and clarify more about the process of this cytotoxicity and visually monitor the nanoparticle's first contact

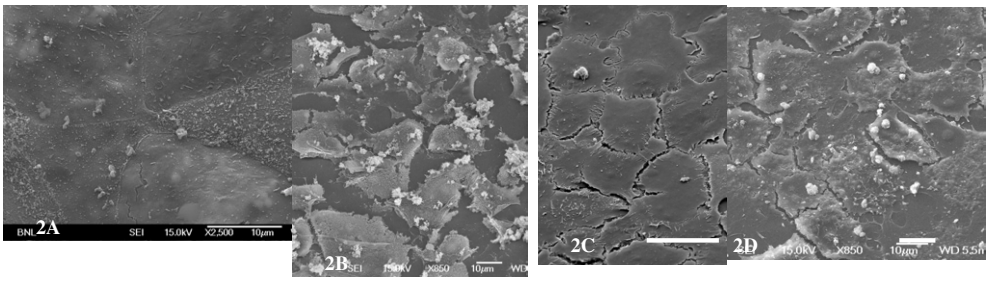

Figure 2. Control (A) and 100 μM Carbolex exposed (B) colon cells. In (A) cells are clearly joined together with normal microvilli and cell membranes, as compared to the Carbolex disrupted monolayer (B) with missing cells, loss of cell–cell attachment and abnormal surface morphology. (C) Normal lung cell monolayer with confluent surfaces and intact surface membranes, compared to (D) Carbolex exposed cells showing changes in the surface morphology of the cells, missing cells in the monolayer and gaps in cell–cell attachments. scale bars = 10 μm.

Table 1. Viability of human cells exposed to Carbolex.

Sample	Mean cell death/12.4 $\times$ 10^3 cells
Colon Caco2-cells	
3 h control	34.3 ± 4.5[a]
2 h Carbolex 100 μM	65.7 ± 2.0[a]
3 h Carbolex 100 μM	80.6 ± 7.8[a]
3 h Carbolex 10 μM	84.0 ± 11.3[a]
Lung NCI-292 cells	
3 h control	56.8 ± 5.4[a]
3 h Carbolex 100 μM	83.0 ± 7.3[a]

[a] Std. error.

with the cell surface, and the process of nanoparticle interaction at the ultrastructural level, identical specimens were examined by TEM and FESEM. Using these imaging methods, questions about whether nanoparticle attachment or phagocytosis induced cell damage, which type of nanoparticles entered the cells (intact SWCNTs, nanoropes, degraded nanotubes, metal particles...), and the fate of the remaining living cells following exposure, could be visually documented.

4.3. Electron microscopy

Control cell monolayers showed better than 90% confluency and attached cells. However, colon cell monolayers exposed for 2 h to 2 μl 100 μM Carbolex suspension revealed a loss of cell–cell contact in more than 40% of the cells, with some loss of basal adhesion to the substrate when compared to normal controls (figure 2).

When lung monolayers were incubated with 100 μM Carbolex (2 μl dose) for 3 h (figure 2(D)), the monolayers seemed intact, with cells attached to the substrate and to one another when imaged by light microscopy and low magnification FESEM. At higher magnification (figure 3(A)), cells with attached carbon nanotubes (arrows) showed a loss of attachment to adjacent cells, and the formation of large cytoskeletal appendages, membrane ruffling (R) and elaboration of a mucoprotein material (MP) on the apical surface (stress response) (figure 3(A)). Cells with no nanoparticles visible on the cell surface exhibited normal morphology with an intact, smooth plasma membrane.

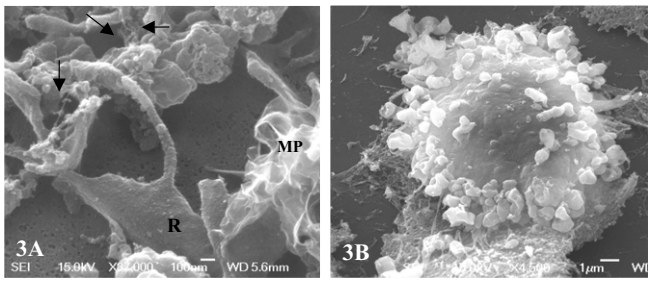

Figure 3. Lung cell surface following Carbolex incubation. (A) In response to direct contact with carbon nanoparticles (arrows), the lung cells produced mucopolysaccharide (MP), large cytoskeletal ruffles (R), and filiform membrane extensions. (B) At a higher nanoparticle dose, the lung cells exhibited apoptosis with separation from neighbouring cells, extensive surface blebbing and impending cell death.

