Sensors as tools for quantitation, nanotoxicity and nanomonitoring assessment of engineered nanomaterials

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The discovery of fullerenes in 1985 has ushered in an explosive growth in the applications of engineered nanomaterials and consumer products. Nanotechnology and engineered nanomaterials (ENMs) are being incorporated into a range of commercial products such as consumer electronics, cosmetics, imaging and sensors. Nanomaterials offer new possibilities for the development of novel sensing and monitoring technologies. Nanosensors can be classified under two main categories: (i) Nanotechnology-enabled sensors or sensors that are themselves nanoscale or have nanoscale materials or components, and (ii) Nanoproperty-quantifiable sensors or sensors that are used to measure nanoscale properties. The first category can eventually result in lower material cost, reduced weight and energy consumption. The second category can enhance our understanding of the potential toxic effects of emerging pollutants from nanomaterials including fullerenes, dendrimers, and carbon nanotubes. Despite the enormous literatures and reviews on Category I sensors, there are few sensors to measure nanoscale properties or sensors belonging to Category II. This class of nanosensors is an area of critical interest to nanotoxicology, detection and risk assessment, as well as for monitoring of environmental and/or biological exposure. This article discusses emerging fields of nanotoxicology and nanomonitoring including the challenges of characterizing engineered nanomaterials and the potentials of combining existing analytical techniques with conventional cytotoxicity methods. Two case studies are provided for development of Category II nanosensors for fullerene nanoparticles and quantum dots. One highlights the uniqueness of a portable, dissolved oxygen electrochemical sensor arrays capable of detecting the ENMs as well as provide rapid nanotoxicological information. This review has shown that addressing the complex and critical issues surrounding the environmental transformation and toxicity of ENMs must be accompanied by the creation of new approaches or further developments of existing instrumentation.

1. Introduction

Nanotechnology—the science of assembling or controlling matter atom-by-atom at the nanoscale level and its potential applications—presents both opportunities and challenges. Opportunity exists to create novel materials based on the enhanced catalytic, optical, magnetic and electrical properties of nanomaterials. Some

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engineered nanomaterials (ENMs) have already been incorporated into a range of commercial products, including pharmaceuticals, automobile additives, personal care, such as sunscreens and cosmetics, clothing, sporting equipment, tires, detergents, and stain-repellants.[1] ENMs are also being used as probes for ultrasensitive molecular sensing and diagnostic imaging, agents for photodynamic therapy (PDT) and actuators for drug delivery, triggers for photothermal treatment, and precursors for building solar cells, electronics and light emitting diodes.[2] Despite the increasing applications of engineered nanomaterials, a complete understanding of the size, shape, composition and aggregation-dependant interactions of nanostructures with biological systems is limited. Also, there are a number of important, unresolved questions concerning the safety of ENMS. The potential exposure scenarios, and their interaction with the biological and environmental systems are largely unknown. Hence, subdisciplines of nanotechnology such as nanotoxicity, nanomonitoring and/or nanomeasurements are emerging. Nanotoxicology focuses on the characterization and categorization of the health effects caused by engineered nanomaterials in order to correlate the nanoparticles structure/function with toxicity.[3] Nanomonitoring or nano-measurement refers to the science of isolating, detecting, characterizing, and quantifying ENMs in complex environmental, biological or ecological samples.

Recent studies have shown that some of the special properties that make nanomaterials useful may also cause them to pose hazards to humans and the environment.[4] For example, silver nanoparticles are harmful to the environment because they may destroy environmentally benign bacteria that are used for waste water treatment[5,6] although they are widely used in different products in the market such as detergents, wound dressings and disinfectants. The effects of engineered carbon nanotubes and metal oxide nanoparticles on diverse microbial communities,[7] algae, plants, and fungi as well as on aquatic invertebrates have also been reported.[8] The toxicity mechanisms of such nanomaterials has been attributed to the production of reaction oxygen species and accidental release of metal ions.[8] Smaller particles in ambient air have been demonstrated in inhalation studies to exhibit adverse health effects due to deeper penetration into lungs and large surface areas.[9] These particles are capable of bypassing the blood-brain barrier through the olfactory bulb. Some metal oxide nanoparticles have been reported to affect the inflammatory processes of the central nervous system.[10] Further, a set of predictive measures of nanomaterial toxicity have also been identified[11–14] relying on the detection of the generated reactive oxygen species (ROS),[15,16] mitochondrial perturbation, inflammation response pathways, lipid peroxidation, protein denaturation and degradation, and DNA damage. Nel *et al.*[16] have stressed the importance of pragmatic and mechanism-based approach in testing the potential harmful effects of engineered nanomaterials. Oberdörster *et al.* have outlined three key elements of nanoparticle toxicity screening strategies:[17] (i) physicochemical characterization (size, surface area, shape, solubility, aggregation), elucidation of biological effects involving (ii) *in vitro* and (iii) *in vivo* studies. In that respect, a broad array of analytical tools and methods are needed to perform such characterizations.

Chemical and biosensors are well suited to complement standard analytical methods for detection of environmental toxins due to their low cost, sensitivity, portability and simplicity. The present review focuses on the development of nanosensors for assessing the toxicity of engineered nanomaterials and case studies are described for the development of nanosensors for fullerenes and quantum dots. This article also provides a short summary of the different techniques which are currently used for the characterization and/or absolute quantification of engineered nanomaterials and nanotoxicity.

2. Toxicity of engineered nanomaterials

Currently, the number of engineered nanomaterials on the market is large and is expected to increase with advances in synthetic and technological developments. As shown in Fig. 1, a large number of engineered nanomaterials have been synthesized to date. This figure also shows that since the first two papers on this topic appeared in 1991, the number of publications did not increase significantly until 2004, when the total number of publications rose to 754. From 2005 to August 2009, there were 3,454 articles published thus bringing the total number of papers published to date to 4,208.

While nanomaterials have numerous applied uses and the benefits of nanotechnology are widely publicized, the discussion of their potential effects on human health and the environment is just beginning. In contrast to the comparatively large number of articles on nanomaterial synthesis (4208), a similar search on the Web of Science with the search word: "nanotoxicology" produced only 171 hits to date. These include 29 review papers, 103 articles, 16 meetings, 13 editorials and 10 news articles. Clearly, there is an increasing interest in the toxicity of engineered nanomaterials.

In general, nanoscale particles, whether termed ultrafine, engineered, intentional, or incidental, pose challenges for physical, chemical and biological characterization. Meantime, these new materials could have a number of potential causes of toxicity or concerns: (1) nanostructures have been demonstrated to have electronic, optical, and magnetic properties that are related to their physical dimensions, and the breakdown of these nano structures could lead to unique toxic effects that are difficult to predict. (2) Nanostructured surfaces are utilized in many

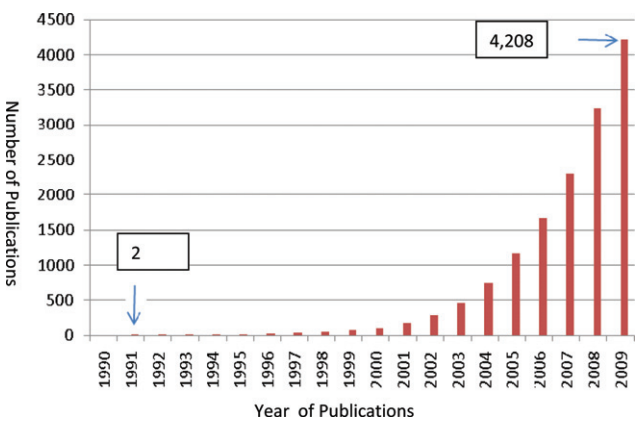

Fig. 1 Published papers on nanomaterials synthesis from 1990–2009. These statistics were generated from the ISI Web of Science using a combination of search terms that represent "nanomaterials and synthesis".

catalytic and oxidative reactions. If these reactions induce cytotoxicity, the toxicity can be greater than what is observed for a similar bulk materials because of the enhanced surface area-to-volume ratio for nanoscale materials, (3) some nanostructured materials contain metals or compounds with known toxicity and thus the breakdown of these materials can elicit similar toxic responses to the components themselves.[2] For example, the removal of tetrahydrofuran (THF), the solvent used in preparing ~30 nm–100 nm particles of C_{60} resulted in a loss of toxicity: *Biomaterials* **27** (29): 5049–58, 2006. (4) Synergistic and/antagonistic reactions of ENMs with other chemicals may be difficult to isolate and (5) ENMs could be used as potential chemical/biological weapons.

The first steps to identify a number of critical risk assessment issues regarding manufactured nanomaterials[9] began at a 2004 workshop cosponsored by the National Science Foundation and the National Institute of Environmental Health Services. Critical issues identified include exposure assessment, toxicology, ability to extrapolate, environmental and biological fate, transport mechanism, persistence, transformation, and overall sustainability. This workshop was instrumental in bringing about the National Science and Technology Council (NSTC). The NSTC is the principal means by which the Executive Branch coordinates science and technology policy across the diverse entities that make up the Federal research and development enterprises. A subcommittee of the NSTC is the Nanoscale Science, Engineering and Technology (NSET), which is responsible for coordination of the National Nanotechnology Initiative (NNI). Recently, as part of a national effort to stimulate new research and knowledge in this area, the National Institute for Occupational Safety and Health (NIOSH) collaborated with NSET to co-sponsor the workshop on Human and Environmental Exposure Assessment of Nanomaterials from Feb. 24–25, 2009 in Bethesda, MD.

The cytotoxic potential, detection and characterization of nanomaterials are dependent on factors such as functionalization, geometry, and material. One study evaluated a variety of carbon nanomaterials and found single walled nanotubes (SWNT) to be much more cytotoxic than fullerenes or multi-walled nanotubes (MWNT).[14] Conversely, another study evaluating MWNT, carbon nanofibers and carbon black found that the smaller the size of the nanomaterial, the greater the toxicity.[18] A key nanotoxicity study worthy of note is that reported by A. Tagaki *et al.* (2008).[152] These authors demonstrated that intraperitoneal administration of MWNT led to the induction of mesothelioma in p53 heterozygous mice after these model animals were injected with micrometer sized MWNTs, with lengths reaching tens of micrometers that correspond to the size and shape of asbestos. The result of this study points to the possibility that carbon-made fibrous or rod-shaped micrometer particles may share the carcinogenetic mechanisms postulated of asbestos. Some researchers have also shown that functionalization can change the cytotoxicity of nanomaterials. For example, the cytotoxicity of fullerenes can be decreased after fullerenes were hydroxylated with 24 hydroxyl groups,[19] while the cytotoxicity of carbon nanomaterial may be enhanced if it was functionalized with carboxylic acid moieties which promote aqueous phase dispersion.[7] These studies, as others, have primarily been tested *in vitro* and the results may not accurately translate to *in vivo* interaction. These and many similar contrasting reports make it difficult for researchers and policy makers to determine which nanomaterials to study and how to regulate the industry.

