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Perspective

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Culture Medium-Associated Physicochemical Insights on the Cytotoxicity of Carbon Nanomaterials

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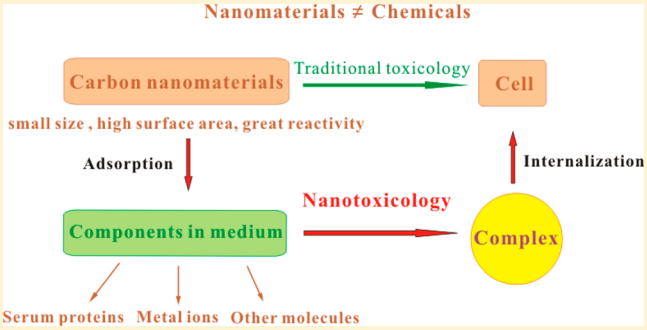
ABSTRACT: Carbon nanomaterials are the most studied materials in nanotechnology. There have been numerous studies on cytotoxicity assessments of carbon nanomaterials, which, however, often lead to controversy. It is generally considered that chemical and physical properties of carbon nanomaterials should have specific biological outcomes. More recent studies have identified the significance of environmental factors surrounding nanomaterial-treated cells. In this perspective, we mainly review culture medium-associated physicochemical insights on the cytotoxicity of carbon nanomaterials, which are largely based on studies in our laboratory. These studies established the close relationship and interplay among the physicochemical properties of the nanomaterials, culture medium, and their toxicological responses.



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1. INTRODUCTION

Over the past 15 years, we have seen an exponential rise in the number of papers on nanotoxicology,[1−3] and the initial screening for nanotoxicity is preferably performed in vitro.[4,5] However, certain standard cytotoxicity assays that are well suited to assess chemical toxicity generate conflicting results when nanomaterials are assessed.[6−8] We consider that the novel physicochemical properties of nanomaterials may generate specific biological outcomes. For the past 10 years, our research group has been performing a series of studies on the unique physicochemical properties of nanomaterials and the resultant impact on bioeffects. Here, we mainly review our work on the culture medium-associated physicochemical insights on the cytotoxicity of carbon nanomaterials.

2. STRUCTURES AND PROPERTIES OF CARBON NANOMATERIALS

Carbon nanomaterials are the most celebrated products of nanotechnology, encompassing fullerenes, nanotubes, graphene, nanodiamonds, and a wide variety of related forms.[6] Fullerenes are carbon allotropes which roll up to form closed-cage, hollow spheres. The most readily available member of the fullerene family, C_{60}, is a truncated icosahedron formed by 12 pentagons and 20 hexagons where each atom is placed at a vertex.[9] Their unique structures lend them several properties including photosensitivity, redox property, high chemical reactivity, and excellent catalytic and magnetic properties. By introducing proper hydrophilic residues, water-soluble fullerene derivatives have received much attention for their potential biomedical applications.[10,11] The Nobel Prize in chemistry in 1996 was awarded jointly to Kroto, Curl, and Smalley for their landmark discovery of fullerenes.

Carbon nanotubes (CNTs) are well-ordered, all-carbon hollow graphitic nanomaterials with a cylindrical nanostructure. They are either single-walled nanotubes (SWNTs) or multi-walled nanotubes (MWNTs). SWNTs are constructed of a

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single sheet of graphite (diameter 0.4−2 nm), while MWNTs consist of multiple concentric graphite cylinders of increasing diameter (2−100 nm). The length of both of them can be up to a few micrometers. CNTs possess high tensile strength, ultralight weight, rich electronic properties, and excellent thermal and chemical stabilities.[12,13]

Graphene is a monolayer of carbon atoms that are tightly packed into a two-dimensional honeycomb lattice structure.[14] Since the seminal work of Geim and Novoselov on freestanding graphene in 2004,[15] various forms of graphene sheets such as graphene oxide (GO) and reduced graphene oxide (rGO) have been actively explored.[16] This extremely thin nanomaterial possesses very high mechanical stiffness and elasticity, as well as extraordinary electronic and thermal transport properties.[15−18] Geim and Novoselov's discovery of graphene earned them the 2010 Nobel Prize in chemistry.

