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In vitro assessments of nanomaterial toxicity

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1. Introduction

The rapid expansion of scientific, technological and commercial interests in sub-micron materials, assembly, and properties unique at this size-scale has spawned the fields of nanoscience and nanotechnology. Over 500 consumer products currently on the market claim to contain elements of nanoscience and nanotechnology with new entries coming daily [1, 2]. This market annually requires metric tons of raw nanomaterials, ranging from nano-sized metals and metal oxide particles to carbon nanotubes [3, 4]. Such manufacturing and consumer utilization then produces multiple different sources of release of these materials into the environment, eco-system, water [5] and food supplies, and other routes of non-voluntary entry into the human body [6]. Demand for nanotechnology in medical products will grow by more than 17% annually to reach an estimated $53 billion market in 2011, with the largest share of this opportunity in pharmaceutical applications, expected to reach $18 billion in 2014 [7]. Moreover, the US National Science Foundation predicts that half of the pharmaceutical industry product line will comprise central nanotechnological design features by 2015. At least 12 nanomedicines are already approved, and progressively more are seen entering active development stages [8]. Hence, a steady succession of new nanotech-based drugs, imaging agents, and diagnostic products are anticipated to seek (and possibly gain) regulatory approvals and subsequent access to human-prescribed use. Therefore, deliberate point-sourced (i.e., drug-dosed) as well as uncontrolled, inadvertent environmental nanomaterial exposure to humans will undoubtedly increase through many different routes.

While nanomaterials are attractive for both basic science and technological reasons, both deliberate (i.e., medicinal or therapeutic) and inadvertent or uncontrolled (i.e., environmental), increasing human exposure to nanomaterials together with the distinct properties of these materials all mandate development and validation of accurate nanodevice and materials characterization protocols and predictive toxicity and hazard capabilities. These methods must reliably predict and assess the possible spectra from benefits to possible risks and health hazards associated with exposure to nanomaterials as they become more widespread, pervasive agents in manufacturing and medicine. The inter-agency National Toxicology Program recommends the classification of a new entity according to its plausible risks, according to the data extant concerning the new entity. Accordingly, the entity is interrogated by a set of tests designed to characterize a given risk and characterize mechanisms for related outcomes [9]. Such an

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assessment regime would best consider traditional pharmacology and toxicology approaches to dose response, acute and chronic exposure, as they correlate to rigorous physicochemical characterization [10] and various levels of physiological reactivity (e.g., molecular, cellular, tissue, organ toxicity) in making risk-benefit analyses of these materials both in various manufactured forms and incorporated within new technologies [9]. This need becomes compelling if future studies continue to bolster recent preliminary findings that claim biological responses to carbon nanotubes not unlike those observed for the well-known carcinogen/irritant, asbestos [11–13].

Importantly, new evidence showing *in vivo* immunomodulatory cell processing of intravenous nanoparticles acutely dosed for antigen presentation and vaccine induction suggests specific uptake, transport and cell processing of nanoparticles [14, 15]. That such immune surveillance is also affected by non-deliberate, potentially harmful or chronic low-level nanoparticle processing remains to be shown. To this end, many materials testing methods well-established for macroscale biomaterials and for soluble pharmaceutical products are simply adapted in a variety of ways to discover correlations between nanomaterial properties and the biological responses *in vitro* to cells and *in vivo* to animal preclinical models. This requisite analysis precedes any human *in vivo* materials applications: such monitoring falls broadly under the scope of "biocompatibility testing" (i.e., ISO 10993), although no definitive or scientific metrics exists for such assessment except the FDA-mandated safety and efficacy requirements. However, to date, two notable deficiencies in such testing exist, namely that little long-term chronic nanomaterial exposure data is available in any form, and that connections between extensive acute exposure cell-based testing models *in vitro* with any *in vivo* response are generally lacking. Implanted biomaterials might indeed be known to do no harm to a host in various bulk forms (e.g., sheets, fabrics, milled large pieces as implants), but could be decidedly and distinctly reactive and harmful if presented to the host in a different form, e.g., as a colloid or particulate [16] A particular challenge is to assert safety and efficacy for nano-scale biomedical systems, distinct from similar materials in micro- and macroscopic morphologies. Routes of biological exposure, as well as materials chemical and physical properties must be carefully considered in these nano-biocompatibility tests. Each route and each material size and form has its own pharmacological and/or toxicological properties that require unique assessment protocols and criteria for healthful versus harmful exposure outcomes. Hence, there is currently no evidence or confidence in assuming that nano-scale pieces of known biocompatible bulk metals or metal oxides, ceramics or polymers would share accepted biocompatible properties of their bulk materials. Similar presumptions might also be made about the biocompatible relationships between micron and nanometer-sized dispersions of these materials, or various colloidal formulations of sub-micron materials (i.e. as pharmaceutical formulations for parenteral injections, inhalation, or oral administrations). Therefore, the current nanomaterials revolution in human dosing, exposure and toxicological assessments must be accompanied by an equally ambitious biomedical research effort to develop new tools, methods, pharm/tox protocols, biocompatibility and safety standards and host exposure qualifications for different nanomaterial classes.

There are indeed concerns specific to nano-scale materials in biological systems: as materials dimensions approach the nano-scale, certain properties become scale-dependent, especially in particles below 20nm diameter. These include capillary forces, optical effects/color, melting points, conductivity, ionization potential, electron affinity, magnetism, and, significantly, surface energy and reactivity. Specific surface areas for micron-sized particles (e.g. fumed silicas and commercial carbon blacks) are typically 60–80 m^2/g, a considerable surface-to-mass ratio many times greater than their macroscopic counterparts. Commercial CB-1 carbon black and single-wall carbon nanotubes – a major current nanotechnology interest for both therapeutics and consumer products – have specific surface areas approaching 1000 m^2/g. Similar area scaling effects are seen with miniaturization of surface topology, porosity,

texturing, and high-density fabrication in sub-micron features. Hence, surface structure and composition, and therefore intrinsic reactivity, are perhaps the dominant structure-determining properties in nanomaterials. Surface effects must also be considered a unique and very significant set of functional nano-properties that requires both control and careful characterization enroute to exploitation in specific nanotechnologies [9, 16].

Such high specific surface area materials have a high interfacial chemical and physical reactivity of many types that translates to biological reactivity. Compared to bulk-phase atoms, two important, distinguishing features of surface atoms in nano-systems are (1) their lower coordination number and (2) their increased exposure to reactive species in the environment. These features translate to intrinsically higher surface atom reactivity than bulk atoms and manifestation of this reactivity in some usual but also other very unique ways. As particle size decreases, the surface properties of the atoms dominate, leading to significant changes in particle reactivity. The relative fraction of surface atoms to bulk atoms in a structure, called *dispersion*, exhibits a power law scaling in the nanoscale regime. While less than 1% of a *micro*particle's atoms occupy surface positions, over 10% of the atoms in a 10-nm diameter metal particle reside on its surface (and 60% in a 2-nm particle!) [17]. This huge fractional surface presence in nanomaterials contributes to a change in surface physical and chemical properties as materials are reduced in size below 20nm [18]. This presents unique challenges for the materials science and associated surface analytical communities to characterize and control surface properties, as well as asserts quality control specifications at the nanoscale that would help standardize different nanomaterial properties. Deploying these materials *in vivo* has even more challenges.

Significantly, the question of whether or not these nanomaterial properties, or other effects (e.g., intrinsic colloidal instability and aggregation phenomena in aqueous milieu, bio-accumulation in the environment or tissues, contaminant adsorption and transport) would permeate, become persistent and influence biological systems remains to be determined. Currently, there is no consensus about the intrinsic risks, tolerance, hazards, toxicity or dose-response relationships for almost all classes of nanomaterials. A search of the current literature can easily provide diametrically opposed opinions on safety and tolerance of the same nanomaterial in model *in vitro* test systems (e.g., cell lines) and in small animal *in vivo* testing models. Nano-tolerance and nano-toxicology are emerging fields with unique constraints placed by the size and surface properties of the materials under analysis. Few tools and methods exist that can adequately track nanomaterial properties and reactivity in biological or physiological systems. Importantly, these methods are primarily *in vitro* assays to date and, as for many biocompatibility tests conducted for related macrophase materials, could have little if any correlation or validation to *in vivo* materials tolerance [19]i.