To test the effect of higher nanoparticle dose on cells, 15 μl of the same 100 μM Carbolex solution was incubated with lung cell monolayers. At this higher dose, the cells lost their intercellular junctions disrupting the monolayer, when compared to the 2 μl samples (figure 2(D)). At higher concentrations, many apoptotic cells were found in various stages of self-destruction, exhibiting reduced cell volume, extensive surface membrane blebbing, and pulling away from the substrate (figure 3(B)).

Figure 4(A) shows the initial response of Caco-2 colon cells following 2 h exposure to 10 μM Carbolex suspension. In response to contact with the nanomaterial, the apical surface elaborated a mucoprotein material on the apical surface (arrows), that was not evident on control cells. This elaborated material, in response to the presence of Carbolex, caused the entrapment of nanoparticles (nanotubes, nanoropes and graphene) on the cell surface and onto adjacent microvilli (M) (figure 4(B)). Following 2 h exposures, the microvilli (M) were still morphologically normal, but after longer contact (figure 4(C)), the microvilli showed signs of swelling and plasma membrane damage (black arrows) by TEM and FESEM at the sites of contact with the carbon nanoparticles (CNT material). Figure 4(D) shows protein decorated nanoropes and nanotubes (arrows) directly attached to the colon cell surface after 3–4 h exposure (10 μM). Here severe cell surface damage was apparent, with numerous small holes and tears in the plasma membrane, exposing the underlying cytoplasm (large white arrow). By TEM these Carbolex treated colon cells showed direct uptake of small nanoparticles into the cytoplasm without any evidence of phagocytosis or membrane bound vacuoles. In many cells, nanoparticles (white chevrons) were seen surrounded by nuclear material within intact colon cell nuclei (figure 4(E)). On the surface of the colon cells, areas closest to the carbon nanoparticles (CNT) exhibited disruptions in the plasma membrane lipid layers (black solid arrows, figure 4(F)).

The membrane damage observed by TEM and FESEM when carbon nanoparticles were touching the plasma membrane may be the result of membrane lipid peroxidation reported by Sayes *et al* [14] and Oberdorster *et al* [33]. In this case, since the plasmalemma is a bimolecular lipid–protein leaflet, the peroxidation of the lipid would compromise and disrupt the integrity of the membrane (figure 4(F), black arrows), by causing the loss of lipid where the plasma membrane and carbon nanoparticles are in close proximity or in contact. Normally osmium, post-fixation and lead staining preferentially increase contrast of the plasma membrane, but in figure 4(F) there are electron lucent breaks in the plasma membrane adjacent to the carbon nanoparticles, consistent with the loss of the electron dense osmium and lead staining of the