Nanodiamonds (NDs), a new member of the carbon nanoparticles family, have a truncated octahedral architecture that is typically about 2 to 8 nm in diameter.[19] Because of the high surface free energy of a single particle, NDs dispersed in aqueous solution usually spontaneously form clusters of tens to hundreds nanometers with a lower free energy.[20] They exhibit various superior characteristics such as optical transparency, chemical inertness, and extremely high hardness, stiffness, and strength.[21,22] In addition, detonation of carbon-containing explosives can be used in an inexpensive synthesis of NDs on a large scale.[23]

In general, carbon nanomaterials with small size have ultrahigh surface area. For example, the typical value of the surface area of SWNTs is around 300 m^2/g.[24] The two-dimensional structure endows graphene with a large theoretical surface area of about 2600 m^2/g. In fact, a Brunauer−Emmett−Teller (BET) measurement showed that the surface areas of graphene with different layers range from 270 to 1550 m^2/g.[25] Therefore, these carbon nanomaterials can efficiently adsorb various biomolecules such as drugs, peptides, proteins, and nucleic acids.[26−31] NDs dispersed in aqueous solution spontaneously form clusters with large particle size; however, there are many nanoscaled pores and vacancies in the interior of the ND cluster. Various molecules can be adsorbed in these nanoscaled pores in the interior of ND clusters or on the surface of ND clusters by noncovalent interactions.[32]

3. OVERVIEW OF CYTOTOXICITY STUDIES ON CARBON NANOMATERIALS

Early studies of carbon nanomaterial toxicity have produced apparently conflicting results. For example, in 1994, Scrivens et al. showed that C_{60} fullerenes had no effect on the proliferation of immortalized human keratinocytes or fibroblasts.[33] Similarly, Fiorito et al. found that C_{60} fullerenes did not stimulate the release of NO by murine macrophage cells in culture and possessed a very low toxicity against human macrophage cells.[34] On the contrary, in the work of Sayes et al., they showed that C_{60} was cytotoxic to human skin (HDF) and liver carcinoma (HepG2) cells. Water-soluble functional groups on the surface of a fullerene molecule decreased the toxicity of pristine C_{60}.[35] Subsequently, they evaluated the fundamental cytotoxic mechanisms of C_{60} fullerenes and demonstrated that lipid peroxidation and resultant membrane damage were responsible for their cytotoxicity.[36]

Shvedova et al. reported the first cytotoxicity study on CNTs in 2003, in which they investigated the effect of unrefined SWNTs on human epidermal keratinocytes (HaCaT). Results showed that exposure to SWNTs resulted in accelerated oxidative stress, loss in cell viability, and morphological alterations to the cellular structure.[37] Similarly, Monteiro-Riviere et al. found that MWNTs incubated with human epidermal keratinocyte (HEK) cells for up to 48 h resulted in pro-inflammatory cytokine release and loss in cell viability in a time- and dose-dependent manner.[38] However, in the work of Kam et al., human promyelocytic leukemia (HL60) cells were incubated with SWNTs for 24 and 48 h, and no appreciable cell death was observed.[39] In another study, Dumortier et al. found that SWNTs were internalized by B and T lymphocytes as well as macrophages and did not influence the functional activity of immunoregulatory cells.[40]