This review identifies current methods commonly used to assess nanomaterial surface and bulk properties and biological reactivity in model *in vitro* systems. These assays are important to characterizing nanomaterial applications in biotechnology, ecosystems, agri- and aqua-culture, biomedical applications and toxicity screening. In large part, these methods represent direct extension of methods known for up to 4 decades for other macroscopic biomaterials compatibility or soluble drug toxicology assessments, adapted to nanoscale colloids. Few of these methods are specifically discriminatory to nanoscale properties, sizes or physical states, and many do not report sensitive information about the nanomaterial behaviors in biological systems: they simply report gross materials behaviors and assay signals averaged over the system employed. Few analytical methods provide direct information on nanosystems within biological milieu (solutions of proteins, or cells, or tissues). Lacking the proper tools and sensitivity, current nanotoxicology is in many ways analogous to a blind man describing an elephant: the possible dimensionalities of what scientists seek and need to define for nanophased materials in the environment or living host are enormous. At the same time, the

analytical tools available to accurately and reliably determine the pharmacological and toxicological fate of these materials are primitive enough in such complex bio-systems to provide only small, often equivocal, pieces of the resulting huge parameter space.

1.1. Pre-biological Materials Characterization

Before biological responses to nanomaterials may be assayed to any degree with any real scientific validity, materials properties as supplied (with probable contamination), cleaning procedures, batch-batch variability, and solution properties such as intrinsic aqueous stability, aggregation and flocculation must be thoroughly screened. This is a materials science exercise in quality control, but critically important before meaningful results may be interpreted from later *in vitro* or *in vivo* testing. Bulk materials analysis should involve purity certification, aqueous leachables, and electron microscopy of bulk nanophase morphology, polydispersity, intrinsic aggregation, solubility, and when appropriate, bulk phase thermal analysis, conductivity or redox behavior, and spectroscopy (i.e., fluorescence, vibrational) to provide some quality control of the expected materials physical and chemical states, and stability.

1.1.1. Surface contamination—Surface contamination of biomaterials is a thermodynamically driven process of surface energy reduction through the adsorption of adventitious air- or water-borne contaminants or renovation of the biomaterial surface through chemical processes. Similarly, the surface contamination of nanomaterials may range from adventitious adsorption to surface oxidation, corrosion, charging or electron transfer reactions of the nanomaterial itself. Due to the highly reactive nature of nanophase surfaces, surface adsorption of many types of molecules in the milieu is expected to reduce surface free energy of the solid interface. Finely dispersed materials (e.g., diatomaceous earth, alumina, silica, activated charcoals) are already used as adsorbent beds to remove dissolved species. Nanomaterials reproduce this same scenario but with substantially increased surface areas. Hence, contamination of nanomaterials in both air and in aqueous milieu is likely by spontaneous adsorption from ambient phases, and at a degree commensurate with 200–1000 m^2/g surface areas. This means that a few hundred milligrams of carbon nanotubes contaminated with a monolayer of unintentional adsorbate could introduce over a milli-mole of possible surface leachate to the solution to which they were introduced. Hence, nanomaterial surfaces could unintentionally introduce high levels of contaminants by reversible adsorption of air- or solution-borne species. That this might occur to transport high levels of unintended or toxic adsorbate species into human beings via adsorption to nanomaterials is one possibility. A second issue is alteration of nanomaterial colloidal stability by such solute or surfactant adsorption to induce aggregation and altered physical properties of the nanomaterial compared to its ‘ideal’ monodisperse, ‘clean’ state. A third concern is that assays will not distinguish toxic endogenous adsorbates from intrinsically toxic nanomaterials without careful control experiments and surface analysis. Deliberate surface modification agents used to stabilize nanomaterials (surfactants, steric and electrostatically charged stabilizers) could also slough in biological systems or partition into serum proteins or membranes (as many drugs do) to produce confounding issues with nanotoxicity or nanomaterial compatibility. Because cell-mediated particle uptake (at least at the micron-size scale) is influenced by surface properties and chemistry, surface contamination and aggregation resulting from surface contamination and/or adsorption in biological systems can affect these mechanisms of cell processing, providing a basis for skewed outcomes for impure materials used for *in vitro* and *in vivo* testing. Therefore, surface composition and its control are requisite to understanding nanomaterial interactions with living systems.

Possible sources of surface contamination of nanomaterials are diverse. The adventitious adsorption (e.g., of bacteria-derived ubiquitous endotoxins – pyrogens or lipopolysaccharides (LPS) – or polyaromatic hydrocarbons – PAHs – is one concern. Surface oxidation, corrosion,

charging or electron transfer reactions of the nanomaterial are also important [19] Left uncharacterized, these various forms of nanomaterial surface contamination and alterations represent a substantial and confusing variable to understand the results of any experimental outcome of nanomaterials simply introduced into *in vitro* or *in vivo* pharm/tox models. In fact, it is likely that nanomaterials will carry significant adsorbed contamination into test systems under most all conditions, with the exception rather than the rule being a clean, unreactive, or at least consistent, pure material surface chemistry. Therefore, understanding and controlling this interface is a key challenge to understanding nanomaterial biocompatibility, risk assessment and possible toxicity mechanisms. This parallels the well-known surface analytical paradigm in macroscale biocompatibility materials and device testing [19]. The number and varieties of different biomaterials surface characterization methods commonly implemented to assure some quality control standards to implanted materials chemistry and physical conditions is extensive [20]. This chapter lacks the breadth necessary to sufficiently describe the numerous well-known materials characterization tools used prior to any *in vitro* or *in vivo* assay. Nevertheless, such nanomaterial quality-control reporting should be expected to be essential in order to understand the material characteristics prior to exposure to biological systems.

1.1.1.a. Adsorbed endotoxin contamination: One example surface contaminant is ubiquitous bacterial endotoxin, lipopolysaccharide (LPS). As a component of gram negative bacterial cell walls, LPS remains one of the most common surface-adsorbed contaminants of serious concern for all biomaterials [21]. As a ubiquitous, heavily glycosylated, phosphorylated lipid often present on skin, on all that skin has touched, in all milieu capable of supporting bacteria, and in most water sources (except expensive pyrogen-free water), LPS surface activity favors its adsorption to hydrophobic surfaces, while its phosphate groups allow it to bind to positively charged surfaces [22–24]. These properties allow the endotoxin to contaminate virtually any surface. Endotoxin affects biological systems strongly as a known pyrogen by raising organismal body temperature or inflammatory cellular cytokine production through its presentation to host cells of many types. Hence, reliably discriminating its activity distinct from the nanomaterial's intrinsic inflammatory activity is important. Given the exaggerated surface-to-volume ratio for nanophase systems, the possible amounts of adsorbed endotoxin (e.g., grams adsorbed endotoxin per gram of material) are significant. Titanium dioxide (TiO_2) particles have been shown to bind LPS in such a way as to reduce *in vitro* cellular inflammatory response to LPS [25]. Other nanoparticles have been shown to bind endotoxin [26] and some have been evaluated for their ability to bind endotoxin from aqueous solution [27]. Moreover, investigations of particle-endotoxin interactions in relation to orthopedic wear particles have found that particle-adsorbed endotoxin can effect implant loosening through localized inflammation [28, 29]. Therefore, endotoxin contamination is possible on a variety of nanoparticles, producing an inflammatory response that is magnified by nanomaterial presence to levels that would warrant concern over endotoxin's potent inflammatory reactivity *in vivo* and with many cell lines [25]. The presence of adventitious endotoxin contamination in the cell cultures or on the nanomaterials applied to cells could activate cells and generate inflammatory cytokine responses essentially indistinguishable from any response generated by the nanomaterial exposure. Additionally, such cellular activation could also alter other cell signaling pathways for cell-based assays with unintended consequences. Consequently, endotoxin contamination of nanomaterials, nanomaterial stock solutions, lab glassware, ultrafiltered lab supply water, and cell culture materials should be regularly assayed [30], controlled, and remediated before any cell response assay to nanomaterials is attempted [31].

The Limulus Amebocyte Lysate (LAL) assay [32] is the current standard for detecting soluble endotoxin *in vitro* because it is considered the best assay currently available and the established means for endotoxin testing in spite of its inherent inconsistencies (i.e., assay signal is relative to fixed LPS standards that may or may not reflect diverse sources or reactivity of LPS). The

colorimetric variation of the LAL is widely used in biomaterials testing, particularly in pre-clinical settings, and is more accurately defined as a measure of endotoxin activity rather than endotoxin concentration. Thus, it is generally accepted that endotoxin may only be detected by these assays if it is soluble or readily displaceable (i.e., not permanently surface-bound), but some methods have been developed to correlate detectable solute endotoxin to surface-bound endotoxin for the same system [32, 33]. For such methods to be reliable, adsorbed endotoxin concentrations would need to be shown to depend on known LPS exchange or desorption rates under the relevant *in vitro* conditions where LPS undetected by the assay in surface-bound form produced a reliable answer when surface-displaced in biological milieu. That is, irreversibly adsorbed endotoxin might be indirectly correlated by knowing its soluble, reversibly adsorbed, assayed LPS fraction in biological milieu. This rigorous analysis is simply not done on nanomaterials to date, despite the possibility of LPS being one of the most likely biologically reactive contaminants present at high levels from routine materials processing and manufacturing. Hence, biological tracing of cause and effect of adsorbed LPS and its partitioning between nanophases and biological milieu is unknown.