3 Environmental detection and characterization of ENMs-EPA perspectives

The mission of the United States Environmental Protection Agency (EPA) is to protect human health and the environment. EPA must have a sound scientific basis to carry out this mission. EPA conducts and supports programs that address human health and the environmental effects of substances; assesses potential risk management approaches; and finds innovative, cost-effective ways of reducing risks. According to the EPA Nanotechnology White Paper, "a challenge for environmental protection is to help to fully realize the societal benefits of nanotechnology while identifying and minimizing any adverse impacts to humans or ecosystems from exposure to nanomaterials".

Emerging areas of concern for the EPA include understanding health and environmental effects of ENMs and being able to communicate the exposure (health and safety) risks to the public. Extensive research is needed which provides for accuracy, precision and sensitivity of analytical techniques for understanding ENMs. Research areas focus on the engineering factors involved in the transport, transformation and longevity of ENMs (Draft Nanomaterial Research Strategy (NRS), EPA/600/S-08/002). EPA's National Exposure Research Laboratory (NERL) Environmental Sciences Division (ESD) is responsible for conducting studies for detecting, characterizing, quantifying, and monitoring of nanotechnology and emerging contaminates in the environment. Areas of research include source, fate, and transport, exposure, preventing and mitigating risks of nanomaterials through identifying technologies that can be applied to measure and minimize exposure to ENMs. ESD concern is greatly vested with the transport of nanomaterials in the environment which requires understanding the mechanisms of environmental media, the various environmental parameters, and the various nanomaterial parameters. Nanomaterial parameters include particle size and charge, chemical or elemental composition, and surface modifications. Environmental media parameters include pH, ionic strength, flow rate, composition, and the presence of naturally occurring (such as dissolved organic carbon) and anthropogenic contaminants.[20] EPA is seeking to discover and define previously unknown and poorly understood vulnerabilities and provide quantitative data to exposure risk assessors which would make it possible to predict estimates of nanomaterials that most likely would be of concern (US EPA Nanotechnology White Paper, EPA 100/B-07/001).

4. Conventional methods for assessing nanotoxicity

As described earlier, the increased prevalence of nanomaterials in consumer goods has laid the foundation for the emerging field of nanotoxicology and nanomonitoring. This has brought attention to the research needs regarding the toxicological assessment of ENMs. A tremendous amount of activity has been witnessed in this field as reviewed by Marquis *et al.*[3] However, it is important

to recognize that particle or agglomerate size distribution can, and often does, change as a material is prepared and used in toxicological studies. The size and shape of the material interacting with an organism may differ dramatically from its original form. Thus, the size distribution "as doses" might be quite different from that of "as-generated" or "as-received" material.[3] Conversely, hardly any single assay is readily available on quantification, measurement and monitoring technologies for nanomaterials. Techniques are best used in concert with each other to provide a more complete understanding of uptake.

In this section, we summarized the existing methodologies for detection of nanotoxicity including microscopy, spectrometry, classical cell culture techniques, sensors as well as computer-assisted molecular modeling techniques. As shown in Fig. 2, there is an intersection between the characterization techniques for ENMs and the detection of its nanotoxicity. Relevant properties that could be measured include dose, purity, particle size & distribution, shape, surface chemistry, surface charge, surface area and surface activity as well as their ability to penetrate the biological systems.

4.1 Microscopy techniques

Microscopy is one of the most powerful techniques to provide valuable information regarding the size, shape, and morphology of nanomaterials. Examples include electron and proton microscopy, atomic force microscopy (AFM), scanning probe microscopy and scanning tunneling microscopy (STM), and each has specialized skills. Table 1 provides a summary of the microscopic techniques for characterizing nanomaterials. The advantages and drawbacks of each technique are also highlighted. Specifically, TEM has provided the most detailed information regarding *in vitro* nanoparticle uptake and localization by allowing both visualization of nanoparticle location within a cell or tissue and, in conjunction with spectroscopic methods, characterization of the composition of the internalized nanoparticles[3,21,22–25] before and after exposure to cells. High resolution transmission electron microscopy (HRTEM) can especially be the best choice to identify the crystalline structures of particles, as reported by Petri-Fink *et al.*[26] for super-paramagnetic iron oxide nanoparticles and by Warheit *et al.* quartz particles.[22] However, HRTEM is subject to artifacts caused by sample preparation or special analysis conditions. For example, it requires high vacuum and thin sample sections or particles of limited diameter to allow the electron beam to penetrate through the sample. Tissue sample preservation, fixation, and staining require skills to preserve the sample details and to avoid introduction of artifacts.[27] The SEM can be used to image the sample surface by scanning it with a high-energy beam of electrons in a scanning pattern. Lin *et al.* has examined the phototoxicity of ZnO nanoparticles by *Lolium perenne* (ryegrass) using SEM.[23] The results indicated that ZnO nanoparticles greatly adhered onto the root surface. Scanning proton microscopy (SPrM) is an analogue of electron microscopy and enables the unique feature of elemental mapping down to the parts-per-million level with high accuracy. However, the technology for focusing protons is technologically more complex than for electrons due to the much higher momentum of protons. Tong *et al.*[28] has probed the cytotoxicity of nanoparticles (ZnO, Al_2O_3 and TiO_2) and organic compounds using SPrM on a *T lymphoblastic* leukaemia Jurkat cell lines.

As a result of continuous developments in sample preparation, imaging techniques, and instrumentation, AFM is regarded as a companion of both X-ray crystallography and electron microscopy. AFM has also evolved into an imaging method that yields structural details of biological samples such as proteins, nucleic acids, membranes, and cells in their native environment. It has been used in various bio-medical applications including the testing of cellular toxicity of nanoparticles and carbon-nanotubes.[29] In addition, AFM is used to measure the surface area of nanoparticles, which is thought to play an important role in the toxicity of nanoparticles.[54–56,153] Chen *et al.* used AFM to

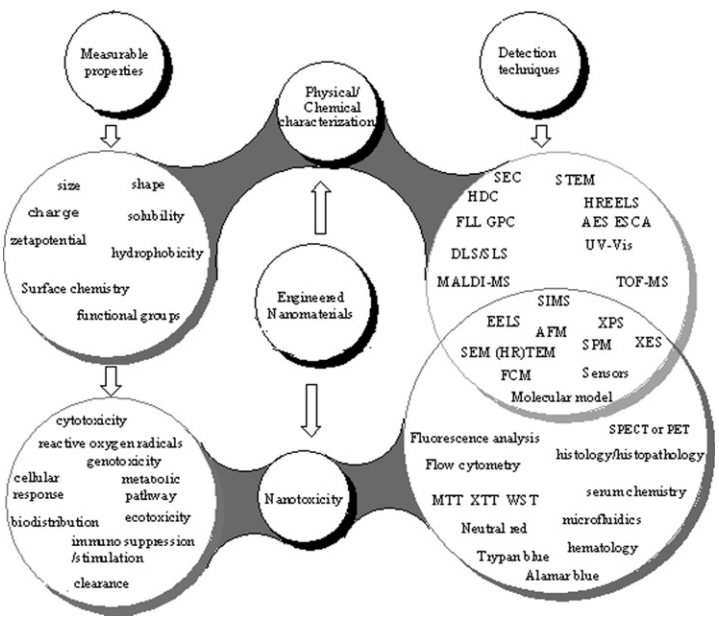

Fig. 2 Major techniques employed for the characterization of engineered nanomaterials and for assessing nanotoxicity.

Table 1 Microscopic Techniques for Characterizing Nanomaterials[a]

Method/References	Applications	Nanomaterials	Advantages and drawbacks
TEM[3,21,23–25]	*In vitro* uptake/localization	Organic, inorganic & hybrid nanomaterials	• Visualization of ENMs •TEM images provide poor resolution of diffuse electron materials
HRTEM[22,26,27]	Crystallization of particles	Iron oxide, quartz particles	• Individual particles and agglomerates can be resolved • Provides valuable information on size, charge and morphology • High resolution microscopy is subject to artifacts caused by sample preparation conditions • Requires thin sample sections or particles of limited diameter to enable the electron beam to penetrate through the sample
SEM[23]	In vitro/in vivo surface morphology	Phototoxicity of ZnO, Al_2O_3, TiO_2	•Requires high vacuum conditions • A specimen is normally required to be completely dry
SprM[28]	T lymphoblastic leukaemia Cell lines	Organic, inorganic & hybrid nanomaterials	•Nuclear microscopy can quantitatively map all elements in the periodic table • Provides simultaneous structural imaging • Provides unique features of elemental mapping • Suitable for parts-per million level sensitivity • High momentum of protons creates a more complex focusing problems for protons than for electrons
AFM[29]	Biomedical Imaging & cellular toxicity of nanoparticles	Carbon nanotubes	• Allows the determination of surface area of ENMs • Provides sub-nanometer resolution at a reasonable signal-to-noise ratio under physiological conditions • AFM is regarded as a companion of both X-ray crystallography and electron microscopy and has evolved into an imaging method that yields structural details of biological samples such as proteins, nucleic acids, membranes, and cells in their native environment
AFFM[30]	Biomedical imaging		• Provides biochemical identification
SNUL	Imaging nanoparticles		• Applicable for imaging
Fluorescence microscopy[31,32]	Cellular cytotoxicity	Imaging of SWCNTs	• Qualitatively determines the binding and uptake of nanomaterials
	Nanoparticle uptake and localization	Dendrimer ENMs	• Visual inspection of cells with bright field microscopy for changes in cellular or nuclear morphology • Quantitative assessment can be achieved in a manner similar to ICP-AES through use of bulk fluorescence or on a cell-to-cell basis using confocal fluorescence • Cellular uptake is a necessary prerequisite

[a] *Abbreviation key:* TEM: transmission electron microscopy, SEM: scanning electron microscopy, SPrM: Scanning proton microscopy, AFM: atomic force microscopy, AFFM: atomic force fluorescence microscope, SNUL scanning near-field ultrasonic holography, SWCNTs:single walled carbon nanotubes.

determine the shape and size of copper nanoparticles, and then calculated the average surface area per gram. However, the most common method to measure the surface area is Engelhard, the multipoint Brunaeur, Emmett and Teller (BET) method. In this case, the gas absorption to the surface of these particles is first measured, and then the surface area can be calculated out using BET method. Instruments for BET method are commercially available from Quantachrome Instruments, Beckman Coulter and Micromeritics. Besides, scanning mobility particle sizer (SMPS), transmission electron microscopy (TEM), and diffusion charging (DC) have also been reported for measurements of some nanoparticles.[154] Atomic force fluorescence microscope (AFFM) has been developed that combines the high-resolution topographical imaging of AFM with the reliable (bio)-chemical identification capability of optical methods.[30] A related scanning probe microscopy technique called scanning near-field ultrasonic holography can now image nanoparticles buried below the surfaces of cells, which could prove useful in nanotoxicology.[31,32]

4.2 Classical cell culture techniques

In vitro cytotoxicity assays are the major alternative methods to animal testing for basal cytotoxicity assessment of chemicals, typically indicating the number of cells which are dead or alive after exposure to test chemicals. Conventional *in vitro* cell-based assays are commonly used to screen cytotoxic effects induced by chemicals in a variety of cell systems. Examples include biochemical methods such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT), neutral red uptake (NRU), ATP and lactate dehydrogenase (LDH) measurement, Sulforhodamine B (SRB) assay, WST assay. Others include growth assays such as colony forming efficiency (CFE), cytokine

assay, phagocytosis assay, nitric oxide assay, and glutathione assays. These techniques are equally applicable to measure the cytoxicity of nanoparticles. Several recent peer-reviewed publications have focused on a set of predictive measures of cytotoxicity of nanomaterials[11–14] through the detection of the generated reactive oxygen species (ROS),[15,16] focusing on oxidative stress, mitochondrial perturbation, inflammation response pathways, lipid peroxidation, protein denaturation and degradation, and DNA damage. Table 2 provides a list of the classical cell cultures and other techniques with utilities for assessing the risks posed by engineered nanomaterials.