For the case of graphene and its derivatives, most of the work reported that they induced toxic effects on bacteria or mammalian cells.[41−43] For instance, Akhaven and co-workers found that GO and rGO caused bacterial membrane damage and that rGO was more toxic than GO.[42] Zhang et al. demonstrated that graphene induced concentration- and shape-dependent cytotoxic effects to neuronal PC12 cells, and the reactive oxygen species (ROS) generated by graphene was proposed as one of the main mechanisms for the cytotoxicity.[43] On the contrary, Ryoo et al. reported that graphene and GO were highly biocompatible and improved gene transfection efficacy in NIH-3T3 fibroblasts.[44] In the work of Ruiz et al, they demonstrated that GO did not have intrinsic antibacterial, bacteriostatic, and cytotoxic properties in both bacteria and mammalian cells. Interestingly, it seemed that GO acted as an enhancer of life, increasing not only mammalian cell growth but also bacterial growth.[45]

So far, various studies show that NDs are consistently tolerated by a variety of cells.[46−48] Schrand et al. first reported that NDs showed a minimal toxic effect on cell viability (by MTT and ATP production assays) and did not cause any significant production of ROS.[46] When compared with other carbon-based nanomaterials including carbon blacks (CBs), MWNTs, and SWNTs, NDs showed the best biocompatibility on neuroblastoma and macrophage cells.[47] Similarly, Liu et al. measured the toxicity of NDs and CNTs on human lung A549 epithelial cells and HFL-1 normal fibroblasts, and the results showed that NDs did not reduce the cell viability or alter the protein expression profile, while CNTs showed a significant toxicity to the cells.[48] However, conflicting results have also been reported. In the work of Dworak et al., they demonstrated that starting from a concentration of 50 μg/mL, ND-mediated oxidative stress contributed to DNA damage limiting their biocompatibility to lymphocytes and thus inhibited cell proliferation and induced apoptotic cell death.[49]

4. INTERACTIONS OF CARBON NANOMATERIALS WITH COMPONENTS IN CULTURE MEDIUM AND THEIR BIOEFFECTS

In the past 10 years, our research group has been performing a series of studies on nanotoxicology with the aim of evaluating possible effects of unique physicochemical properties of nanomaterials on cytotoxicity assessment. Typically, mammalian cells are cultured in cell culture medium, containing 10% fetal bovine serum (FBS). In this medium, there are large amounts of serum proteins and a variety of organic and inorganic components. The ultrahigh adsorption ability of carbon nanomaterials may allow for efficient loading of these components, and then, interference with toxicity assessment results. Herein, we summarize our main progress in this field.

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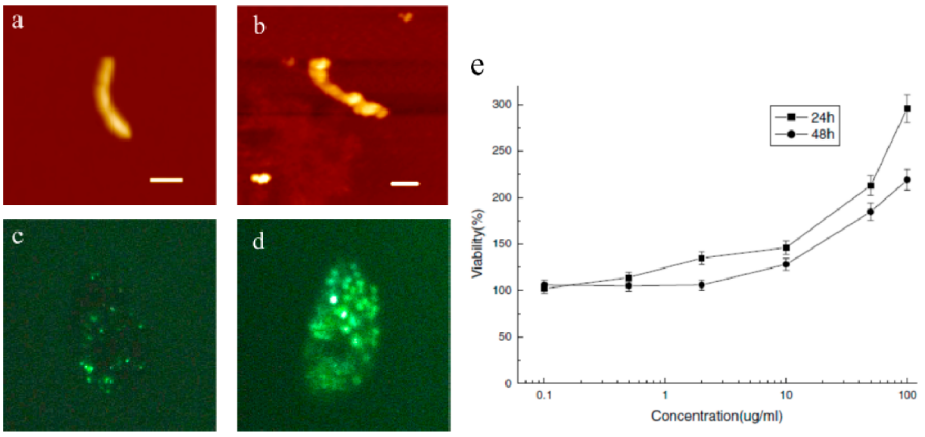

Figure 1. MWNTs showed growth stimulation to *Tetrahymena pyriformis*. (a,b) AFM images of MWNT samples deposited on the SiO_2 substrate. (a) Oxidized MWNT prior to conjugation with peptones and (b) after conjugation to 10 mg/mL of peptone. (c, d) Fluorescence images of *Tetrahymena pyriformis* after incubation in (c) fluorescently labeled peptone solution alone and (d) peptone–MWNT solutions for 2 h (peptones labeled to be green fluorescent). (e) The viability of *Tetrahymena pyriformis* after incubation at various concentrations of MWNTs in proteose peptone yeast extract (PPY) medium.