1.1.1.b. Other surface contaminants: Other adsorbates expected as surface-adsorbed contaminants are no less problematic. Polyaromatic hydrocarbons (PAHs) – known carcinogens [34] – commonly adsorb to surfaces exposed to ambient air, in which PAH exist ubiquitously in trace amounts. Production of carbon-based nanomaterials is now known to generate substantial toxic by-products including at least 15 PAHs [35]. Additionally, carbon nanomaterials are well-known to actively adsorb volatile PAHs co-produced as part of the carbon nanophase manufacturing process [36]. Other common laboratory surface-active contaminants include volatile hydrocarbons and silicones from pump exhausts and various volatile plasticizing additives (e.g., alkylated phthalates such as ubiquitous surface-active plasticizer, dioctylphthalate). Additionally, catalyst species or unreacted synthesis reagents residual from nano-synthesis are common contaminants in nanosystems [37] such as the Fe, Co, or Ni catalyst or amorphous carbon soot retained by carbon nanotubes following their synthesis [38]. Simple water rinsing or suspension will not remove these adsorbate layers easily or completely. Catalyst removal from carbon nanotubes is routinely performed by stirring in acid for ~2 days, while amorphous carbon is oxidized and removed by 20–70hr treatment with H_2O_2 or by thermal treatment [39]. Additionally, the surface-active components in biological fluids (proteins, lipids) can promote exchange and desorption of these adlayers when other cleaning methods may not have removed them. Hence, surface-adsorbed contaminants on nanomaterials may be off-competed, replaced with biological, surface active substances. This can produce reactions to the solubilized form of surface contaminant in the test organism. Alternatively, surface-contaminated nanomaterials may elicit substantially distinct biological responses *in vitro* and *in vivo* compared to purer or cleaner phases of the same materials. These distinctions must be carefully understood.

1.1.1.c. Detection of surface contamination: Nanoscale-specific surface analysis methods are still few in number, but surface contaminants may often be detected by one or more of many well-established surface analysis techniques including time-of-flight secondary ion mass spectrometry (ToF-SIMS), x-ray photoelectron spectroscopy (XPS) [40].as well as x-ray fluorescence (XRF) and related energy dispersive x-ray analysis (EDX), and surface-enhanced Raman spectroscopy (SERS)[41]. ToF-SIMS uses a beam of accelerated ions to sputter ionized atoms and molecules from the surface (<1nm depth) of a material under ultra-high vacuum (UHV) to produce a complex yet sensitive mass spectrum of the sample surface with a spatial resolution as fine as 40nm [36]. XPS focuses a beam of x-rays on the sample under UHV resulting in the emission of core level electrons whose photokinetic energy is measured. The energy of the surface-emitted electrons and relative proportions of various energized electrons yields surface chemical information concerning the origin of the electron's atomic environment and the relative proportions of those atoms in the top 9nm of the surface, respectively. Spectral

information include atomic composition, bonding, oxidation state of the surface, including any elements save H and He, with spatial lateral resolution as fine as 8 micrometers for some instruments [36]. In XRF, voltage-accelerated x-rays or gamma rays are focused into a sample where they generate secondary fluorescent x-ray photons characteristic of the atomic environment where they are produced, yielding absolute quantification of all elements (except H, He, & Li) present in the sample [36]. Energy-dispersive x-ray fluorescence scans only the top 5 micrometers of the sample, but provides the entire spectrum simultaneously, while wavelength-dispersive x-ray fluorescence scans up to 150 micrometers into the sample, providing the spectrum sequentially [42]. Though atomic absorption spectroscopy and some forms of mass spectroscopy generally have lower limits of detection, synchrotron radiation XRF is capable of elemental detection in the femtogram (10^{-15}g) range [42]. In SERS, intense (laser) light is focused upon the surface of which a small fraction loses or gains energy by inducing vibrations in the surface of the sample and then leaves the sample at a different frequency (the Raman spectral shift). This energy change reflects the chemical composition of the sample surface. When this technique is applied to a roughened metal surface, molecules on the surface enhance the Raman signal intensity more than 10^6 times leading to the distinction of 'surface enhancement' compared to traditional Raman spectroscopic methods [43].

1.1.2. Particle sizing and aggregation—By definition, nanomaterials possess at least one dimension below 100 nm and because many unique properties of nanomaterials stem from their size nanoparticle sizing is a critical aspect of pre-characterization. Additionally, because of intrinsic high dispersion and elevated surface energy, nanoparticle aggregation is thought to be common in complex experimental conditions such as biological media, although surprisingly few studies report much actual data. At the nanoscale, aggregation is extremely difficult to discern, especially in biological milieu (e.g., serum containing 50–70mg/ml of protein), but could exert a pronounced effect upon nano-specific material properties interacting with cells and tissues. Important properties affected by aggregation include colloidal stability and homogeneity, electronic and optical behavior, and cell or bacterial uptake/targeting properties, especially in the presence of proteins. Consequently, particle sizing and aggregation stability assays in various solution conditions relevant to biology are an important early-phase component of nanomaterial characterization. Commonly used methods of particle sizing currently include transmission electron microscopy (TEM), scanning electron microscopy (SEM), optical spectroscopy (UV-VIS), dynamic light scattering (DLS), and fluorescence polarization. These methods are often not conclusive when applied to nanomaterials in complex systems and are therefore best utilized in pre-experimental characterization. Additionally, each method possesses its own inherent uncertainties, making corroboration of results with one or more additional methods the desirable means of sizing or aggregation determination.

1.1.2.a. Electron microscopies: TEM is a well-established technique for micro- and nano-imaging of material features: morphology, particle size distributions and aggregation. Therefore, many studies have relied upon TEM for particle shpae, sizing and aggregation information [33, 36, 44–48]. Particle sizing by TEM is capable of yielding the most unequivocal sizing information available via direct electron imaging, but is generally used only for metallic samples (though the energy filtering variation of the technique, EFTEM, may be used to image non-metallic materials [44, 49]). Though TEM is a robust and widely accepted particle sizing technique, requires ultrahigh vacuum (UHV) conditions and specialized equipment. Additionally, *ex situ* particle aggregation information observed by TEM is not necessarily representative of *in situ* aggregation states because sample preparation of a nanoparticle solution for TEM analysis most often requires careful sample dessication. Many aqueous preparations upon drying can produce artifacts and particle aggregation due to increasing ionic strength and surface tension influences upon drying. Aggregation is a common artifact. Hence, sample preparation of a nanoparticle solution for TEM analysis fails to adequately control

surface tension effects of the evaporating liquid, confounding interpretation of the final particle states. Flash freezing and desiccation techniques require practice, but can eliminate some of these TEM preparation artifacts [50]. It is also quite tedious to get TEM replicates assessed for statistical significance. Therefore, TEM solution aggregation information should be corroborated by other methods (i.e., particle sizing and zeta-potential [51], spectrophotometry, and gel electrophoresis [52]). While aggregation information obtained from TEM images may be inconclusive, selected-area electron diffraction by TEM is a powerful tool to identify or confirm crystalline particulate chemistry [53] and to examine material crystal habit/aggregation [45, 47, 54] (e.g., nano-fullerene aggregate samples were distinguished from nano-fullerene crystalline samples in resin-fixed and freeze-dried cells examined by TEM micro-diffraction [44]).

SEM is a powerful surface imaging technique yielding surface features of materials in addition to material sizing and aggregation information [55, 56]. Traditional SEM also requires UHV conditions and dried samples producing some experimental uncertainty on its ability to accurately represent actual *in situ* material characteristics. However, recently commercially available environmental SEM (ESEM) allows the sample to be scanned and imaged in hydrated conditions, while maintaining UHV conditions around the electron gun. Moreover, routine ESEM successfully images only the water surface and objects near the surface, but a modification (wet scanning transmission electron microscopy 'wet STEM') of standard ESEM protocols to utilize a Peltier element (for evaporation control) and the transmission mode of the instrument expands ESEM capabilities beyond the liquid surface to include the imaging of emulsions and particle suspensions [57, 58].