Table 2 List of Instrumental and Classical Cell Culture Techniques for Characterizing Nanomaterials[a]

Abbreviation	Methods/Definitions
Classical cell culture	
MTT[3,11,155]	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS[156]	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NRU[155]	neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride) uptake
XCT[157]	X-ray Computed Tomography
LDH[155]	Lactate dehydrogenase
SRB[158]	Sulforhodamine B assay
WST-1[159]	(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)
CFE[160]	Colony forming efficiency
FCM[161]	Flow cytometry
Assays and kits	
Cytokine assay[162]	A blood test to detect interleukins
Phagocytocis assay	Engulfing and destroying of fungi and bacteria
Nitric Oxide assay	A Kit for the Quantitative Colorimetric Determination of Nitric Oxide
Gluthathione assay	
Tryphan Blue assay	
Fluorescence assay	
Instrumental techniques	
GPC/SEC	Gel Permeation Chromatography (GPC)/Size Exclusion Chromatography (SEC)
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
NMR	Nuclear magnetic resonance
XRD	X-Ray Diffraction
XPS	X-ray photon scattering spectroscopy
TGA	Thermogravimetric analysis
FFF	Field-Flow fractionation
ICP-AES(OES)	Inductively coupled plasma atomic emission spectroscopy
TIRF	Total internal reflectance fluorescence
EELS	Electron Energy Loss Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MALDI	Matrix Absorption Laser Desorption Ionization
EDS	Energy Dispersive Spectroscopy
PET	Positron Emission Tomography
XPS	X-ray Photoelectron Spectroscopy
SRT	Synchrotron radiation techniques

[a] Readers are referred to the cited reviews and articles for detailed descriptions of the advantages and drawbacks.[3,11,156–163]

4.2.1 Colorimetric assays. Colorimetric methods are the major tools employed in cytotoxicity assessment throughout published nanomaterials studies. These colorimetric methods can be further categorized into tests that measure plasma membrane integrity and mitochondrial activity.

Exposure to certain cytotoxic agents can compromise the cell membrane, which allows cellular contents to leak out. Viability tests based include neutral red. Neutral red, or toluylene red, is a weak cationic dye that can cross the plasma membrane by diffusion. The dye tends to accumulate in lysosomes within the cell. If the cell membrane is altered, the uptake of the dye decreased and can leak out, allowing discernment between live and dead cells. Cytotoxicity can be quantified by taking spectrophotonic measurements of the neutral red uptake under varying exposure conditions. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is among the most versatile and popular assays used for in vitro toxicology. Mitochondrial activity can be tested using tetrazolium salts as mitochondrial dehydrogenase enzymes cleave the tetrazolium ring and this reaction only occurs in living cells. Reduction of water-soluble MTT salt by metabolically active cells leads to the formation of MTT-formazan crystals. The insoluble MTT-formazan is deposited in mitochondria, in the cytoplasm, and in the regions of plasma membranes. Reduction of MTT in isolated cells is regarded as an indicator of "cell redox activity". This technique has many advantages when compared to other toxicity assays because it requires minimal physical manipulation of the model cells and yields quick, reproducible results requiring simple optical density acquisition. Other tetrazolium-based assays used to test the cytotoxicity are the MTS, XTT or WST assay. The number of living cells can be determined similarly by quantifying the production of soluble formazan. In assays that produce insoluble formazan dyes (such as the MTT assay), exocytosis of the crystalline product can skew results; therefore assays that produce soluble dyes (such as MTS, XTT or WST-1) are preferred. A summary of colorimetric methods in vitro toxicological techniques used in the assessment of nanotoxicity has been reviewed.[3,11]

Although understanding nanoparticle effects on mitochondrial activity is important, it is just one of many relevant cellular functions. The major problem with using traditional toxicology assays is that nanomaterials may interfere directly with the signal transduction based on the increased reactivity of their surface sites compared to bulk materials. In one example, Ag nanoparticles were found to interact directly with the tetrazolium salt of the MTT assay, suggesting greater than 100% viability of nanoparticle-exposed cells based on the ability of the nanoparticles to generate the colored formazan product. Using a cell-free system, it was also reported that polyoxyethylene sorbitan monooleate-suspended SWCNTs interfered less with MTT assays than sodium dodecyl sulfate-suspended SWCNTs. Moreover, depending on the purification procedure of SWCNTs, they were able to convert MTT into its MTT-formazan insoluble form in the absence of any living system.[33]

In addition, Trypan blue, a diazo dye, is only permeable to cells compromised membranes, therefore dead cells are stained blue while live cells remain colorless. The amount of cell death can be determined *via* light microscopy. More extensive cytotoxity studies have provided supportive information for nanoparticles studies such as cobalt, magnetic, TiO_2, Al_2O_3, Silica, silver, carbon nanotubes[12,34–41,11,42] by examining the extent of DNA damage using several methods. This includes flow cytometry, micronuclei assay, comet assay, mutation frequency, and 8-ooxo-dG assays as well as DNA microarray studies. Whole genome microarray analysis of the early gene expression changes induced by 10- and 500-nm particles showed that the magnitude of change for the majority of genes affected correlated more tightly with particle surface area than either particle mass or number.[17,43]

4.2.2 Fluorescence assays. Optical imaging techniques have recently attracted a lot of interest for medical applications due to its non-invasive procedure, high temporal resolution and relative low cost. Fluorescence imaging in the visible-wavelength range is routinely used for conventional and intravital microscopy.[44] Because cellular uptake is a necessary prerequisite, fluorescence microscopy has been used to qualitatively determine the binding and uptake of nanomaterials. One simple cytotoxicity test involves visual inspection of the cells with bright field microscopy for changes in cellular or nuclear morphology. Fiorito *et al.* first used this technique when evaluating the cytotoxicity of single-walled carbon nanotubes (SWNTs).[45] Quantitative assessment can be achieved in a manner similar to ICP-AES through use of bulk fluorescence or on a cell-to-cell basis using confocal fluorescence. Total internal reflectance fluorescence (TIRF) has great potential for real-time imaging of fluorescent nanoparticles as in the work by Lee *et al.* examining uptake and localization of dendrimer nanoparticles for gene delivery.[46] Real-time NIR fluorescence imaging has been developed for the quantitation and biodistribution of semiconductor quantum dots.[47] Two important fluorescence dyes (calcein acetoxymethyl (calcein AM) and ethidium homodimer) have been used to test the live/dead viability test when exposed to the nanomaterials, such as fullerenes and gold nanoshells. When excited at 495 nm, calcein AM and ethidium homodimer emit distinct fluorescence signatures at 515 nm and 635 nm, respectively.

Alamar Blue has been recently applied to nanotoxicological studies by assaying cellular redox potential.[48] Alamar Blue is reduced to a soluble fluorescent product, resorufin (λem 590 nm), providing simpler sample preparation compared to the MTT assay. However, interpretation of Alamar Blue results is difficult because the biochemical mechanisms of Alamar Blue reduction have not yet been explored. Additionally, nanoporous silicon has been found to react with Alamar Blue in the absence of cells.[49] In the case of cytotoxicity, it is important to recognize that cell cultures are sensitive to changes in their environment such as fluctuations in temperature, pH, and nutrient, in addition to the concentration of the potentially toxic agent being tested. Therefore, controlling the experimental conditions is crucial so as to ensure that the measured cell death corresponds to the toxicity of the added nanomaterials *versus* the unstable culturing conditions. In addition, as nanoparticles can absorb dyes and be redox active, it is important that the cytotoxicity assay is appropriate. Conducting multiple tests is advantageous to ensure that valid conclusions are drawn. It seems clear that in vitro cellular systems will need to be further developed, standardized and validated (relative to in vivo effects) in order to provide useful screening data about the relative toxicity of nanoparticles. In that respect, standard reference materials (SRMs) of engineered nanomaterials are needed for validation, just as there are SRMs for urban air and diesel exhaust particles. Currently, only one type of SRM exists: the "gold nanoparticle standard" developed at the National Institute of Standards and Technology (NIST).

4.3 Methodologies developed for the qualitative analysis of nanomaterials

As described in the above section, microscopy techniques and traditional cell culture techniques could be used for the characterization of nanoparticle uptake and localization, bio-distribution and qualitative analysis of nanotoxicity wherein human and environmental samples are exposed to nanoparticles. Quantifying the amount of nanomaterial is however an important issue in view of potential toxicity of nanomaterials. Several methods were addressed here including electron-dispersive X-ray analysis (EDS), ICP-AES/OES/MS, isotope labeling, synchrotron radiation techniques. Table 2 provides a list of relevant instrumental techniques for quantifying engineered nanomaterials.