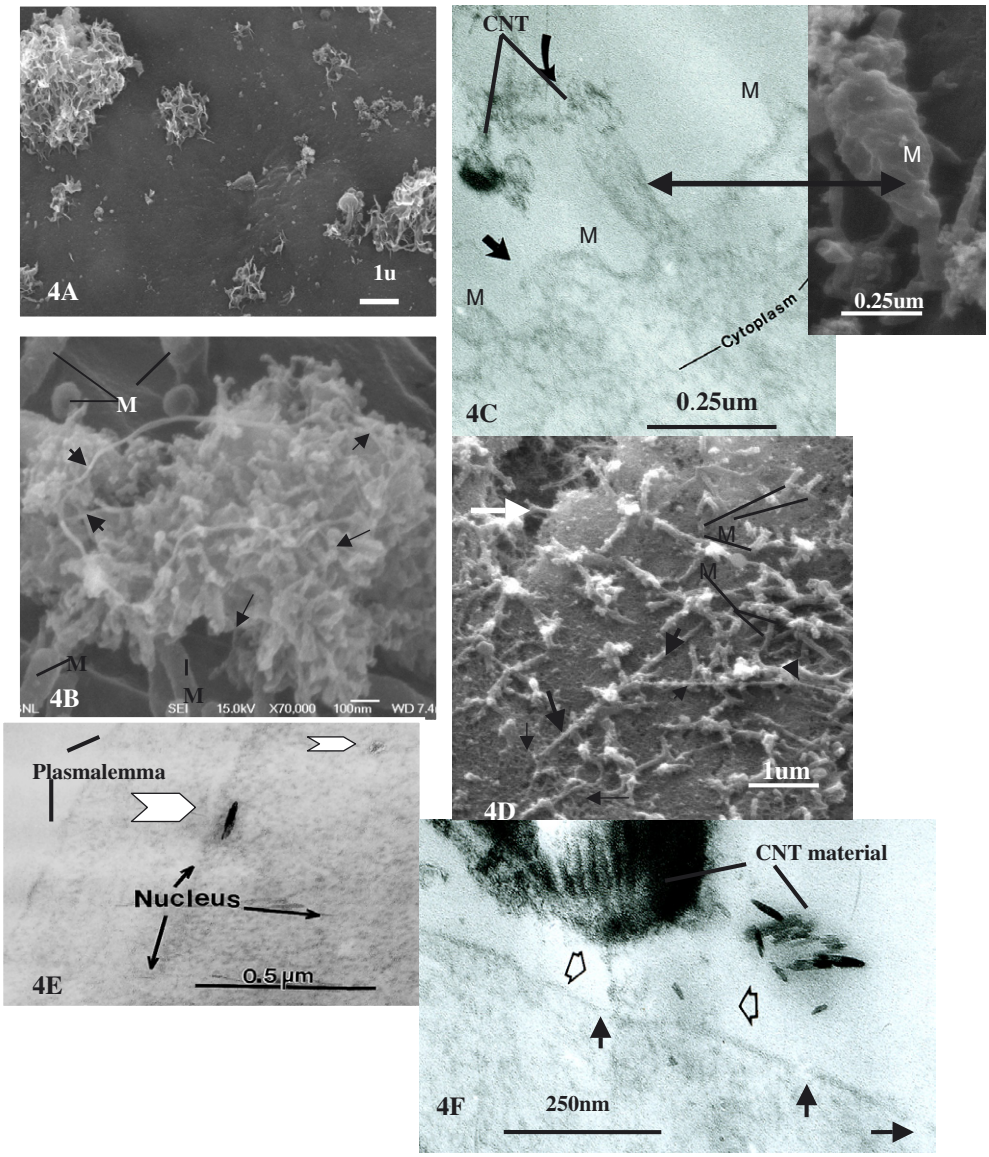

Figure 4. Colon cells exposed to Carbolex. (A) Early (up to 2 h) response to Carbolex nanoparticles shows mucoprotein elaboration on the apical cell surface. (B) Nanoparticles (arrows) entrapped on surface-elaborated material, holding nanoparticles in contact with the apical membrane and microvilli (M). (C) Direct contact of nanoparticles (CNT) with microvilli (M) caused swelling of the microvilli and damage to their outer membranes. (D) At 3–4 h nanoparticle exposure, areas of nanoparticle contact with cells (black arrows) showed holes and tears (white arrow) in the apical membrane surface. (E) Concurrently with apical surface membrane damage, nanoparticle material was observed within the cell nuclei and cytoplasm. (F) Breaks in the plasma membrane (black solid arrows) were seen in areas with attached Carbolex nanoparticles (CNT material). These disruptions in plasmalemma continuity may be visual evidence of plasma membrane lipid peroxidation.

membrane. Lipid peroxidation associated with nanoparticle contact offers an explanation for the holes and tears seen in the apical membranes of the human cells (figure 4(D)) with attached

nanoparticles, imaged by FESEM. Neighbouring cells without attached carbon nanotubes or nanoropes showed normal surface membranes similar to controls, without signs of damage. In these human epithelial cells, direct contact with the carbon nanoparticles may be necessary for cytotoxic damage to take place.