4.1. Carbon Nanoparticle–Protein Interaction. In 2006, we examined the influence of MWNTs on the growth of the unicellular protozoan *Tetrahymena pyriformis*. Contrary to the findings from most other investigations, we found that MWNTs showed growth stimulation rather than growth inhibition to *Tetrahymena pyriformis*.[50,51] Further experiments indicated that peptones (a major nutrient for cells in culture media), which were bound to and delivered into cells by MWNTs, were responsible for the growth stimulation (Figure 1).[51] This result provided new insights into the importance of culture media components in understanding how CNTs affected living systems.[52]

On the basis of this work, we studied the effects of serum proteins in culture media on the toxicity of MWNTs and three kinds of CBs to mammalian cells. Results showed that serum proteins adsorbed on carbon nanomaterials attenuated the inherent cytotoxicity of carbon nanomaterials and that the extent of toxicity attenuation increased with increasing amounts of serum proteins adsorbed on carbon nanomaterials.[53] Consistent with this work, we demonstrated that CNTs with different surface modification also showed much lower cytotoxicity in the serum-containing cell culture medium when compared to that in serum-free medium.[54] Similarly, in the work of Herzog et al., they demonstrated that serum proteins adsorbed on carbon nanomaterials greatly reduced their toxicity to cells too.[55] Furthermore, Ge et al. investigated the interactions of SWNTs with several kinds of serum proteins including BFG, Ig, Tf, and BSA and found a competitive binding of these proteins with different adsorption capacities, which led to much reduced cytotoxicity for these protein-coated SWNTs, according to their respective adsorption capacities.[56]

In the follow-up work, we carried out systematic investigations on the interactions between GO nanosheets and mammalian cells.[30] Results indicated that at low concentrations of FBS (1%), human cells were sensitive to the presence of GO and showed concentration-dependent cytotoxicity. However, at 10% FBS, the concentration usually employed in cell culture medium, the cytotoxicity of GO was greatly mitigated. We considered that large amounts of serum proteins adsorbed on GO nanosheets attenuated the cytotoxicity of GO arising from direct interactions between the cell membrane and GO nanosheets.[30,41] All of these findings will help further guide the design of safe carbon nanomaterials by comprehensive preconsideration of their interactions with serum proteins.

4.2. Carbon Nanoparticle–Ion Interaction. Five years ago, we evaluated the biocompatibility of the detonation NDs. Consistent with the results reported in the literature,[46−48] we found that NDs in complete cell culture medium showed no apparent toxicity on a variety of cells, whereas in serum-free medium, NDs exhibited apparent cytotoxicity.[32] Subsequently, we demonstrated that the cellular response of NDs in serum-free medium is related to ND–sodium ion interaction. In this medium, a large amount of sodium ions were adsorbed and delivered into the cell interior by NDs (Figure 2). Excessive sodium ions inside the cells induced osmotic stresses followed