First, qualitative elemental analysis techniques can be performed on biological samples exposed to nanoparticles using an electron microscope coupled with a microanalysis system to identify the chemical composition of the nanoparticles present in the sample.[50] For example, EDS was used to confirm the presence of silver nanoparticles within cells,[27] and electron energy loss spectroscopy (EELS) was used in conjunction with TEM for elemental confirmation of carbon nanotube uptake.[51] Inductively coupled plasma atomic/optical emission spectroscopy (ICP-AES/OES) is widely regarded as a powerful technique for the quantification of elemental nanoparticle (NP).[52] The first complete quantitative in vivo pharmacokinetics study on QDs was achieved by using ICP-AES to quantify quantum dot distribution showing the mononuclear phagocyte system (MPS) uptake with no excretion, a useful outline of techniques applicable to tracking QDs in vivo.[53] Subsequently, their use in the quantitative analysis of nanomaterials was reviewed by Marquis *et al.*[3] Recently, it was reported that the toxicity of nanoparticles increases with decreasing particle size on a mass basis or production of ionic metals.[54–56,153] This observation may be true for certain type of nanoparticles, but there does not appear to be evidence that this is true for all types of nanoparticles. To understand this phenomenon, inductively coupled plasma mass spectrometry (ICP-MS) techniques were carried out to explore how they produce toxicity *in vivo*. The results suggest that when the sizes of particles such as copper decreases down to a nanoscale, copper becomes extremely reactive in a simulative extracorporeal environment. The underlying chemistry relies on the enhanced chemical reactivity at the nanoscale. Chemical reactions occurring between a solid and liquid phase always initiate at the interface of the two phases. Hence, the surface molecules can directly influence chemical reactivity. In accordance with the collision theory, large surface area must lead to a high

probability of effective collision, which determines the ultrahigh reactivity during molecular interaction. Certain chemical reactions are thermodynamically possible but are too slow kinetically. However, when the particle size reduces to the nano-scale, the large specific surface area will sharply speed up the chemical reaction and may eventually cause nanotoxicity that micro-scale substance does not allow. Hence in this case, the nanosized copper particles consume the hydrogen ions in the stomach much more rapidly than the micron-sized coppers leading to massive generation of cupric ions which are highly toxic *in vivo*.[54–56]

However, there are a few recent examples of nanoparticle-enabled MS that, though not explicitly linked to nanoparticle toxicity studies, demonstrate the potential of this approach. Kong *et al.* used carboxylated/oxidized diamond nanoparticles to extract proteins from blood, centrifuged the nanoparticles, mixed them with a MALDI matrix, and performed MS analysis with 2 orders of magnitude improvement in sensitivity over analysis done without nanoparticles.[57]

In addition, isotopic labeling is a technique for tracking the passage of a sample of substance through a system. Wang *et al.*labeled the water-soluble hydroxylated carbon single-wall nanotubes with radioactive ^{125}I atoms, and then the tracer was used to study the distribution of hydroxylated carbon single-wall nanotubes in mice. This study, for the first time, affords a quantitative analysis of carbon nanotubes accumulated in animal tissues.[58] Two other different nuclear imaging modalities have been used for the quantitative analysis of quantum dot including the single photon emission computed tomography nuclear imaging (SPECT) and positron emission tomography (PET). For SPECT, the most commonly used gamma-emitting radionuclide tracer includes 99-metastable-technetium (99mTc), iodine-125 (^{125}I) or indium-111 (^{111}In).[47] Recently, Colvin *et al.* revealed an important fact that the bioactivities of fullerene derivatives were altered largely with the change of outer modified hydroxyl groups.[59] Since traditional measurement methods such as XPS and NMR were not precise enough to determine the exact number of hydroxyl groups, a further measurement of the hydroxyl number was performed using synchrotron radiation X-ray photoemission spectroscopy. Through the binding energy spectra of C1s electrons for C=C and C–OH in Gd@C82(OH)x, intensities for the non-functionalized and hydroxylated carbons were obtained.[60]

4.4. Molecular modeling techniques

A computer assisted prediction approach could help to identify the effects of physicochemical factors of nanoparticle and how these factors play their role on toxicology, and correspondingly in the systematic analysis and prediction of biological effects in various media. Physiologically based pharmacokinetic (PBPK) modeling has played a significant role in guiding and validating *in vivo* studies for molecular chemical exposure and can serve as a significant tool in guiding similar nanotoxicity studies. Shelley *et al.* has directed the first attempt to model the *in vitro* effects of a nanoparticle exposure, in this case, aluminium (80 nm) and its impact on a population of rat alveolar macrophages[61,62] was evaluated.

Another elementary step towards a quantitative assessment of the risks of new compounds to the environment is to calculate their predicted environmental concentrations (PEC). Mueller *et al.* described a study utilizing a life-cycle perspective to model the quantities of engineered nanoparticles (the flows of TiO_2, Ag and CNT) released into the environment.[63] The method was applied to the engineered titanium dioxide nanoparticles, including the nanoparticles of silver, carbon nanotubes, fullerenes, ZnO and carbon black. The quantification was based on a substance flow analysis from products to air, soil and water. The PEC-values were then compared to the predicted no effect concentrations (PNEC) derived from the literature to estimate a possible risk. The results of this study make it possible for the first time to carry out a quantitative risk assessment of nanoparticles in the environment and to suggest further detailed studies of nano-titanium dioxide. The following parameters were used as model inputs: estimated worldwide production volume, allocation of the production volume to product categories, particle release from products and flow coefficients within the environmental compartments. To cope with uncertainties concerning the estimation of the model parameters (*e.g.* transfer and partitioning coefficients, emission factors) as well as uncertainties about the exposure causal mechanisms (*e.g.* level of compound production and application), probabilistic methods, sensitivity and uncertainty analysis was applied.

In the toxicity study of nanoscale Titania, what did correlate strongly to cytotoxicity was the phase composition of the nanoscale Titania instead of sample surface area. Anatase TiO_2, for example, was 100 times more toxic than an equivalent sample of Rutile TiO_2. The most cytotoxic nanoparticle samples were also the most effective at generating reactive oxygen species; ex vivo Reactive Species (RS) generation under UV illumination correlated well with the observed biological response. These data suggest that nano-TiO_2 samples optimized for RS.[15] Also, in a study entitled "*Nano–C_{60} cytotoxicity is due to lipid peroxidation*", by changing the number of hydroxyl groups on the fullerene surface resulted in a reduction of toxicity by several orders of magnitude.[59] Also as discussed earlier, functionalization, which often change part or the entire structure of nanomaterials can greatly influent their toxicity. These studies showed that the toxicity of nanomaterials could be highly correlated with their structures. In addition, potential toxicity of the fullerene nanoparticles was lowered significantly by using these *in vitro* assays to target chemical aspects of the nanomaterials that contribute to toxicity, indicating that *in vitro* testing provides a cost-effective means for such studies, and high-throughput *in vitro* assays[59,64,65] are well suited for developing mechanistic models to inform material development.

5. Sensors for quantitation and nanotoxicity of engineered nanomaterials

Nanomaterials offer new possibilities for the development of novel sensing and monitoring technologies. Nanosensors can be classified under two main categories: (I) sensors that are themselves nanoscale or have nanoscale materials or components, and (II) sensors that are used to measure nanoscale properties. The first category can eventually result in lower material cost, reduced weight and power consumption. The second category can enhance our understanding of the potential toxic effects of engineered nanomaterials. This is an area of critical interest to

detection and risk assessment, as well as for monitoring of environmental exposure.[66,67] This section provides an overview of the use of engineered nanomaterials for sensing (Category I sensors) and quantitation, nanotoxicity assessment of engineered nanomaterials using category II sensors.

5.1 Category 1-Nanotechnology-enabled sensors or sensors that are themselves nanoscale or have nanoscale materials or components

5.1.1 Nanomaterials and nanotechnology for sensing. As nanotechnology has come to prominence for a wide range of devices, it also brings new possibilities for chemical and biosensor construction. The use of nanoscale materials such as nanoparticles, nanowire, nanoneedle, nanosheet, nanotube, nanorod, nanobelt and nanocomposites for sensing have seen explosive growth in the past 5 years, since the report for low-potential detection of NADH using carbon nanotube-modified electrodes by Wang *et al.*[68] and the first use of gold nanoparticles as labels for electrochemical immunosensors by Limoges *et al.*[69] Dozens of reviews are available which partly deal with the use of nanomaterials for nanobiosensors,[70–72] more detailed reviews on carbon nanotube-based sensors[73–83] and metal oxide and quantum dots nanoparticles-based biosensing[84–87] can be found. Nanowires as sensing materials have also been reviewed.[88]

Due to the electrical, magnetic, optical properties of these materials, the nanomaterials-based biosensors has been categorized into electrochemical,[89] optical or photo-electrochemical, magnetic and mechanistic and surface plasmon resonance enhanced sensing types *etc.* Their dimensions are on the same scale as biomolecules, which unveils exciting possibilities for their interaction with biological species, such as microbial, tissue, cells, antibodies, DNA and other proteins. Extensive research papers and reviews using nanomaterials for electrochemical bioassays have since been published and this continues to show an increasing tendency. They have been used for the construction of enzyme sensors, immunosensors and genosensors to achieve direct wiring of enzymes and relative components to electrode surface, to promote spectroelectrochemical reaction, to impose barcode for biomaterials and to amplify signal of biorecognition event.

5.1.2 Application of nanomaterial-based chemical and biosensors. The development of portable nanotechnology-based sensors has the potential to meet the needs for low cost, rapid, high-throughput, and ultrasensitive bioassays for biomonitoring an array of chemical markers.[90] The resulting nanobiosensors have been applied in the detection of glucose,[91] food pathogens,[92] biomedicine, antioxidant capacity assessment[93] *etc.* Nanostructured membrane has been developed for the fabrication of implantable nano-biosensor and applied in clinical diagnostics[94] *etc.* Recent advances in chemical analyte detection and optical imaging applications using gold and silver nanoparticles has been reviewed by Murphy *et al.*[95] The successful coupling of the functional group on the quantum dots have made it possible to increase the power of a number of biochemical, molecular biological and physiological experiments, including tracking the movements of proteins within the cell, characterizing the expression of cell determinant markers on the cell surface, and other studies illustrating the utility in understanding the biological response to exposures, potentially in whole organism studies,[96,97] significantly reduces the application of toxic indicator and reference dyes to the cells.[98] Nanomaterials offer unique properties that can be exploited in environmental sensors[99,100] to detect environmental toxins including pesticides, hazardous industrial chemicals, toxic metals, pathogenic bacteria and undesirable vapors and liquids at the particle's surface. Recently, application of advanced nanomaterials for environmental monitoring have been reviewed systematically by Andreescu.[101] Nanobiosensors based on individual olfactory receptors are developed,[102] which are used to mimic the way human and animal noses respond to different odors and allow for rapid and noninvasive assessment of VOCs, that can constitute a signature of metabolic states or diseases, participate in aromas in food, and be associated with drugs and explosives or to domestic and environmental pollutants.