To verify that the reactions to the Carbolex were not due to the Ni associated with this commercial preparation, purified air oxidized nanotubes were also tested at 10 and 100 μM concentrations with the colon Caco-2 cells. Colon Caco-2 cells exposed to air oxidized nanotubes showed slightly higher cell necrosis than the necrotic damage seen with Carbolex (table 1), with increased membrane blebs and blisters in areas of direct contact (3 h 10 μM, 93.8 $\pm$ 8.3 mean cell death/12.4 $\times$ 10^3 cells; and 3 h 100 μM, 105.7 $\pm$ 12.0 mean cell death/12.4 $\times$ 10^3 cells). This indicates that the carbon nanoparticles can directly cause these cytotoxic effects.

When the thin sections of cells (exposed to Carbolex) containing intranuclear nanoparticles were analysed at 200 kV by high resolution field emission TEM (JEOL 3000F), the nanomaterial inclusions seen in figure 4(C) produced lattice structures. This observed lattice and the diffraction pattern were not consistent with those of carbon nanotubes. *With the integrity of the outer plasma membrane compromised by lipid peroxidation (which by TEM appeared as breaks in the apical surface membrane), nanoparticles having the appropriate charge, and affinity for intracellular cytoplasm, could freely enter the cell.* Although there were many carbon nanotubes and nanoropes in the Carbolex preparations, these were observed by TEM and FESEM to remain outside the cells, at the cell surface; however, the crystalline carbon nanoparticle was able to freely enter the cell cytoplasm and was the only type of nanoparticle to be seen within cell nuclei.

5. Discussion

Many investigators have found that there was a dose and exposure time requirement for carbon nanoparticles to produce damage in living biological cells and tissues [5–9, 29, 46, 47]. Similarly here, we found that Carbolex nanoparticles showed increased cytotoxicity when the concentration and exposure time were increased, with damage reaching a maximum for both doses at 3 h exposure. These results suggest that the duration of exposure may be even more predictive of damage than the nanoparticle concentration. If the nanoparticle damage process to human cells is initiated by the production of reactive oxygen species, which in turn cause irreversible damage the cells, with lipid peroxidation of the exposed plasma membranes of the cells (causing the loss of cellular integrity and consequent membrane function), anything that can reduce the ability to generate reactive oxygen species, and prevent lipid peroxidation, may prevent permanent damage [37, 44, 49].

In our *in vitro* colon cell monolayer preparations, the junctional complexes joining cells together began to separate within 2 h of Carbolex nanoparticle exposure, and some cells lifted away from the monolayer substrate, indicating an alteration in cellular attachment protein function. This finding coincides with the molecular findings of Cui *et al* [29] using human kidney cells, that demonstrated the decreased expression of adhesion associated proteins (cadherin, laminin and fibronectin), following SWCNT exposure.

We found that colon and lung cells produced surface mucoprotein material that resulted in the trapping of the Carbolex nanoparticles (carbon nanotubes, nanoropes, graphene, nickel) on the apical cell surface, which facilitated the destructive contact between the nanoparticle and membrane surface (figures 4(A), (B) and 5). Those cells devoid of attached nanoparticles showed no apical mucoprotein material, and had normal membrane and intracellular ultrastructure by TEM and FESEM. Contact between the nanomaterial and