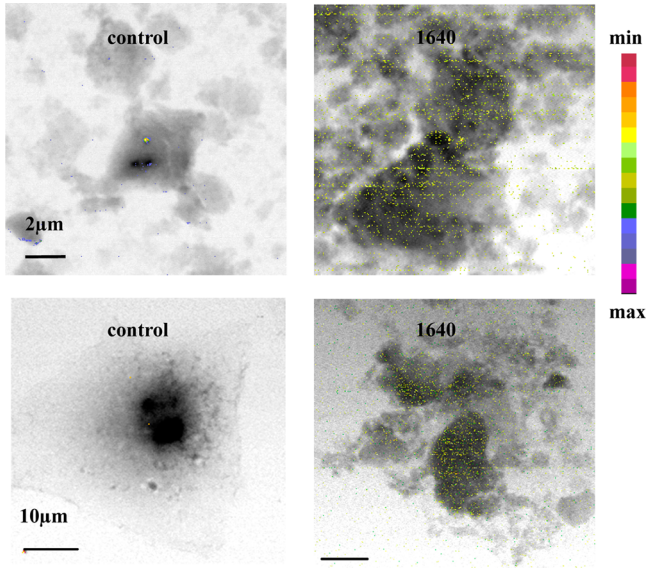

Figure 2. Observation of NDs-Na complexes in RPMI-1640 medium (upper) and HeLa cells (lower) by STXM techniques. The scanning step was 50 nm. The range of quantities noted by the color bar is from 4.1×10^{-6} to 1.9×10^{-5} in (upper, left), from 5.2×10^{-6} to 1.4×10^{-5} in (upper, right), 5.6×10^{-6} to 7.6×10^{-6} in (lower, left), and from 2.0×10^{-5} to 5.5×10^{-5} in (lower, right).

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by cell swelling and an increase in the intracellular levels of calcium and ROS, which led to severe cellular damage. In complete culture medium, however, serum proteins wrapped around the NDs effectively prevented the sodium ions from adsorbing onto the NDs, and as a result, the NDs showed no cytotoxicity.[57]

At present, some reports show that carbon nanomaterials with oxygen-containing functional groups are well suited for adsorption of double-charged metal ions;[58,59] however, there is limited insight about the cytotoxicity triggered by nanoparticle−ion interactions. Recently, we employed both experimental and theoretical approaches to investigate the interactions between NDs and various kinds of metal ions. Results showed that the adsorption capacity of NDs for different metal ions was different, and among them, the adsorption for Cu^{2+} was the most strong. These different ND−ion complexes had different cytotoxicities by influencing the subsequent cellular responses. Detailed investigation of cell responses of ND−Cu^{2+} complexes demonstrated that NDs played the Trojan horse role by allowing more Cu^{2+} accumulate into living cells followed by subsequent release of Cu^{2+} in the low-pH intracellular environment leading to higher ROS level.[60] Our findings provide the evidence of the cytotoxicity triggered by nanoparticle−ion interactions and open new ways in the interpretation and understanding of nanotoxicity.

4.3. Carbon Nanoparticle−Phenol Red Interaction. In addition to serum proteins and metal ions, some reports showed that phenol red can be adsorbed onto carbon nanomaterials, leading to attenuation of their UV/visible spectroscopic characteristics or the removal of their characteristic color by centrifugal ultrafiltration.[61−63] However, little data is still available concerning the cell responses triggered by nanoparticle−phenol red interactions.

We studied the interaction of phenol red in culture medium with carbon nanomaterials (MWNTs and three kinds of CBs) and its effects on cytotoxicity (Figure 3). Results indicated that phenol red, commonly employed in toxicological studies, was nontoxic because of its poor membrane permeation and low concentration in culture medium. Interestingly, when it is highly enriched on and delivered into cells by carbon nanomaterials, pronounced cytotoxicity exhibited. In particular, CBs were nontoxic themselves even after internalization into cells from serum-free culture medium without phenol red, whereas as carriers of phenol red, they played an essential role in the cytotoxicity induced by phenol red.[64] Our results suggest that some constituent components in cell culture medium whose biological activity can be negligible in conventional toxicity assays will interfere with toxicity assessments in nanotoxicology.