5.1.3 Detection of biotinylated fullerenes and carbon nanotubes. Detection of fullerene and carbon nanotubes using Surface Plasmon Resonance (SPR) biosensor, was recently reported by Kirschner *et al.*[118] SPR exists when polarized light reaches the interface between a thin metal film and a high density medium in Kretschmann geometry. At this point, the alternating electric field within the light causes oscillation of the firmly held electrons in the dielectric substance. This oscillation produces evanescent waves that are non-propagating spatially decaying fields which in turn causes oscillations in the free delocalized electron density of the metal–the so called "surface plasmons".[118,119] In aqueous solution, fullerene and carbon nanotubes interact with proteins in solution and bind to the surface while their structures remain unaltered. This is the basis of Kirschner *et al.'s*[118] biosensor protocol as shown in Fig. 3. First the gold surface was cleaned by removal of macroscopic dirt particles. Next streptavidin was adsorbed to the gold sensor and a biotinylated C_{60}-fullerene derivative was attached. Phosphate buffer solution was flowed before the sensor was primed to detect binding proteins to C_{60}. Proteins with fullerene affinity, monoclonal anti–C_{60} antibody, was flowed across the surface causing change in binding index of refraction[118] which was then correlated to the quantity of fullerenes. It is important to note that for the sensors described in this section, the antibodies directly bind to the biotinylated, water soluble fullerenes. It appears that this sensor can only sense

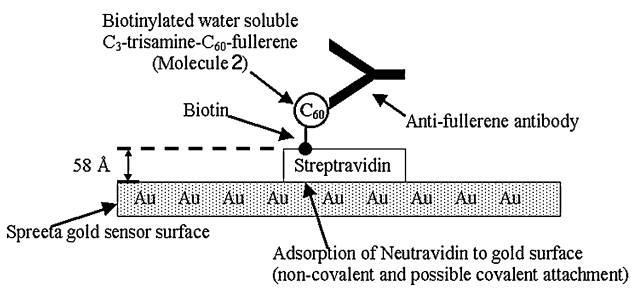

Fig. 3 Schematic of biosensor developed for fullerene from reference 118.

biotinylated fullerenes. Thus is it better categorized as Category I nanosensor.

5.2. Category 2-Nanoproperty-quantifiable sensors or sensors that could be used for detecting nanoscale properties

As described in the previous section, category II sensors represent a developing area of critical interest to detection and risk assessment, as well as for monitoring of environmental exposure[66,67] due to the explosive application of engineered materials. However, compared to the large number of publications on the sensors in the first category, sensors in the second category are very few and/or some of them are not currently available. This section provides a quick overview of on-going work in this category.

5.2.1 Detection of metal and metal oxide nanoparticles

5.2.1.1 Silver nanoparticles (AgNPs). A recent report in this category focuses on the detection of metal nanoparticles (NPs) such as silver nanoparticles (AgNPs). AgNps are known for their antimicrobial activity and for that reason they have been used in water treatment[103] and other applications such as baby pacifiers and food storage containers.[101] Their bactericidal activity is shape and size dependent, with particles of sizes less than 100 nm showing optimal antibacterial activity.[101] Knowledge about silver's ability to kill harmful bacteria[104] has made it popular in creating various consumer products. Despite these useful applications, AgNPs have been reported to be toxic.[105] For example, Asharani *et al.* synthesized AgNPs using starch and bovine serum albumin (BSA) as capping agents to study their deleterious effects and distribution pattern in zebra fish embryos (*Danio rerio*).[105] These authors observed concentration-dependent increase in mortality and hatching delay for the Zebra fish embryos treated with AgNPs.[105]

There are many excellent chemical sensors for silver ions including ion-selective electrodes, optodes, and fluorescent sensors. Plasma emission spectroscopy, Atomic absorption (AAS), and anodic stripping voltammetric methods have been used to measure trace levels of silver.[106] However, none of these have been applied for AgNPs detection. As of now, only one article has appeared reporting their application for AgNPs. The article published by Chatterjee *et al.* on "selective fluorogenic and chromogenic probe for detection of silver ions and silver nanoparticles in the aqueous media".[107] The chemistry of their sensor was based on Rhodamine B derivative **1** as the fluorogenic and chromogenic probe for Ag^+/AgNPs in aqueous media (Fig. 4).

Probe **1** forms colorless and non-fluorescent solution in 20% ethanolic water. With the oxidation of AgNPs by hydrogen peroxide, silver ions were generated. The presence of Ag^+ ion leads to the development of a pink color (λmax: 558 nm) and a strong orange fluorescence (λmax: 584 nm) of Probe I(Fig. 4B), indicating that the Ag^+-promoted ring opening takes place readily[107](Fig. 4B). The fluorescence increased linearly depending on the concentration of AgNPs demonstrating the usefulness of probe **1** for the indirect quantification of AgNPs. The detection limit (LOD) was reported as 14 ppb. This protocol was applied to quantify AgNPs in sanitizer gel and fabric softeners containing unspecified amount of AgNPs.[107] What is most unique about the work discussed here is that the silver ion sensing was applied to silver particles upon silver oxidation with hydrogen peroxide. The most serious problem limiting use of ion-selective electrodes and other existing sensors is interference from other undesirable ions. Some of these sensors are not completely ion-specific; all are sensitive to other ions having similar physical properties.

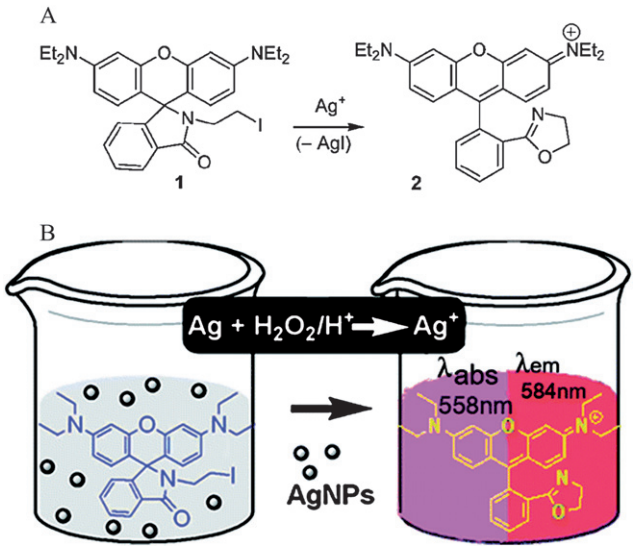

Fig. 4 (A) Ag^+ Promoted Spirolactum Ring Opening of Probe (**1**).[107]**Fig. 4(B)**: Schematic illustration of the sensing mechanism promoted by Ag^+-coordination to the iodide of the probe.[107]

5.2.1.2 Gold nanoparticles (AuNPs). Recently, detection of AuNPs was reported using Surface Plasmon Resonance (SPR) technique. Plasmon resonances in metallic nanoparticles are due to the collective oscillation of conduction electrons against their matrix.[108] Such resonances play a central role in the optical properties of metallic nanoparticles[109] and therefore are useful in detection metal nanoparticles such as AuNPs. Lindfors *et al.* reported detection and spectroscopy of gold nanoparticles using super continuum white light confocal microscopy.[109] They illuminated the sample with super continuum laser light generated in a photonic crystal fiber (PCF) through a cascade of nonlinear effects that gave rise to a spectrum extending from the visible to the near infrared. Using this technique, these scientists were able to detect a single gold particle down to a nominal diameter of $D = 5$ nm. The authors pointed out that this was the first detection of individual gold nanoparticles below 10 nm using a fully optical technique.

Another optical technique for detecting AuNPs is the photothermal detection of gold nanoshells using phase-sensitive optical coherence tomography (OCT) as reported by Adler *et al.*[110] OCT is a high-resolution biomedical imaging modality that produces cross-sectional and three-dimensional images of tissue microstructure by interferometrically measuring the amplitude and echo time delay of backscattered light.[111] Typically, at low temperature gradients the technique is suitable for *in vivo* use[110] and represents a new method for detecting AuNP contrast agents with excellent signal-to-noise performance at high speeds using OCT.

Most recently, Absil *et al.* published a paper on "Full field imaging and spectroscopy of individual gold nanoparticles".[112] Their imaging method was based on a guided laser illumination associated to spatial modulation which allowed the detection of AuNPs down to 10 nm. The fascinating thing about their method was that, they used an imaging spectrometer and white incoherent illumination for the same system and delivered individual spectroscopy of several gold beads simultaneously, allowing a fast and selective discrimination between individual metal particles, aggregates or dust. The signal measured on the camera is directly proportional to the field scattered by the particles that varies as d^3.

In addition, carbon-fiber microelectrode amperometry is implemented to measure the dynamic secretion of chemical messenger molecules from nanoparticle-exposed cells. This method facilitates detection of a specific molecular target based on applied potential, sub millisecond time resolution, and quantitation of endogenous concentrations of chemical messengers released during exocytosis.[114,115] In the presence of AuNPs, carbon-fiber microelectrode amperometry was used to characterize serotonin exocytosis from murine peritoneal mast cells co-cultured with fibroblasts and the results suggest that nanoparticles interrupt the dense-core biopolymer intergranular matrix and present the potential for systematic studies showing how exocytotic function is influenced by nanoparticle size, shape, and composition.[115]

5.2.1.3 Metal oxide nanoparticles. Metal nanoparticles such as ZnO, TiO_2, CeO_2, ZnO and TiO_2 have been used as sunscreens for many years because of their ability to filter UVA as well as UVB light, giving broader protection than other sunscreening agents. For example in 2005, out of 1200 sunscreens authorised by the Austrian Government Department of Health and Ageing Therapeutic Good Administration (TGA), 228 contained ZnO, 363 contained TiO_2 and 73 contained both.[116] It can be inferred that these materials are widely used though they have been theoretically tagged as potentially toxic.[116] Methods for detecting these metal nanoparticles are relatively few. A biosensor has already been reported for the detection of AuNPs. AuNPs in the earlier section was also used for detection of metal oxide nanoparticles (ZnO, or Fe_3O_4).[113] This is perhaps the only nanosensor found for metal nanoparticles. Other available methods are the conventional characterization of nanomaterials. For example, Tyner *et al.*[117] compared up to 20 existing conventional methods for detecting and characterizing metal oxide nanoparticles in unmodified commercial sunscreens. Their findings showed that only varied-pressure SEM, AFM, laser-scanning confocal microscopy and X-ray diffraction were found to be viable complementary methods for detecting and characterizing nanoparticles in sunscreens. However, none of these has been used to detect and characterize ENMs fully without complementary methods.

5.2.3 Potential sensors for the detection of nanoparticles. The global demand for biosensors capable of rapidly detecting the toxic effect of metals or nanoparticles is exploding. For example, according to a recent British Broadcasting Corporation technical market research report, the U.S. *in vitro* toxicity testing (predictive toxicity) market had a value of $765 million in 2006 and will double by 2011, reaching $1.5 billion (http://www.biophagepharma.net/index.php). A significant amount of research effort is being devoted to this field. The following section describes some potential chemical and biosensors which have been used for detection of the different toxins. Although these have potentials for being used in quantitation and nanotoxicity assessment of nanomaterials, no specific data for such applications have been reported.