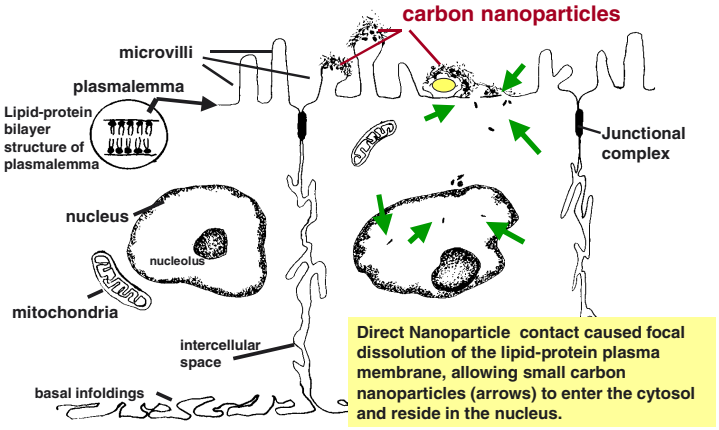

Figure 5. Human colon cells incubated with carbon nanoparticles (consisting of SWCNTs, nanoropes, graphite) showed no damage ultrastructurally unless the nanoparticles became entrapped on the cell surface mucoprotein secretions (surface structure with ellipse), or directly contacted the plasma membrane of the cells. The focal dissolution of the plasmalemma caused by nanoparticle contact permitted entry of very small nanoparticles (large arrows) into the cell. These particles passed through the cytosol and nuclear membrane, ultimately residing within the nucleus.

the apical cell membrane were predictive of plasma membrane focal damage. By electron microscopy, both colon and lung cells showed focal damage to the microvilli (swelling and surface membrane dissolution figures 4(C), and 5) and apical plasma membrane at sites of nanoparticle attachment (figures 4(B), (D) and 5). Holes and tears in the apical membrane appeared only on those cells with nanoparticles, although by 3–4 h exposure the monolayers had been significantly disrupted (due to detached cells, necrosis and apoptosis of many of the cells). TEM of cells with nanoparticles attached at the cell surface showed small areas of dissolution of the lipid–protein bilayer of the plasma membrane (figures 4(E) and 5), suggestive of lipid peroxidation and oxidative stress [14, 33, 49, 50].

Unlike the results of Monteiro-Riviere *et al* [9] using MWCNTs, Carbolex treated cells showed no phagocytic uptake of SWCNTs, nanoropes or other nanoparticles in vacuoles. Although SWCNTs and nanoropes remained outside the cells, a smaller crystalline carbon nanoparticle was found within the cytoplasm and the nuclei of still viable cells (figures 4(E) and (F)). Figure 5 schematically shows this early response to carbon nanoparticle exposure in colon cells.

At higher magnifications these particles had no surrounding vacuolar membranes, but were free in the cytoplasm or nucleoplasm. Using a JEOL 3000 field emission TEM, the small carbon nanoparticle inclusions (figures 6(A) and (B)) did not produce SWCNT electron diffraction patterns, nor appear as typical carbon nanotubes or nanotube bundles (figure 6(C)). In stained epoxy embedded thin sections, these intracellular nanoparticles (figure 6(A)) revealed a lattice structure (figure 6(B)).

There were also abundant crystals of these nanoparticles seen at the cell surface adjacent to larger accumulations of SWCNTs and nanoropes (figures 4(E) and (F)). Colon cells incubated with purified air oxidized SWCNTs also showed these small nanoparticles, suggesting that these were not unique to raw Carbolex preparations. Because this type of small nanoparticle displayed the capability to enter and freely travel within cell cytoplasm and nuclei (without vacuolar transport), the possibility for long-term cytotoxic effects is significant, especially in cells with carbon nanoparticles in contact with nuclear DNA. There is also a possibility

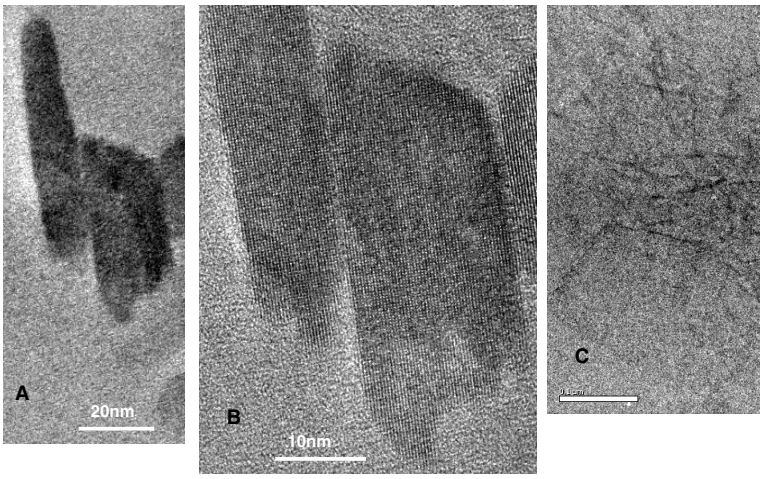

Figure 6. In plastic embedded thin sections of colon cells exposed to raw Carbolex nanomaterial, carbon nanoparticles were seen within, and adjacent to, the human colon cells (A). These nanoparticles exhibited a lattice structure (B). In comparison, plastic embedded thin sections of SWCNTs (C) had a very different morphology and were observed on the cell surface or entangled in the extracellular matrix at the cell surface, but were not found within the cells or cell nuclei.