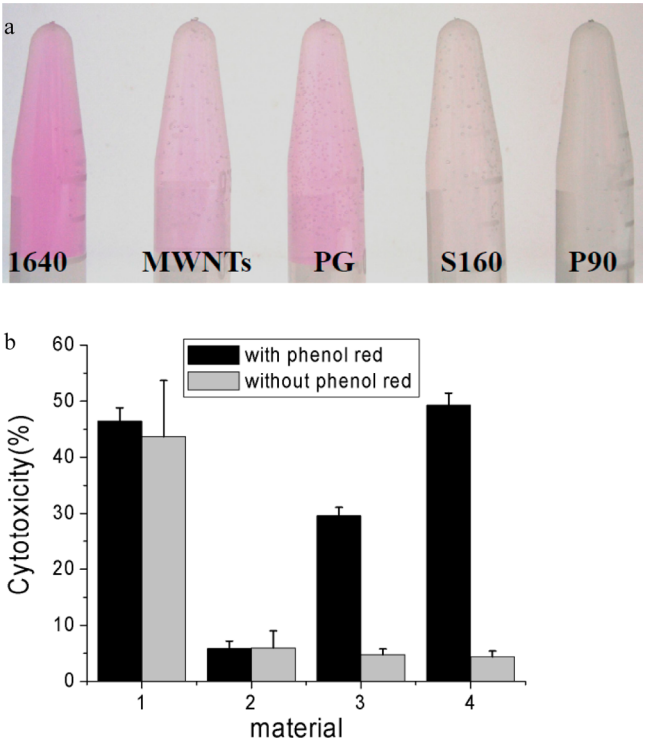

Figure 3. Carbon nanomaterial−phenol red interaction and its cell responses. (a) The color change of phenol red in supernatants of carbon nanomaterials after 2 h of incubation in serum-free medium. (b) Cytotoxicity assessment of HeLa cells exposed to MWNTs, PG, S160, and P90 (denoted by 1, 2, 3 and 4, respectively) in serum-free medium with or without phenol red.

5. CONCLUSIONS AND PERSPECTIVES

From the work mentioned above, we concluded that nanomaterials are different from chemical molecules due to their exceptional features. First, their small size induces permeability changes in cell membranes, enhancing cellular uptake. Second, high surface area, which may produce great chemical reactivity, leading to toxicological responses, positive or negative, from adsorbed components in culture medium such as serum proteins, metal ions, or other molecules, transported into the cell interior during endocytosis of the nanomaterials. Therefore, nanotoxicology is a distinct category of toxicology. Standard toxicity assays, initially developed for the evaluation of direct interaction between chemicals and cells, are often inadequate for nanotoxicity assessment. In nanotoxicology, we should consider the indirect interaction between nanomaterial-component complexes and cells.

To investigate the potentially endless number of biophysicochemical interactions at the nano/cell interface and their toxicological responses, we have developed a two-step toxicity determination method.[53] Briefly, cells were incubated with nanomaterials suspended in designed solution [medium containing 10% FBS, serum-free medium, phenol red-free medium, phosphate buffered saline (PBS), etc.] for several hours and then washed with PBS to remove the uninternalized carbon nanomaterials. Subsequently, cells were incubated in complete cell culture medium for designed times, and viability was determined by the WST/INT/XTT assay. This approach allows for analysis of the cytotoxicity of various kinds of nanomaterial-component complexes as well as pristine nanomaterials. In future, we suggest that detailed studies on physicochemical-associated nanotoxicity should be carried out with an emphasis on establishing a relationship, if any, between the physicochemical properties of the nanomaterials and the toxicological responses.

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Notes

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■ ABBREVIATIONS

CNTs, carbon nanotubes; SWNTs, single-walled carbon nanotubes; MWNTs, multiwalled carbon nanotubes; GO, graphene oxide; rGO, reduced graphene oxide; NDs, nanodiamonds; BET, Brunauer−Emmett−Teller; HDF, human skin cell; HepG2, liver carcinoma cell; HaCaT, human keratinocyte; HEK, human epidermal keratinocyte; HL60, human promyelocytic leukemia cell; ROS, reactive oxygen species; CBs, carbon blacks; FBS, fetal bovine serum; PBS, phosphate buffered saline

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