5.2.3.1 Environmental sensors based on protein engineering (FRET output). In the last decades, many types of biosensors have been under continuous development, integrating biological components such as proteins, nucleic acids, membranes cells and even tissues acting as receptors, and different signal transducers devices including microbalances, electrodes, optical components and semiconductors. Such instruments have been applied into diversity of fields but especially for the detection of contaminants in foods and environment. More recently, new types of protein-only biosensors are being explored by incorporating a novel protein engineering algorithm to create site-directed mutants.[120] It has been possible to engineer specific binding sites for a series of targets ranging from L-Lactate and serotonin to toxins such as trinitrotoluene, soman, and the potentially toxic gasoline additive methyl tertiary butyl ether (MTBE).[121,122] Detection of analyte binding to the mutant binding proteins is accomplished through optical techniques. One commonly used output is fluorescence resonance energy transfer (FRET), which involves the transfer of energy from one fluorophore to a second located in close physical proximity—the fluorescence of the second probe being inversely proportional to the distance between the two probes. When the two lobes, each with a fluorophore tag, move relative to each other upon analyte binding the degree of energy transfer is altered and can be easily observed. An alternative approach is to use a single fluorophore tag placed in a location which undergoes a change in microenvironment on analyte binding which in turn results in an alteration of probe intensity. This tactic, like that ion channel based sensors, is highly amenable to the creation of sensor arrays.

A PDS® prototype biosensor has been developed by Biophage (Biophage Pharma Inc.),[123] which can rapidly detect (in less than 1h) the harmful effects of certain toxic metals like mercury and cadmium on mammalian cells. This versatile biosensor provides a user friendly, rapid and safer *in vitro* toxicological first screening detection method. It represents a viable, cost effective alternative for toxicity testing and at the same time offers customers a "politically correct" solution which addresses the controversial issue of animal testing. These solid proof-of-concept results open completely new markets for the biosensors in environmental risk assessment.

5.2.3.2 Cellular and molecular based electronic and optical biosensors. A cellular and molecular-based electronic biosensor can evaluate toxicity in real time by monitoring cellular impedances *in vitro*. Recently, a continuous online technique based on electric cell substrate impedance sensing (ECIS) was demonstrated for measuring the concentration and time response function of fibroblastic V79 cells exposed to nanomaterials such as QDs and fluorescent gold nanoparticles.[124]

The $ECIS_{50}$ values agreed well with the results obtained using the standard neutral red assay. Cadmium selenide quantum dots showed direct cytoxicity with the ECIS assay. Blanc-Béguin *et al.* reported the ability of a GMI bio sensor detection of magnetite nanoparticles incorporation into rat prostate adenocarcinoma cells (MatLyLu), which was a high sensitivity magnetic bio-sensor based on the giant magneto-impedance (GMI) effect.[125]

An inherently array-based approach uses cell based biosensors through optical imaging. The premise behind these biosensors is that bacterial strains can be engineered to respond to distinct toxin exposures with well-defined alterations in cellular processes associated with reporter constructs for optical readout. The major distinction of this approach relative to other sensor techniques is that the readout relates to the functional effects of the toxicant rather than its exact identity. Cell arrays have been created in which tens of thousands of individual cells are placed at the tips of an imaging fiber bundle to detect responses to toxic metals and genotoxins.[126,127] This approach allows for exponential growth in the complexity of the system and allows for sensing of undefined vapors by analogy to benchmark patterns.

Real-time cell electronic sensor (RT-CES)® System is a novel cell-based assay system[46] to monitor cellular events by measuring the electronic impedance of sensor electrodes integrated on the bottom of microtiter E-Plates. Based on measured impedance, a dimensionless parameter, Cell Index is derived and reported to provide quantitative information about the biological status of the cells, including cell number, viability, morphology, and cytoskeletal dynamics. Wilson Roa has pointed out to develop new method with the novel RT-CES array to measure the combinative toxicity of engineering nanoparticles and identify specific toxicity induced by nanoparticles from the effects of the drugs or biomolecules that attached on the medicinal particles. Potential harmful effects when these nanomaterials are used in some medical situation, such as X-ray, UV or ultrasonic will be also investigated. This method has been selected as one of standard assays for U. S. Environmental Protection Agency's ToxCast Program.

5.2.3.3 Microfluidic devices—MEMS/SOMS/ion channels. It is currently possible to develop micro- and nano-scale arrays-primarily based on affinity reagents-that can detect specific sets of harmful agents in the environment. MEMS devices are manufactured using similar microfabrication techniques as those used to create integrated circuits. Interest in MEMS for biological applications (BioMEMS) is growing rapidly, with opportunities in areas such as biosensors, pacemakers, immunoisolation capsules, and drug delivery. Suzuki *et al.* have developed a nanoparticle manipulator using a MEMS based structure, where nanoparticles can be selectively injected based on mechanical vibrations, thermal heating, and electromagnetic waves and these results demonstrated direct physical control of the interaction between yeast cells and nanoparticles in liquid for the first time.[128] Wang *et al.* use a MEMS bioreactor to evaluate toxicity of quantum dots and CNTs to intestinal epithelial (Caco-2) cells, which enable small-scale studies to explore the dose *versus* toxicity relationship of exposing nanoparticles to human cells.[129]

The Self Organizing Molecular Systems (SOMS) mercury/phospholipid biosensor technology developed by Dr Andrew Nelson offers considerable potential as a systematic nanotoxicity analysis tool for use alongside conventional toxicology methods,[130] which has been used for studying β-sheet peptide/phospholipid interactions and are important for an understanding of the folding of β-sheet rich membrane proteins and the action of antimicrobial and toxic peptides.

Cell-based microfluidic devices, the application of microfluidic technology to cell culture-based assays, are also described as "cell chips," "cell biochips," or "micro-bioreactors." These microscale cell assay devices are now becoming practical tools for the rapid screening of chemicals and drugs, and several have been developed specifically as toxicity screening assays. A microchip device (HμREL) with integrated dissolved oxygen sensors was designed to allow the scientist to test a compound within a matrix of different cell types and to see whether a nanomaterial is effectively targeted to a particular organ or cell, and whether it has detrimental effects on organs such as the kidney, liver, or heart. Using this novel technology will save not only human and animal lives, but also time, money, and resources.[131]

Channels and pores that respond directly to molecules or to physical stimuli are found within sensory systems. Recent nanotechnological developments in the miniaturization of electronics and wireless communication technology have led to the emergence of environmental sensor networks (ESN), among these are sensors inspired by decades of work on the conductance properties of ion channels. A sensor modeled on an ion channel has two defined states—occupied and unoccupied (open and closed)—readily distinguished by a readout of the channel conductance and so are capable of translating an analog "random" signal (analyte presence) into a digital "on/off" signal. These stochastic sensors have been tailored to monitor divalent metals, anions, and a broad range of organic molecules, such as different metal ions (detection of Zn^{2+}, Co^{2+}, or a mixture of the two) have distinct kinetics of interaction with the channel filter.[132] Other groups have sought ways of improving electrical biosensors. Ion channel switches are engineered to interact with highly specific antibodies or ligand receptors in the bilayer rather than binding analyte directly, facilitating their adaptation to a broad range of analytes.[133] These sensors have been applied for detecting a broad range of hormones, such as thyroid stimulating hormone, and pharmacological agents such as gramicidin, digoxin, and amiloride.[134] Another electrical sensing approach uses microfluidic chips to detect particles moving through a small channel 7–9 lm long and 1 lm wide. When a colloidal particle moves through the channel, there is a change in the conductance across the pore; when the colloid has an affinity ligand bound, the increased volume further alters the conductance, yielding a quantifiable signal.[135]

Clearly, a number of different research groups are developing approaches to create sensor devices with the goal of developing largely automated platforms for the quantitation and nanotoxicity of engineered nanomaterials. In this aspect, these technologies are all in the early stages of development. Electrical Dekati Industrial Hygiene Particle Sensor (EDIPS) (Dekati Ltd.) offers real-time particle measurements to insure workplace

safety, thus minimizing the need for cleaning and maintenance, but it is still in a micron range.[136] Innovative approaches to introduction of samples from the environment, fractionation and delivery to the sensor itself must developed in order to make this extension to field deployable devices that can be used of individual risk assessment.

6 Case studies for category II nanosensors and for nanotoxicity assessment

6.1 Sensors for detection of nanoparticles

As indicated earlier, Category II sensors should be able to detect and accurately quantify nanomaterials in complex environmental samples. Recently, our laboratory showed that if *beta-cyclodextrin*(*beta*-CDs) are immobilized on an appropriate transducer such as a piezo-electric crystal, nanosized organics could be isolated and detected based on the ability of the CDs to complex the organics according to size exclusion mechanism.[137] The central idea is to utilize the ability of the CDs to complex a comparable sized fullerene (*e.g.* C_{60}), exclude this from the mixture of other fullerenes such as C_{70} hence measure the mass changes on a quartz crystal microbalance (QCM) according to Sauebrey Equation[138]

Clearly, as shown in Fig. 5, at the molecular level, fullerenes C_{60} with smaller diameter (0.7 nm) than beta-cyclodextrin (0.79 nm) can be captured by *beta*-cyclodextrin while excluding fullerene C_{70}(diameter 0.8 nm). On the QCM, these events can be monitored as change in frequency (ΔF) and converted to mass (Δm). To achieve this chemistry, specific protocol was followed.[138] Briefly, *beta*-CD was converted to amino-*beta*-CD (solid) *via* known synthetic route[139] and dissolved in deionized water. Next, gold QCM was cleaned in piranha ($H_2SO_4 + H_2O_2$, $v/v = 3/1$), dried under nitrogen and immersed in 3,3′-dithiodipropionic acid. *beta*-CD assembly was achieved through N-Dicyclohexylcarbodiimide/N-hydroxysuccinimide (DCC/NHS) immobilization chemistry.[140] Finally the *beta*-CD modified QCM was exposed to the mixture containing fullerenes C_{60}, C_{70} and toluene (control) and the Δm correlated to the number of C_{60} fullerene nanoparticles captured. Cyclodextrin-fullerene interaction chemistry is based on the size of *beta*-CD hydrophobic inner cavity which complexes with C_{60}[138,141] and not with C_{70} or higher analogues.[139] Using this sensor, we have shown that fullerene C_{60} could be quantified selectively from C_{70} and control. Sensor selectivity was assessed using similar surface chemistry but with *gamma*-cyclodextrin, γ-CD immobilized onto the QCM transducer for the detection of fullerene C_{70} which has larger diameter to facilitate its capture.[137] Sensor surface characteristics have been confirmed by Nuclear Magnetic Resonance, Atomic Force Microscopy and Secondary Ion Mass Spectroscopy. Results confirmed that the sensor chemistry is selective for desired fullerene (C_{60} or C_{70}) depending on the nature of the immobilized CD.