that the described nanoparticles may actually be a combination of carbon nanomaterial and cellular protein [49], which would allow the carbon nanomaterial to enter and reside in the cell, without triggering lysosomal and cell-protective responses. We are doing further experiments to identify and characterize these nanocrystals, and to determine if those nano-inclusions found in the nucleoplasm of viable cells have the potential to alter nuclear function, resulting in changes in protein expression which ultimately may result in future pathology.

6. Conclusions

Biological cells respond to nanoparticles in very specific and complex ways that are dependent on the inherent nanoparticle characteristics (smallness of the particle size, charge, surface chemistry, shape, functionalization, core composition (carbon, metals, Si), ability to aggregate, degradation components), as well as the type and age of the exposed cells. Added to the inherent physico-chemical characteristics of these particles is their ability once inside a biological environment to redistribute, agglomerate, self-functionalize with biological macromolecules [29, 38], or become biodegraded, forming new and sometimes toxic materials, or posing a threat in terms of bioaccumulation and failed elimination from the organism's body, or environment. More studies need to be done to characterize the reactive profile and toxicity of the ever-increasing range of commercial and academically produced nanoscale materials [1, 49]. Nel *et al* [49] suggest that biological nanomaterial activity may depend on physicochemical characteristics that are not considered in most screening studies, and therefore nanotoxicity testing 'must attempt to characterize the nanomaterial with respect to size (surface area, size distribution), chemical composition (purity, crystallinity, electronic properties etc), surface structure (surface reactivity, surface groups, inorganic/organic coatings, etc), solubility, shape and aggregation'. Nanoparticle–protein complexes forming within biological systems following nanoparticle exposure may produce such biological responses as platelet aggregation, fibrotic changes, translocation of particles, and protein-initiated bioaccumulation, causing

organ failure or severe systemic inflammatory-immune responses. Because of the small size and unique characteristics of these nanoparticles not only are *in vivo* and *in vitro* studies of bio-reactivity to specific nanoparticles needed, but also many more environmental studies. A case in point is a NIOSH study [48] revealing that agitation of unprocessed SWCNT material in a laboratory setting, showed a bimodal distribution of particles in the range of 10–1000 nm, where the particles less than 100 nm in size were composed predominately of nanoropes, with the larger airborne particles consisting of compact non-tubular carbonaceous material [48]. This type of study provides industrial workers, researchers and students using these nanomaterials with specific information for choosing appropriate personal protective equipment and designing processing strategies to maintain safe nanomaterial handling. For each type of nanomaterial used, doing the research to understand the potential toxicity is essential. Until more is known about these materials, and in the face of ever more development and production of new types of nanoparticles, it is important to use universal safety precautions and to avoid direct contact, aerosolization or airborne distribution of these nanoparticles, even though many nanoparticles may be designed to pose no danger to biological systems.

Each type of biological cell may also respond differently to nanoparticles, because they too have very different surface characteristics (charge, surface ligands, surface recognition factors, mucopolysaccharide coatings, age-related surface changes, antigenic sites, etc) and these can significantly alter how one cell may interpret an interaction with a foreign material. What may be benign to one type of cell may produce an inflammatory response in other cells or tissues. In humans, individual reactivity and immune responses of cell types can vary from individual to individual as a result of genetic inheritance and mutation (susceptibility) [49]. Therefore, nanoparticles and new nanomaterials need to be tested, and their biocompatibility fine-tuned to be optimized for safety at the earliest days of nanomaterial design and development for the protection of researchers, industrial workers, the environment and any exposed living organism.

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