1.1.2.b. Optical spectroscopy: Depending on metallic electronic and lattice structures, interband electron physics and excitation energies, certain metal nanoparticles and nanorods exhibit size-dependent absorption and scattering of light through excitation of the metal's plasmon band electrons by incident photons of the correct wavelength or through scattering of incident photons of the correct wavelength according to Mie theory [59, 60]. Free electron physics is essential for plasmon excitation. Efficient electronic coupling of metal lattice energies and excitation interband energies are also required. These requirements are found in a few pure metals: Pb, In, Hg, Sn, Cd Ag, and Au are free electron metals that exhibit plasmon excitation bands. In most metals, the plasma frequency is in the ultraviolet, making them shiny (reflective) in the visible range. These are less interesting for nanoparticle applications for this reason. Some metals, such as copper and gold, have electronic interband transitions in the visible range, whereby specific light energies (colors) are absorbed, conveniently yielding their distinct color of interest. Other metals and metal oxides exhibit plasmons in non-visible optical energy regions, making plasmon excitation and detection of the plasmon extinction or band shifts challenging. Metal oxide formation and intrinsic surface metal lattice mis-match with excitation energies frequently distort energetic interband coupling and free electron physics, diminishing plasmon coupling effects for most metals, except those essentially oxide-free (i.e., more noble-like). Changes in surface plasmon band extinction occur in the metal by adsorbate binding, altering surface interband electronic states as manifested in altered refractive index. Surface oxide and thick adsorbed surface-stabilizing adlayers (e.g., organic films/coatings) can mask other adsorbate electronic influences with the metal. Surface plasmon resonance (SPR) optical effects are dependent upon shape, diameter, surface adsorbates, and distance between plasmonic particles [61]. Consequently, gold/silver nanoparticles –currently most commonly studied -- are routinely sized by measuring the extinction wavelength(s) of incident light [62, 63] Platinum, palladium, copper and other metals also possess plasmonic optical properties if protected from substantial contaminating oxides (i.e., copper nanoparticles within silica nanoshells) [64]. Importantly, plasmon peak absorbance progressively decreases in intensity and red shifts to higher optical wavelengths as the average particle diameter increases [65]. This yields a characteristic plasmonic optical peak absorbance for each size of metal

nanoparticle defining its size [65]. Similarly, adsorption of ligands (e.g., contaminants, stabilizing layers, proteins, DNA) onto the nanoparticle (also quantum dot) surface will also red-shift the extinction wavelength from that of the clean material by a few nanometers [62, 66]. Additionally, inter-particle distances smaller than average particle diameter will result in a shift in sample absorbance [53]. Significantly, this may be used as an online indicator of particle aggregation. For instance, gold colloidal solutions color shift from red to blue indicates particle aggregation [62]. However, in biological mileu where non-specific adsorption induces particle aggregation, effects of particle surface adsorption and resulting aggregation are difficult to distinguish.

1.1.2.c. Dynamic light scattering: Dynamic Light Scattering (DLS) has been widely applied for direct determinations of particle sizing in solution [67, 68]. DLS of nanoparticles of similar dimensions to soluble proteins in protein milieu, or dilute serum is very complicated: few controls or calibrations are ever reported. ASTM Grade I (e.g., ultrapure) water systems use 200nm filters to remove particulates. This means that this research-grade purified water contains endogenous populations of particles below 200nm that confound DLS sizing of introduced nanoparticles. For example, the Millipore Simplicity® Ultrapure Water System has a final filter of 50nm, leaving smaller particles in the water [52]. DLS is theoretically better applied in micro-scale size regimes in which particles scatter much more light than in nano-size regimes [69]. Consequently, DLS data are easily skewed by the presence of small amounts of contaminants.

Additionally, nanoparticle solutions are notoriously sensitive to changes in salt, protein, or surfactant concentration. Colloid stability decreases with increasing salt concentration as Debye length-dependent electrostatic particle-particle repulsions decrease below the order of attractive van der Waals interactions [63]. Also, charge-stabilized or charged ligand-stabilized particles will aggregate rapidly in the presence of an oppositely charged protein (such as the aggregation of lysine-capped gold nanoparticles by human serum albumin (HSA) [70]. Conversely, addition of non-polar adsorbates (polymer, surfactant, peptide) will increase colloid stability through steric stabilization, but also change sizing and modeling fits [71]. DLS yields particle hydrodynamic size in solution, allowing measurements of particles bearing adsorbed coronas of molecules: particles adsorbed with proteins are especially relevant in this respect because of their size and abundance in physiological systems. One DLS study asserts the capability of DLS to differentiate between end-adsorbed, (111) face, and shaft-adsorbed, (110) face, bovine serum albumin (BSA) on gold nanorods [72]. However, such nano-sizing analyses by DLS require a high degree of skill to perform, careful instrument calibration, positive and negative controls, attention to reagent purities and some knowledge of optical data modeling algorithms to predict a sizing outcome consistent with many assumptions about the optical scattering physics in the solution. Many particle sizing studies, spanning metals, metal oxides and polymers, treat the technique blindly as an automated method with default scattering models and curve fitting assumptions that are neither described, justified nor validated for various sample types. In fact, the result of the above DLS study of albumin-adsorption has been contrasted by the results of a spectroscopy-based study which found no appreciable length change for gold nanorods (of similar size to those in Ref. 72) following exposure to human serum albumin [73].

Loose adsorbates and unwanted solutes in nanoparticle systems are typically removed by dialysis [74] or centrifugation [63]. Loose particle aggregates are often broken up by bath sonication of the nanoparticle solution [68, 75]. However, care is warranted when employing conventional sonication treatments (bath and probe) since they are difficult to standardize (density, dose, power, local heating) [33] and sometimes have unwanted side effects, such as the addition of metal particles typically shed from the probe tip during sonication treatment, and oxidation of surface-active, stabilizing alkylthiol coatings on nanoparticle surfaces [76].

Given the foregoing, DLS sizing data, when uncontrolled and uncorroborated by other sizing techniques, are generally viewed with some skepticism since the method has great potential for skewed interpretation or ambiguity because of its dependence on user experience and model assumptions. Therefore, DLS studies of nanoparticles should be carefully designed, including sample calibration with sizing standards under relevant conditions and subsequent, thorough descriptions of the data analyses employed. Recently released standards now exist to aid researchers both experienced and novice to the area of particle sizing by light scattering. These aids include the ASTM Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (ASTM E2490 – 08) and NIST gold nanoparticle standard materials (NIST RM8011, NIST RM8012 and NIST RM8013). However, complicating biological milieu (ionic strength, surfactants, proteins) should compel researchers to seek confirmation of results using other sizing methods.

1.1.2.d. Fluorescence polarization: Currently, time-resolved fluorescence polarization anisotropy (TRFPA) is used in nanosystems for the purpose of sizing. Observed fluorescence polarization decay time is correlated to fluor or particle size (hydrodynamic radius [77]) according to the Stokes-Einstein-Debye rotational equation for particle motion. Using this method, 1–10 nm particles may be sized with 0.1 nm resolution [78]. RFPA utilizes sub-nanosecond-resolution laser pulses and detectors to excite fluors in assay milieu, often high-throughput screening multi-well plates; these fluors could be nanoparticles. Optical excitation induces fluorescence that can be distinguished in free-floating versus receptor-or surface-bound states by emission anisotropy. Monitoring the decay of the fluorescence polarization provides information that distinguishes particle or fluor binding (i.e., to receptors, membranes, proteins) from assay components. While TRFPA is a widely used technique in drug discovery assays to monitor “hits” by drug-target binding signals,[79].

it has not yet found wide application in analogous particle or nanomaterial characterization assays. Prospectively, this technique may yet be analogously adapted to relate fluorescence anisotropy of labeled nanoparticles to nanoparticle-cell receptor interactions and elucidate pathways of toxicity through correlation with toxicity endpoints.