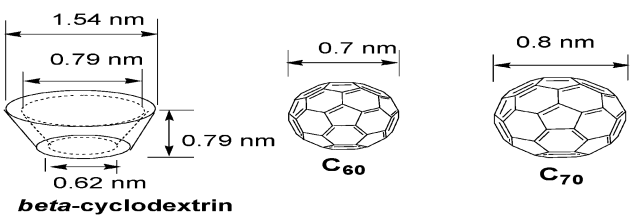

Fig. 5 Schematic representation of beta-CD and the fullerenes used as guest molecules with their dimensions.

6.2 Evaluation of nanotoxicity using dissolved oxygen sensor (DOX)

A prototype multichannel system was utilized that enables the simultaneous, quantitative and continuous measurement of dissolved oxygen using a 96-well electrode biosensor prototype (Fig. 6) known as the DOX-96 device. DOX is fully automated, portable, equipped with a multipotentiostat, and can be connected to a computer. The latter enables external control of the instrument, on-line recording of experimental parameters, graphical presentation, and data storage. The instrument software plots current intensity *versus* time for each well while data are simultaneously processed for 12 channels, each corresponding to 8 sensors. Experimental setup involves the use of 96 disposable electrodes in a three-electrode format (reference, working, auxiliary). These 96 sensors are placed in a conventional 96-well plate in which the cells are cultured.

Our group has reported the development of DOX systems for cytotoxicity assessment. Andreescu utilized the DOX system for measuring the activity of cancerous cells as well as their interaction with chemical toxins based on the level of oxygen consumed by the cells.[142] In another work, we reported that the data from the 96-channels could be coupled to pattern recognition techniques to identify the microbes based on their oxygen consumption.[143,144] Respiration of cells generates a reduction in the concentration of dissolved oxygen, which is determined using electrical current produced. This was achieved by a continuous monitoring of the oxygen consumed by the cells with time. These methods give advantages in terms of assay time and data quality compared to the conventional methods such as XCT, fluorescence, MTT and Trypan Blue techniques. Oxygen is

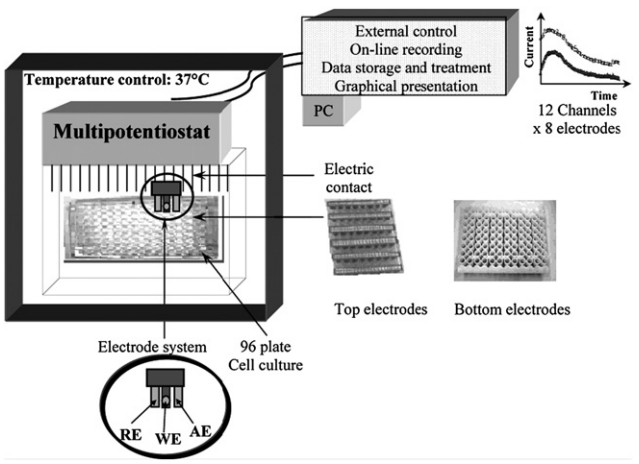

Fig. 6 Schematic diagram of the multichannel DOX oxygen sensor system used for measuring cytotoxicity. Two configurations of the 96 electrodes are available with the electrodes at the top or bottom of the wells. Each sensor consists of three electrodes: reference (RE), auxiliary (CE), and working (WE) electrode.[142]

a requirement for oxidative phosphorylation, and thus respiration in aerobic systems and a good indicator of the metabolic activity of the cells. Thus, the level of oxygen consumed by cells as a result of respiration can provide information regarding viability. The respiration of cells generates a decrease of the current related to the concentration of dissolved oxygen according to the following reaction:

This is a potential method for the detection of nanotoxicity and for nanomonitoring. Consequently, we explored the DOX system for the detection of nanotoxicity of ZnTe quantum dot. The operational procedure using the DOX system consists of measuring the level of oxygen in the tissue culture medium (TCM) in which the cells grow over time. In this case, the plate was incubated in optimum growth conditions at 37 °C under CO_2 atmosphere, and the reduction current was measured for a maximum of 30 min under a constant applied potential (−400 mV) after cell plating or incubation with the quantum dot. The results shown in Fig. 7 demonstrate that ZnTe quantum dot showed an obvious toxicity in a concentration dependant manner. As can be seen, the higher concentration of cells correspond to the lower current intensity (Fig. 7(B)), suggesting a low concentration of dissolved oxygen implying that higher level of oxygen is consumed. This current is also proportional to the concentration of cells present in the medium. The results are comparable with MTT assay (Fig. 7(A)). Continuous monitoring requires more stringent conditions, especially with respect to sterility of the system, CO_2 level, and temperature and electrode repeatability, application potential *etc.*, and optimizing experiment is still underway.

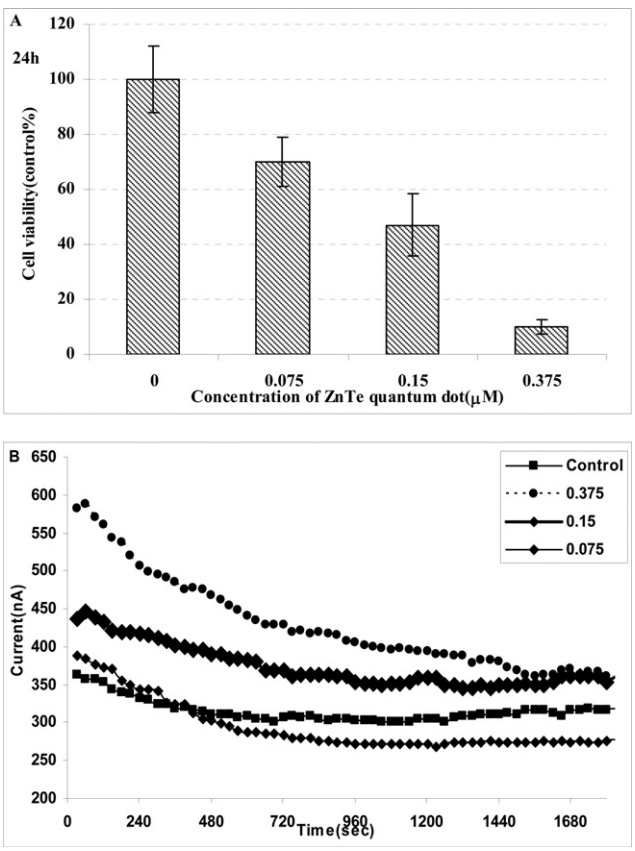

Fig. 7 (A) MTT results of A549 lung cancer cells after one day incubation with ZeTe quantum dot. (B) Typical DOX responses during 30-min monitoring from 0.075, 0.15, and 0.375 μM ZnTe after one day of incubation with A549 lung cancer cells (2.5×10^4 cells/well). Results are the mean of 5 identical electrodes (Quantum dot ZnTe was a gift from Dr Jun Zhang, Chemistry Department, SUNY- Binghamton).

6.3 Nanofiltration

An essential step in the quantitative risk assessment of ENMs is to isolate the materials from complex environmental or biological samples. Nanomonitoring must therefore be accompanied by sample preparation and/isolation from the complex matrix in order to minimize interference and matrix effect. Toward this goal, one approach is to create a template for the immobilization of the ENMs. In that respect, an ideal material must exhibit spatio-selective interaction with the compounds through a 3-dimensional binding capacity, and also provide accessibility to the underlying transducer in the case of Category II nanosensing. Polymeric materials with nanoporous cavities created *via* self-assembly or mechanical synthesis makes ideal material for this purpose because the pores are able to coordinate different engineered materials functionality, thus making it a desired template for detecting these in real time.

Although a lot of work has been performed in the field of nanofiltration, majority of these earlier works have focused on biomolecule separation and detection or elimination of impurities such as proteins, lipids or even virus in water.[167–171] The use of nanofiltration for the removal of environmental pollutants, specifically organic molecules has also been reported.[172] However, few studies have focused on the isolation or separation of nanoparticles particularly inorganic engineered nanomaterials using nanofiltration. Recently, we have successfully prepared a new class of porous, sponge-like and flexible nanocomposite polymeric membranes with the capacity to capture the ENMs.[145,146] These conducting polymer-based nanofilters were tested for the separation of quantum dots from aqueous solutions because the fluorescence of quantum dots provides a quick and easy method for the detection and determination.

The polymer membrane is porous, flexible and nanoporous (Fig. 8A). It was installed as filter paper in a conventional filtration system, while quantum dots solution was injected through a syringe. Fig. 8(B) shows the decrease of fluorescent emission of quantum dots with over 90% quantum dots quantitatively filtered out directly from aqueous media. This method not only provides a detection method for quantum dots but also gives an efficient way to eliminate toxic quantum dots from water sources. When the capacity of the isolating material is reached, the attached ENMs of interest could be quantitatively released during a regeneration step by exposure to fresh plugs of *n*-butanol, or acid washing. This restores the integrity of the material and could therefore be re-usable

7. Future perspectives

Despite the availability of various methodologies for the qualitative and quantitative analyses of engineered nanomaterials and for assessing nanotoxicity, there are still critical gaps in

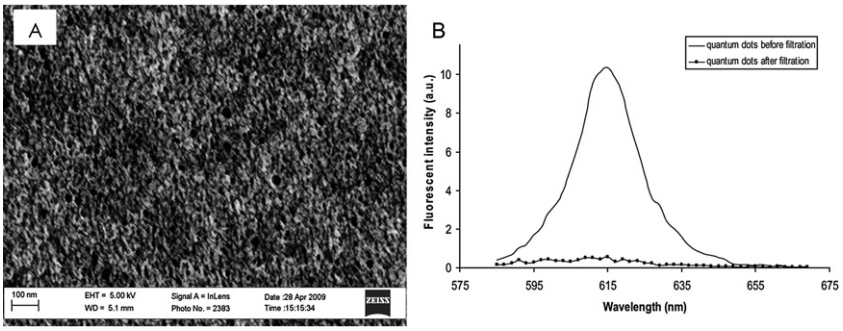

Fig. 8 (A.) SEM micrograph for nanostructured polyamic acid membrane at a magnification of 200 000×. **(B).** Fluorescent emission intensity of 10 nM quantum dots aqueous solution before and after filtration using the polymeric membrane.

knowledge required for risk assessment purpose. We have reviewed this emerging field of nanomonitoring and established the efficient, detection and measurement tools for tracking the release of nanoparticles from a very wide range of production processes, formulation and consumer products. In essence, some of these techniques could be used for environmental monitoring as long as they are coupled with separation or extraction techniques that allow the prior removal of major interferences and their performance characteristics evaluated on a case-by-case basis.[6] Methods have been developed for natural or engineered nanomaterials in simple matrices, which could be optimized to provide the necessary information, including microscopy, chromatography, spectroscopy, centrifugation, as well as sample extraction and filtration and related techniques. However, a combination of these with sampling techniques is often required for the detection and characterization of engineered nanoparticles in complex matrices, *i.e.* water, soil, food or living systems.[147] *In vivo* studies of nanostructures provide new challenges and detection strategies must be capable of quantifying all of the major parts of a nanostructure in tissues and organs since many modern nanostructures are engineered with multiple components. The prerequisite for the accurate quantification is the appropriate sample preparation including the isolation of the nanomaterials from complex matrix.

However, sampling of nanoparticles is another challenging task for several reasons. First, the sampling strategy should ensure that the particle collection methods, including location, represent as accurately as possible the real exposure at the site in question and methods should be developed and chosen according to the size and nature of the particles under investigation. Secondly, because of their small mass, separation of nanoparticles from larger particles by inertial impaction can only be achieved at a relatively high pressure drop, thus necessitating the need for unique isolation materials or approaches. Thirdly, considering the typical ambient atmospheric nanoparticle concentrations, collection of filtered samples for gravimetric analysis and chemical characterization is only feasible with certain high volume sampling techniques.[148] In addition, it is difficult to distinguish ENMs and natural nanomaterials in environmental and biological matrices. New approaches for the isolation of the nanomaterials are thus required. The ideal material must be suitable with small volumes and be able to tolerate minimal clean-up and preparation prior to analysis. Future work should focus on addressing these challenges and identifying which is most important for specific nanomaterials and which measurement methods are most effective.

Airborne engineered nanomaterials present complex exposure measurement challenges. Although there are field-based equipment such as the TSI particulate analyzers,[163] GRIMM air monitors,[164] and Kanomax[165] for monitoring particle number concentrations and size distributions, there are still challenges for continuous multipoint monitoring.[166] In many instances, traditional mass-based sampling and laboratory analysis techniques may not be suitable for evaluating airborne engineered nanoparticulates because of detection limit issues or because they are expensive, bulky volume, require extensive operator training and are not available for in-situ and field-based environmental monitoring. New methods and tools for measuring exposure to airborne engineered nanomaterials will be required to protect the health of workers in nanotechnology-related jobs-estimated to total 10 million people by 2014.[149] For many nanomaterials, it is postulated that the surface area, not the mass, determines toxicity. One approach for evaluating the presence or absence of engineered nanoparticles is to use a direct read instrument, such as a handheld condensation particle counter (CPC),[166] to survey particle concentrations at various locations. Currently, several direct read instruments are commercially available for air sampling for particulate including CPC *etc.*, however, they cannot typically be applied to measure only specific engineered nanoparticles. Therefore, the development of a "universal aerosol monitor" was now advocated capable of providing detailed information on the nature of airborne engineered nanomaterials to which people are exposed. The proposed wearable sampling device would measure aerosol number, surface area, and concentration mass simultaneously and would be low cost. "An economical integrated device will empower small and large nanotechnology industries alike to reduce uncertainty over what their workers are exposed to, and enable them to develop safer working environments" said Maynard.[149] "This will require targeted research into developing new methodologies and new instruments".

Summary and conclusions

To date, the potential impacts of nanomaterials on human health and the environment have been limited by insufficient understanding of the risks associated with its development, manipulation, and wide-ranging applications. This article has reviewed

the emerging field of nanomonitoring and nanotoxicology including the challenges of monitoring engineered nanomaterials, the potentials of combining existing analytical techniques with conventional cytotoxicity methods. Conventional methods for assessing the properties and characteristics of raw nanomaterials focus on the size distribution and effects. They are unsuitable for detection and quantification of complex environmental samples. Hence identification and characterization of these materials is an important first step in assessing their risk. Consequently, the environmental monitoring of nanoparticles is a critical research area; and one which would greatly benefit from novel approaches to detect their presence and characterize their properties.

The advantages and drawbacks of major microscopic techniques for characterizing nanomaterials have been reviewed including TEM, HRTEM, SEM, STM, and AFM. In general, these provide convenient means of imaging and assessing nanoparticle uptake and visualization. Except AFM which allows the characterization of materials under physiological conditions, major drawbacks for most microscopic techniques include the need for high vacuum environments and complex sample preparation. In addition, most of these techniques do not provide information on the toxicity of the nanomaterials. While nuclear microscopic techniques can quantitatively map all elements in the periodic table, the high momentum of protons creates a more complex focusing problems and their capability for nanomonitoring and nanotoxicity could be severely limited.

A large number of classical cell culture and colorimetric assays (*e.g.* MTT, NRU, SRB, LDH, Tryphan Blue *etc*) are currently available for assessing the toxicity of engineered nanomaterials. Conventional cell culture techniques are readily available for characterizing engineered nanomaterials (MTT, LDH, Tryphan Blue *etc*). These are generally more suitable for in-vitro cytotoxicity assays and are convenient alternatives for animal texting. The reagents used in the traditional colorimetric assays such as the MTT have been found to interfere directly with nanoparticles such as silver, suggesting greater than 100% viability of nanoparticle-exposed cells. Methods such as flow cytometry, fluorescence microscopy and DNA microarray assays have been used to quantitatively determine the binding and uptake of nanomaterials. Quantitative assessment can also be achieved in a similar manner using techniques such as ICP-AES. Some of the reagents used in nanotoxicological studies have been found to react with nanomaterials, making the interpretation of results rather difficult. It is thus clear that *in vitro* cellular systems will need to be further developed, standardized and validated in order to provide useful screening data about the relative toxicity of nanoparticles. SRMs of engineered nanomaterials are also needed for validation, just as there are SRMs for urban air and diesel exhaust particles. Currently, only one type of SRM exists: the "gold nanoparticle standard" developed at the National Institute of Standards and Technology (NIST).

Other analytical tools for characterizing nanomaterials such as mass spectrometry and gel permeation spectroscopy are accurate and provide convenient detection. They are however, expensive, require extensive operator training, and are rarely used to quantify complex environmental samples. In order to analyze environmental samples, these must be coupled with extraction procedures and may not be amenable for application in field environment. The difficulties involved in the limited solubility of some nanomaterials may pose significant limitations for using techniques such as GPC[173–175]. Conventional MS are currently not advanced enough to provide quantitative information about nanomaterials or distinguish between single atoms and nanoparticles.[173–175] While GFAAS may readily provide elemental information about inorganic nanomaterials, it is unlikely to distinguish between the bulk inorganic materials and the nanoscale counterparts.

Addressing the complex and critical issues surrounding the environmental transformation and toxicity of nanoparticles must be accompanied by the creation of new approaches or further developments of existing instrumentation. In that respect, sensor technologies provide convenient means of analyzing these materials. Category I nanosensors have been discussed, which partly deal with the use of nanomaterials for nanobiosensors. For a broader description of Category I nanosensors readers are referred to excellent reviews on this subject.[150,151] The development of Category II nanosensors has also been reviewed in detail. This class of nanosensors is an area of critical interest to nanotoxicology, detection and risk assessment, as well as for monitoring of environmental or biological exposure. In general, there are many excellent chemical sensors for silver ions including ion-selective electrodes, optodes, and fluorescent sensors. Plasma emission spectroscopy, Atomic absorption (AAS), and anodic stripping voltammetric methods have been used to measure trace levels of silver. However, none of these have been applied for AgNPs detection. As of now, only one article has appeared reporting their application for AgNPs. Although gold nanoparticles (and AgNPs) have been widely utilized to amplify sensor responses and as labels for sensors, only few sensors have been reported for directly monitoring the nanoscale properties of gold and metal oxide nanomaterials. Other potential sensor techniques such as PDS, ECIS, RT-CES and DOX have been analyzed and presented as viable alternatives. This is because; they are user friendly, rapid and safer for first screening detection and toxicity testing.

As presented here, an essential step in the quantitative risk assessment of ENMs is to isolate the materials from complex environmental or biological samples. Few studies have focused on the isolation or separation of nanoparticles particularly inorganic engineered nanomaterials using nanofiltration. Another case study has been discussed here involving porous, sponge-like and flexible nanocomposite polymeric membranes for the separation of quantum dots from aqueous solutions. Promising results were recorded based on the decrease of fluorescent emission of quantum dots and over 90% quantum dots were quantitatively filtered out directly from aqueous media.

This review has shown that there is hardly any single sensor or other analytical technologies that is readily available for quantitative and simultaneous detection, characterization, and monitoring of ENMs. Existing techniques are best integrated with separation of other methods to provide information on toxicity of engineered nanomaterials, especially in complex environmental media. In a scenario involving complex environmental matrices, including cells (microstructures), bacteria (microstructures), viral components (nanostructures), volcanic ash, pollens, engineered nanoparticles and proteins (on the order of nm), Category II nanosensors are required that distinguish

ENMs and naturally-occurring nanoparticles. An ideal Category II sensor should be portable, robust, require minimal sample preparation and should be low cost. The sensor or sensor system should also exhibit the following characteristics:

(i) Be able to isolate the desired nanomaterials from complex environmental matrices

(ii) Distinguish among different types of nanomaterials (*e.g.* functionalized and unfunctionalized, hybrid organic metal nanoparticles vs inorganic ENMSs)

(iii) Distinguish between intentionally produced nanomaterials ENMs (MWCNTs, QDs *etc*) from ultrafine, incidental nanomaterials (combustion, industrial and other particulates) or naturally occurring nanosized particles (*e.g.* pollens, viral components, dead bacteria, living bacteria, spores, viral components, or fungi) that may be present in the environment.

(iv) be capable of in-situ, remote and continuously reflecting the concentrations of these materials.

With respect to the research needs presented above, case studies are discussed for the development of Category II nanosensors, specifically for detecting fullerenes, quantum dots and for DOX toxicity sensing. DOX has the potential for rapid nanotoxicity and nanomonitoring. This review highlights the use of DOX for monitoring the toxicity of ZnTe Quantum dots and results are comparable with MTT technique. Unlike MTT however, there problems of high background resulting from the interactions of the MTT reagent with the nanoparticles have been obliterated. DOX requires no additional reagents and it simply uses the metabolic activities of the cells in real time. The major advantages of DOX sensor vs conventional methods such as XCT, MTT and Trypan Blue is the portability, short assay time and data quality compared.

In view of the complexity of environmental matrices, another simplified approach to addressing the instrumental and analytical needs for assessing the risks posed by ENMs is to treat the natural particles as interferants and just filter out all cellular matter (which is orders of magnitude larger than the engineered nanoparticles in question). An ultimate goal in utilizing sensors as tools for assessing and measuring the toxicity of nanomaterials is to achieve comprehensive exposure studies in real-time, including remote assessment of exposure. Realizing such a goal will require not only advances in sensor and sample handling technologies but also informatics and remote sensing infrastructures. Such comprehensive exposure studies should extend beyond environmental monitoring to encompass biomonitoring, monitoring at an individual level, ecological and toxicity exposures, as well as tissue distributions of toxins and environmental agents.

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