1.1.2e Other techniques: Several other techniques have been applied to nanomaterials sizing with less frequency. These include :

- large angle x-ray diffraction (XRD) used to differentiate between crystalline and amorphous samples [80];
- multi-angle laser light scattering (MALLS) is used in combination with UV-Vis spectroscopy in field flow fractionation (FFF) for particle sizing and colloidal separation [68];
- small angle x-ray scattering (SAXS) and small angle neutron scattering (SANS) both of which are used to analyze both particle core and shell sizing as well as particle shape, to a spatial resolution of about 10 nm [81];
- inductively coupled plasma-mass spectroscopy (ICP-MS) used to measure trace metal impurities [82]; and
- inductively coupled plasma-atomic emission spectroscopy (ICP-AES) used to measure the concentration of gold (atoms) in cell suspensions (but notably neither size nor shape) [83]

2. *In vitro* biological testing: cell types, selection, and use

All nanomaterials exposed to *in vivo* conditions immediately and continuously encounter a broad array of biological and physiological species including thousands of surface-active

molecules, many different cell types in different tissue environments, and reactive pathological or inflammatory conditions. The interactions, influence, and possible toxicity of nanomaterials with proteins and cells is an essential focus in assessing and understanding nanomaterial compatibility versus toxicity. Cell-nanomaterial reactions of interest include cellular uptake and processing of nanomaterials in various routes, effects on cell signaling, membrane perturbations, influence on the cellular electron transfer cacades, production of cytokines, chemokines and reactive oxygen species, transcytosis and inter-cellular transport, gene regulation, overt toxic reactivity, no observable toxicity, and cell necrosis or apoptosis. Most often, *in vitro* culture of cell lines (commercial, typically genetically altered) or primary cells (tissue-harvested) on plastic plates, either with or without serum, with bolus dosing of nanomaterials and subsequent cell reactivity profiling is the common assessment method [84]. Wide varieties of *in vitro* assays with cells should consistently reflect the variety of possible physiologic responses to nanoparticles *in vivo* and all possible cell processing routes and natural reactions.

Typically, several major cell types are used *in vitro* for testing including phagocytic, neural, hepatic, epithelial, endothelial, and red blood cells and various cancer cell lines. In each case, the specific cell line selected for *in vitro* assay is intended to model a response or phenomenon likely observed or sensitized by particles *in vivo*. However, there are thousands of transformed cell lines to choose from, each with its own inherent shortcomings, many deposited within commercial registries (i.e., American Tissue Type Culture Collection, ATCC) each day. In all such assays, determining meaningful endpoints that reflect physiological stress, toxicity, or therapy or some other phenomenon detectable *in vivo* remains a challenge – this is a common issue involving the consistent disconnect between *in vitro* and *in vivo* results and lack of cell assay predictability of biocompatibility with biomaterials. Cell monocultures as measured by *in vitro* assays rarely react in such isolated pathways in native tissues; tissues comprise multiple, dynamically communicative cell types that produce non-linear and correlated responses to toxins and foreign materials. These effects are not often recapitulated *in vitro* in adherent cell monocultures in dilute serum media on plastic plates. A second major concern is the mounting evidence that secondary cell lines supplied by commercial vendors are either contaminated or no longer consistently or reliably represent the cell phenotype claimed. As few investigators actually validate the phenotype before assay use or against primary cell types, secondary cell lines represent an increasingly confounding, unvalidated source of information claimed to represent *in vivo* cell responses [19]. Lastly, even related cell lines can produce different results. One cell study of carbon nanotubes screened three human lung cancer cell lines, H596, H446, and Calu-1, for their response to MWCNTs, and selected H596 as the model for further study because its viability was the most sensitive to MWNT exposure and yielded the most repeatable results from the three cell lines as evaluated by the MTT viability assay [85]. Thus, specific cell lines within a given series of commonly derived cell line phenotypes may respond to a given stimulus in disparate ways and with varying intensity, making cell line selection an important aspect of experimental design. While cell lines can contribute important insights into select scientific aspects of cell-materials interactions, without consistent phenotyping and validation in culture as justification for their use, *in vitro* results may be increasingly confusing until quality control standards for cell behavior and biological relevance are implemented, compared and enforced.

There are numerous inconsistencies in secondary cell line use that are frequently ignored. Increasingly, primary cell culture use is urged, but primary phagocyte, tissue-specific and tumor cell cultures also have challenges. Isolation of the cell or progenitor of interest often requires differention via specialized media and iducers/growth factors. Such differentiation requires cell phenotyping, making the entire isolation process expensive and tedious. Retention of cell phenotype and its accurate validation during experiments is equally onerous. Primary cultures also require much higher seeding densities *in vitro* and usually need attachment to a

surface for efficient proliferation while many transformed cell lines may proliferate in suspension or without special attention to growth conditions. Immortalized cell lines often exhibit more rapid division and phagocytosis rates and capacities than their *in vivo* counterparts and sometimes a lack of reproducibility arising from passaging or sourcing. By contrast, primary cells obtained from tumor biopsy may provide a closer representation of the *in vivo* tumor cell type. However, 2-D monocultures are generally not representative of the *in vivo* case because the culture lacks the spatial interactions and phenotypic/cell-type cross-talk of the actual tumor. Therefore, co-culture and 3-D cultures are increasingly encouraged to more-closely model the complexities inherent in tumor physiology [19]. Additionally, cells normally refractory to high particle uptake in culture (i.e., primary cells of many types) might, if rapidly dividing, present internalized particles by non-specific capture during cell division. This is rarely controlled by the use of staged or non-dividing cultures with rapidly dividing cells since many assays provide better signal during proliferative phases. Additionally, primary cell culture often requires both animal resources, maintenance fees, and post-harvest animal sacrifice, raising costs, ethical controversies and significant paperwork for the investigator (ex., IACUC and IRB applications) [86]. Nevertheless, potentially misleading and costly outcomes for many poorly planned or unjustified cell culture studies should mandate careful scrutiny of secondary vs. primary cell culture designs. Clearly, all studies should be justified and validated based on careful cell phenotype selection and maintenance, and the intended outcomes for their use for nanomaterial testing. Too often, all of these components are missing from the reported experimental designs, producing serious questions about the significance of the work.

In summary, cell line-based screening for “biocompatibility”, transport and processing pathways, toxicology, drug delivery, or imaging is commonly employed currently. To be useful, the major challenge remains to connect these cultured cell responses with nanomaterials *in vitro* under controlled conditions to predictions about their behaviors in more complex, relevant environmental and organismal systems. A rigorous rationale for selecting and using certain cell lines, validations of their phenotypes under specific culture conditions with further validation of *in vitro* results to *in vivo* correlations is very important.

2.1. Phagocytes

Because nanomaterial toxicity is often associated with materials uptake by cells, (see section 3.3.2) and many early uptake responses to foreign materials introduced by multiple different exposure routes involve sentinel leukocytes and immune modulatory cells (e.g., neutrophils, macrophages, dendritic cells), phagocytic cells (e.g., monocyte and macrophage phenotypes) have been used as experimental models for their ability to actively uptake foreign particles and respond with cytokine signaling cascades or production of reactive oxygen species (ROS). Multiple cytokines are readily assayed from cell culture using commercial kits (e.g., ELISA, arrays) at both message and protein levels, and some cytokines (e.g., IL-6, TNF-alpha, IFN-gamma) are clearly associated with cell-based *in vivo* inflammatory reactions. Characteristic ROS production upon insult by foreign materials [87] produces short-lived highly chemically reactive oxygen/nitrogen radicals of several types, often damaging the surrounding tissue [88]. While direct *in situ* ROS assay is possible, but difficult, many experimental cell models are selected on the basis of observing secondary effects of nanomaterial-cell exposure mediated by ROS injury. ROS production has also been implicated in quantum dot (QD) cytotoxicity through lipid peroxidation and resultant apoptosis through Fas receptor upregulation [89].

Primary phagocytic cells of interest have included peripheral blood monocytes, peritoneal and lung macrophages, and bone-marrow transformed monocytes. Immortalized secondary cells representing this phenotype include RAW264.7, J774.1, IC-21, THP-1, Jurkat, Mono Mac-6, and NR8383. Table 1 shows selected examples of these types of cell uptake assays. This table surveys different particles ingested by different cells in various cultures, indicating the many

chemistries and cell types that participate in particle uptake. What this table (and many of the articles referenced) cannot provide are specific mechanisms of cell uptake that might differentiate cell-particle reactivities. Unfortunately, many phagocytic cell lines are simply chosen for convenience in culture experimentation, including putative phenotype, rapid, reliable proliferation in culture, attachment dependence, and commercial availability. Additionally, increasing skepticism and criticism are cast upon the ability of immortalized secondary commercial cell lines to accurately represent their primary counterparts, or anything else *in vivo* [52, 90–93]. In primary phagocytes, cell differentiation state (i.e., to terminally differentiated macrophages) significantly alters phagocytic and proliferative tendencies. The validity of macrophage differentiation and attachment-dependence in culture and accuracy of cytokine profiles produced compared to *in vivo* responses remains largely unproven. Immortalized cell lines, particularly RAW cells, have at times shown a lack of repeatability stemming from differences in cell passage number or cell sourcing/lineage [94]. Or, as in the case of immortalized hepatic carcinoma cell lines, oncogenic cells divide more rapidly and, consequently, possess altered phagocytic behavior from their healthy counterparts [19].

Cell-based assays of nanomaterials often attempt to place the relevant particle chemistry with the cell phenotype implicated in its processing, both in a culture context. For example, pulmonary exposure to nanomaterials (e.g., by normal inhalation, passive environmental exposure, and/or deliberate use of inhaled nanomedicines) is often modeled *in vitro* by use of either immortalized secondary cell lines putatively representing a phagocyte phenotype (e.g., THP-1, RAW, J7, Mono mac 6 cells or IC-21 lines) [95] or primary cultures of alveolar macrophages that reside at high densities deep in the lung in the alveolar bed and scavenge inhaled foreign materials [96–100]. Moreover, alveolar macrophages are considered to play a key role in particle-mediated inflammation and lung disease [101]. These cells actively phagocytose in culture and are known to produce cytokines in response to materials uptake.

In terms of ROS-induced cell toxicity, Long et al. used BV2 cells (immortalized mouse microglia) to model the characteristic ROS-burst from particle insult to these macrophage-like cells in the context of the brain. As a model for brain injury occasioned by such exposure, they co-cultured these microglial cells with N27 cells (rat dopaminergic neurons) to represent brain neuronal cells most susceptible to DNA, protein, or lipid damage by ROS insult from glial cell ROS production [102, 103]. Similarly, particle overload in the lung has been implicated in ROS production. In culture, human blood-derived monocytes release ROS, consistent with *in vivo* monocyte infiltration and subsequent inflammation in the lung as a result of particle overloading [104, 105]. Furthermore, *in vitro* culture of alveolar macrophages with oil fly ash (OFA), TiO_2, and SiO_2 shows strong induction of ROS production along with high cytotoxicity, suggesting a direct correlation between ROS production and cytotoxicity [91]. Particle-mediated ROS release by polymorphonuclear leukocytes (PMNs) in culture has also been related to the content of Si, Fe, Mn, Ti, and Co as particle-associated, insoluble species introduced to cell culture [106]. Additionally, peritoneal macrophages, collected by murine abdominal lavage and used as an *in vitro* model of phagocytosis, are shown to be highly sensitive to particle coating chemistries [107]. *In vitro* particle uptake of polystyrene particles coated with poloxamer (surface-active FDA-approved triblock copolymer used in drug formulations) has been correlated to *in vivo* particle uptake data [108]. Hence, not only nanoparticle ‘bulk’ chemistry, but nanophase surface chemistry, possibly surface contamination alone, can influence phagocytosis in culture. Much work remains to fully elucidate the inter-relationships between particle exposure, particle uptake, ROS production and toxicity of cells, both those directly involved with particle uptake and those secondarily affected.

2.2. Hepatic and other hematologic cells

Nanomaterials introduced into the blood stream (e.g., via systemic injection, oral absorption, or inhalation) would immediately encounter high concentrations of platelets and red blood cells and would also have frequent contact with the blood-filtering cells of the liver and the mononuclear phagocytic system (i.e, reticuloendothelial system), including Kupffer cells and other phagocytes. *In vitro* model studies of these systems consider several cell types and phagocytes have been described in the preceding section. For circulating particles, the liver consistently plays a prominent role in normal host particle clearance, performed by liver-resident Kupffer cells and not hepatocytes [108]. Cell lines selected as models of liver toxicity include Hep2G [109], and BRL3A, an immortalized rat liver cell line chosen simply for its convenience in modeling liver cell toxicity [110]. Therefore, common cell toxicity endpoints -- cellular morphology, mitochondrial function (MTT assay), membrane leakage of lactate dehydrogenase (LDH assay), reduced glutathione (GSH) levels, reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) are assayed. However, in addition to showing exaggerated phagocytic behavior (as previously discussed), immortalized liver cell lines also exhibit altered ethanol toxicity and transferrin uptake (iron trafficking) behavior from normal hepatocytes [111]. This suggests phenotypic changes in these secondary transformed cell lines that might not reflect accuracy to certain traits found *in vivo*.

Other relevant cell types include endothelial cells (BAECs, HUVECs) and red blood cells (erythrocytes), platelets, and leukocytes in cultures. Platelets are not true blood cells but membrane-bound hematopoietically derived cell fragments without nuclei sourced from marrow megakaryocytes. Platelets contain over a dozen potent chemicals in granules that are excreted in response to platelet activating agents, facilitating blood coagulation. At $1 – 4 \times 10^9$ platelets per liter of blood, and a thousand-fold higher occurence for red blood cells, these blood elements comprise nearly 50% of the packed cell volume and represent major interactive components with nanoparticles in blood, affecting transport, metabolism, and tissue processing. However, these anuclear elements are not typical cells, exhibiting limited metabolism and inability to culture. Primary blood harvests must be treated differently than other cell culture studies: generally only short-term particle incubation studies are possible. Importantly, calcium ion removal (EDTA addition) typically used to prevent blood coagulation in such blood collections are known to chelate other heavy metals (i.e., those introduced from nanoparticle preparations), and prevent platelet degranulation reactions important in these assays.

In terms of particle studies, red blood cells have been contrasted to a phagocytic cell line in order to model non-phagocytotic transmembrane particle movement [112]. Several studies determined that polystyrene particles (200nm, 78nm), gold nanoparticles (25nm), and TiO_2 nanoparticle aggregates (<200 nm) were all able to enter red blood cells, independent of surface charge, but particles or particle aggregates larger than 200nm were found adhered to the cell outer membranes and not inside the cells[113]. Untreated red blood cells and porcine lung macrophages treated with cytochalasin D (to block particle phagocytosis) both showed uptake of TiO_2 ultrafine (22 nm agglomerates of 4 nm particles) and 0.2-micron particles, but not of 1-micron particles. Because untreated macrophages did uptake 1-micron particles, active metabolic particle uptake processes were concluded to be size-dependent [114]. Red blood cells can uptake nanoparticles passively.

Platelet models of blood-related particle toxicity generally use such primary assay endpoints as platelet activation (degranulation) and aggregation. Several forms of nanomaterials were assayed in one relevant study; with the exception of carbon fullerenes, all nanomaterials produced platelet activation, including mixed carbon nanoparticles (amorphous carbon, 7% C_{60}), multi-wall nanotubes, single-wall nanotubes, and standard urban particulate matter. Moreover, introduction of these nanomaterials into a rat thrombosis model accelerated

thrombosis, showing an *in vivo* correlation for the platelet model of nanoparticle toxicity [115].

2.3. Epithelial/endothelial Cells

While phenotypically distinct, the epithelium and endothelium both share common cell-derived physiological barrier properties to the transport of foreign materials into the body, whether through the skin, mucosa, digestive tract or (for injectables) through the walls of the vasculature. This constitutes a very large cell surface area (i.e., intestines ≈ 200m^2; lung epithelium ≈ 100–140m^2) [116] exposed to nanoparticles in both environmental and therapeutic exposure. Consequently, epithelial cells employed for *in vitro* experiments are selected for their similarity to an *in vivo* epithelial phenotype including presence of mucin cell membrane coatings, microvilli, desmosomes, and lamellar bodies [117]. Cell models must distinguish between dermal, oral-gastric, colonic, pulmonary, nasal, kidney and vaginal epithelial characteristics depending upon the analogy sought through *in vitro* experiment and the desired outputs. Often cell lines selected as models of epithelial toxicity are precancerous type II pneumocytic cells [54, 57] since a major result of their chronic inflammation is abnormal growth. Other common transformed epithelial lines include MDCK (kidney), HT-1080, HT29-18-C1 (colonic), and HeLa (cervical) [11]. Moreover, the paucity of culture procedures for some primary cell lines necessitates use of cancer-based transformed cell lines for cell-based assays, though some of these cancer cell lines do not contain the mutated genes common to cancers of analogous tissue. For example, RKO-transformed colon cells does not possess the mutations common to colon cancer and therefore do not reflect an *in vivo* phenotype [84]

Gastric, nasal, oral and urinary tract epithelium are all characterized *in vivo* by viscous mucin production and tissue coatings that slough and regenerate frequently, affecting particle bioadhesion, capture, uptake [19, 118], and aggregation. Mucin thickness is thinner for nasal than for oral or intestinal epithelium, but such details are frequently ignored by cell culture studies most often using non-mucinlyated epithelial cell lines, such as Caco-2, an immortalized non-mucinylated enterocytic colonic cell line used for electrophysiology and cell transport studies. Commercial Caco-2 lines can also be contaminated as supplied [119]. Hence, for most intestinal particle uptake studies, Caco-2 cells do not exhibit an appropriate phenotype, but are used regardless because of their intrinsic capacity to form tight junctions and confluent, sealed cell monolayers in culture. Alternatively, Goblet cell-like MTX-E12 cells are a mucus-secreting cell line that offers a model for mucus interactions in studies of intestinal uptake.

Mucin makes a difference to cell-particle processing. For example, direct comparison of Caco-2 cultures to MTX-E12 cultures with 200 nm polystyrene nanoparticles showed a strong particle hydrophobicity-dependent decrease in particle-cell association (consistent with *in vivo* rat studies that showed decreased polystyrene nanoparticle uptake with increasing hydrophobicity of particle surface coating); this difference effectively disappeared with the pre-treatment removal of the mucus layer from the MTX-E12 cultures. Nevertheless, most of the MTX-E12-associated particles were found to be trapped within the mucus layer that had re-formed during culture with particles. Moreover, 200–300 nm chitosan nanoparticles were found to reach a steady-state association value with MTX-E12 cells in half the time required for Caco-2 cells, due to the strong, electrostatic, chitosan-mucus interactions. Conversely, hydrophilic, 200–300 nm PLA-PEG nanoparticles showed minimal cell association for both cell types [120]. Therefore, especially in the case of hydrophobic and/or positively charged particles, mucinylated gastric epithelial cells are recommended for *in vitro* particle testing, given the high degree of uptake and aggregation state dependence for nanoparticle systems. Studies should therefore focus on ascertaining relevant characteristics of the epithelium *in vivo* relevant to the study design, and produce *in vitro* conditions to recapitulate these traits *in*

vitro as accurately as possible. Cell selection and contextual justification is critical to these determinations.

Endothelial cells tightly line the various vascular conduits within the body and represent the barrier to transport of particles and other species from the blood to surrounding tissues including the blood-brain barrier which severely restricts access of foreign materials and substances to the brain [121]. Due to the varied nature of the circulatory system, endothelial cells vary widely in character from the aortic lining to the blood-brain barrier to the capillary beds of the lung [122]. Therefore, a wide variety of endothelial cells are applied to *in vitro* tests, but relatively few of these have been tested against nanoparticles. Secondary transformed endothelially derived cell lines include COS-7 (kidney) and MS-1 (pancreas). Human aortic endothelial cells (HAECs), human umbilical vascular endothelial cells (HUVECs) and human microvascular endothelial cells -- all commercialized primary cell isolates - have been used as models of endothelial inflammatory response and resultant arthroscleroses inducible by circulating metal oxide particles.

Specifically in this context, alumina nanoparticles were claimed to be toxic to HUVEC cells [123], FeO_3 nanoparticles showed no toxicity to HEACs and induced no significant inflammation at even the highest levels tested while Y_2O_3 and ZnO both induced significant inflammation at the highest doses tested. Only ZnO caused any appreciable cell death, 20% and 50% cell mortality [124]. Moreover, HUVECs and porcine endothelial cells showed dose-dependent up-regulation of the inflammatory adhesion proteins, ELAM-1, ILAM-1, and VCAM-1, in response to alumina nanoparticle administration at surface area-specific doses, with increasing adhesion of activated monocytes [125]. This points to the potential of metal oxide nanoparticles to initiate/exacerbate inflammatory artherosclerotic processes in the absence of cell mortality. PLA-PGA particles also demonstrated adherence to cytokine-inflamed HUVEC as a model for inflamed endothelium *in vivo* consistent with adhesive similarities between PLA-PGA particles and leukocytes (which also adhere to inflamed endothelium) [126].

By contrast, human aortic smooth muscle cells were used to assay uptake of poly (D,L-lactide-coglycolide) (PLGA) polymeric nanoparticles. Particle internalization decreased with increasing concentrations of polymer-associated polyvinyl alcohol (PVA), residual from the NP synthesis [127]. ECV304 endothelial cells (considered relevant for parenteral administration) and 3T3 fibroblasts were transfected with DNA plasmid complexes with both low- and high-molecular weight polyethylenimine (pGL3/LMW-PEI & pGL3/HMW-PEI), respectively, and tested for cytotoxicity with unconjugated HMW-PEI and LMW-PEI. HMW-PEI coated the outer membrane of the cell, inducing necrosis, while LMW-PEI did not adhere to the cell surface. Instead, small LMW-PEI aggregates were found inside ECV304 cells by transmission electron microscopy (TEM) [128].

2.4. Tumor cell models

Aside from common precancerous cells, numerous oncogene-transformed cancerous cell lines (MSTO211H [129], HL60 [130], WTK1 [130], 1321N1 [131], HeLa [75] are employed *in vitro* for materials testing, some for no apparent reason other than convenient culture, rapid expansion and growth, and commercial availability for experimental exploitation, others for their intrinsically high phagocytic abilities for particle uptake [53]. They are also utilized to predict pharmacologic endpoints of specific tissue-derived tumors to nano-based cancer therapies. Specifically, SH-SY5Y human neuroblastoma cells of epithelial origin, were examined for cytotoxicity from functionalized quantum dots (QDs) bearing a variety of surface chemistries. Cell-specific QD cytotoxicity (e.g., to SH-SY5Y cells) reflects both QD limitations for tissue imaging or therapy, but could also allow assessment of novel methods to simultaneously image and then treat neuroblastomas using toxic QDs [130].

Cell-based testing of many other polymer nanotherapeutics uses diverse assortments of cell lines to assay various delivery vehicles: both N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-bound doxorubicin [132] and polyamidoamine (PAMAM) G4 dendrimer-succinic acid-paclitaxel conjugates [133] have been taken up by human ovarian cancer cells (A2780); poly (D,L-lactide-coglycolide) (PLGA) polymeric nanoparticles have been assayed for uptake by human arterial smooth muscle cells (HASMC) [134]; nanoparticles of cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine and Tween-80 were used to transfect human prostate tumor PC-3 cells [135]; poly(D-glucaramidoamine)-compacted DNA nanoparticles were used to transfect BHK-21 (baby hamster kidney) cells [136]; plasmid conjugates of both low- and high- molecular weight polyethylenimine (pGL3/LMW-PEI & pGL3/HMW-PEI) have been used to transfect ECV304 endothelial cells and 3T3 fibroblasts [137]; 2Mb plasmid conjugates, including NaCl-DNA, PLL-DNA, and PEI-DNA were each used to transfect HT1080 human cells [129]; plasmid-PEI-adenovirus conjugates were used to transfect immortalized (DMD) and primary human fibroblasts [138]; histidinylated polylysine-plasmid complexes were used to transfect immortalized cystic fibrosis airway surface epithelial cells (ΣCFTE29o- cells) and airway gland serous cells (CF-KM4 cells) [139]; and plasmid-polygalactosamine (pGL3/pGalN), small (15kDa) and large (100kDa) plasmid-chitosan complexes, and plasmid-lipofectin (pGL3/lipofectin) complexes were used to transfect human-lung carcinoma A549 cells, Hela cells, and B16 melanoma cells [140].

3. Cell-based *in vitro* toxicity assays

Cell-based assays are currently considered central to toxicity testing [9, 141], biomaterials testing [142] and environmental materials exposure testing [143]. Despite the frequent lack of consistency or predictability between *in vitro* models and *in vivo* observations, there is little rational or ethical justification to proceed directly from materials synthesis to animal models, although this appears to be increasingly done in industrial development. Cell models still seek validation as a useful screening bridge between materials quality analysis and *in vivo* deployment. The standard *in vitro* test methods are specified in ISO 10993 [144, 145]. Historically, there is substantial work reported for cell-based assays on various colloids and materials particulates [146]. It follows logically that numerous cell-based assays have also been applied to characterize the response of cells to nanomaterials. Nearly all of these assays have been adapted from other applications. As a result, a degree of ambiguity or inconclusiveness is inherent in some answers from certain assays as applied to nanosystems, sometimes due to intrinsic challenges associated with the analysis of nanomaterials. For example, intrinsic photometric absorbance or fluorescence of nanosuspensions or colloids may alter colorimetric or fluorometric assay reporting. High surface energy and surface area of nanomaterials may also contribute to binding of unanticipated amounts of assay reagent or analyte. Often, ambiguity results from the spectrum of inconsistent or different cell responses observed in these assays frequently interpreted either as toxicity or adverse reactivity. Therefore, corroboration of answers from many different assays is vital to forming valid and credible conclusions about interactions of nanomaterials with cell cultures [97]. Five main assay categories, including ROS production, cell viability, cell stress, cell morphology phenotyping, and cell-particle uptake assays, are central themes in such testing. Challenges in interpreting cellular results in various ‘gray scales’ of toxicity or non-toxic responses include lack of consensus regarding cytokine combinations that reflect acute or chronic toxicity, assay reproducibility and reliability, assay sensitivity, and translation of results from *in vitro* to *in vivo* systems.

One particular concern is a lack of distinction currently between cell assays of nanomaterials in serum-containing media versus those in serum-free media. Serum-free media is often employed to avoid complications of protein-particle interactions in cells. Many studies acknowledge ineffective cellular delivery of nanomaterials (e.g., polymer therapeutics or gene

vectors [142]) in the presence of serum proteins and, therefore, employ serum-free media. However, this introduces questions of relevance and biological equivalence since no *in vivo* exposure would occur in the absence of host proteins. This also has enormous implications for the physical presentation of nanomaterials to cells and possible effects of dispersed versus aggregated materials exposure to cell processing mechanisms and response. Serum proteins comprise several thousand different proteins identified to date amounting to 4–7 mg/ml total protein content in 10% serum media common to cell culture. Many of these are highly surface-active and rapidly promote particle aggregation and even precipitation of colloidal materials introduced into this media. Large particles (micron to sub-100nm size) bind proteins indiscriminately and rapidly from the milieu, changing their surfaces presented to cells and causing flocculation. By contrast, sufficiently small particles (<10nm) are actively bound to, solubilized by, and carried by serum proteins. This facilitates cellular transport mechanisms distinct from bare particles and their aggregates that require phagocytosis or pinocytosis for cell entry. Protein adsorption to particles is extremely likely and pivotal to understanding cellular processing [129]. However, few reliable methods outside of conventional protein radiolabeling and centrifugation and elution protocols have been reported, particularly for full serum analysis. Nonetheless, certain *in vivo* situations experience only transient exposure to plasma or serum. For examples, wound sites clotted with blood and often infiltrated with healing-associated macrophages likely are not effectively perfused with blood proteins. Hence, what cells actually experience *in vivo* in context is not consistent or necessarily modeled by either serum or serum-free culture media. Moreover, nanoparticles in serum-free media bind cytokines more avidly because serum proteins are no longer present to compete for surface sites. This results in reduced levels of detectable cytokines in serum-free media with NP vs. serum-rich media with NP [147], requiring corroborative measurement of cytokine-specific mRNA expression. Additionally, the switch to serum-free media for cultures long-adapted to serum media often requires a specific recipe for each cell type, along with the potential for possible sub-lineage selection [148], phenotypic alteration, decreased survival [149], increased phagocytosis [150, 151], increased cell stress, increased cell transport, altered cell membrane turn-over, altered transcriptional behavior and protein production, and induction of stress responses. Hence, serum-free media used without justification or testing can produce a highly artifactual cell culture condition not particularly relevant to “real world” cell-based testing. Yet, inclusion of serum complicates consistent nanomaterial presentation to the cells and complicates their bioassay [152].

3.1. ROS production assays

Cell-based ROS production was described above in section 2.1 as a consequence of nanoparticle exposure. Since phagocytes are the predominant cell model for nanoparticle toxicity, many cell assays interrogate phagocyte ROS production [153]. (see Table 2). Care must be exercised in ROS-production assays involving carbon black (CB) and TiO_2 as these have both been shown to also generate ROS in cell-free systems [9]. This behavior generally extrapolated over other chemistries that naturally produce ROS precludes accurate determination of ROS produced exclusively by cells. Nevertheless, numerous cell-particle assays have been applied to this endpoint; some seek to quantify the ROS species production by cells directly while others seek to quantify its effects on cell behavior or further production of other cell biochemical reactions.

3.1.1. ROS detection—Direct measurement of ROS in cell media has been accomplished by two similar fluorescein-compound-based tests or by Electron Paramagnetic Resonance (EPR). The reactive fluorescein probes, 2’,7’-difluorescein-diacetate (DCFH-DA) [49, 154] and dichlorodihydrofluorescein diacetate (H2 DCFDA) [68] both fluoresce when oxidized by ROS yielding an optical ROS concentration-dependent response [52, 155]. EPR has the advantage of being an effective radical detection method in either the presence or absence of cells allowing

for the use of a greater variety of experimental controls. For EPR detection of radicals, an adduct-forming, spin-trapping agent (5,5-dimethyl-l-pyrroline N-oxide, DMPO) for hydroxide ($OH^{\cdot -}$) or superoxide ($O_2^{\cdot -}$) radicals or a radical-consuming spin-probe (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) are introduced into the culture or nanoparticle solution for a set amount of time after which the entire supernatant is collected, vortexed, and analyzed on an EPR spectrometer [46, 49, 54]. Additionally, though reactive nitrogen species are studied much less often in cell culture systems, $NO^{\cdot}$ is a radical commonly produced by phagocytes that may react with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$) [156], one of the most reactive oxidizing species. However, $ONOO^-$ readily degrades to $OH^{\cdot -}$ and may thus be detected indirectly. Nevertheless, $NO^{\cdot}$-specific detection and $ONOO^-$ may be accomplished using the EPR spin probe colloid, iron-N,N-diethyldithiocarbamate, Fe-$(DETC)_2$ and 1-hydroxy-3-carboxy-2, 2,5-tetramethyl-pyrrolidine (CPH), respectively [94]. Though Fe-$(DETC)_2$ may not be used in cell solutions [157], $ONOO^-$ may also be detected in cell systems by fluorescent DCF detection (discussed earlier in this section) [157].

3.1.2. ROS effector assays—As stated above in section 2.1, potent but short-lived reactive oxygen species (ROS) are produced by several cell types either as a natural function (e.g., inflammatory oxidative burst response) or in response to other stresses [158]. ROS is produced in response to biomaterials exposure, including particulates [52, 91, 92, 159]. As ROS is indiscriminate in its biochemical oxidative reactivity, most cells have built-in defenses to neutralize ROS by using ubiquitous glutathione, an endogenous, reducing agent depleted by ROS insult. Thus, ROS encountered by a cell may be quantified by the well-known glutathione (GSH) assay [49, 93, 142, 160]. Other assays seek to quantify ROS-effected damage to cell membranes or DNA by essentially analyzing key oxidized species. Immunocytochemistry allows for the detection of specific DNA lesions, such as the OHradical- specific 8-hydroxydeoxyguanosine lesion, a direct measure of ROS involvement in DNA damage [68]. Alternatively, BODIPY-C_{11} is a fluorescent dye that inserts itself into lipid bilayers allowing oxidized and unoxidized lipids to be imaged by their respective green and red colors and quantified fluorimetrically [46]. ROS-induced membrane lipid peroxidation may occur both at the cellular level and organelle level, especially in membranes of highly metabolically active mitochondria, making mitochondrial injury a common indicator of elevated intracellular ROS levels [132]. Compromised cell viability and cell death may result from this insult as well, making this a key analytical marker for ROS-induced oxidative membrane damage.

3.2. Cell viability assays

Cellular metabolism is the focus of many cell viability assays [132]. Assays of live versus dead cells in a sample provide gross estimates of the cell response to an insult and are frequently used as an overall biocompatibility assessment criterion. A number of routine, dye-based cell viability assays reviewed previously are available for this assessment [161]. Most involve differential inclusion, exclusion, or conversion of an added dye or enzymatic conversion of a dye precursor in living versus dead cells that can then be distinguished and quantified colorimetrically. Such dye-dependent assays include neutral red, trypan blue [142], LIVE/DEAD™, lactate dehydrogenase (LDH) [46, 68, 155, 160], formazan-based assays (MTT [46], MTS [19], WST), alamar blue (resazurin) [75], coomassie blue [48], ATP-luciferin luminescence [48, 49], adenylate kinase (AK) release [52], mitochondrial membrane potential (MMP) [48], and thiobarbituric (TBA) assays. These assays possess potential for side reactions and ambiguities. For instance, cysteamine-coated quantum dots have been observed to catalytically reduce MTT to formazan without cellular metabolism [155]; high-density cultures or long incubation times have shown the potential for a secondary reduction of the Alamar blue product to a non-fluorescent, confounding species; silver nanoparticles have been shown to interfere with the Live/Dead™ assay [131], and the Coomassie blue assay primarily reacts with arginine, making it composition-dependent and may be additionally confounded by small

amounts of SDS in solution [162]. Therefore, all such dye-based assays require careful calibration in the presence of high specific surface area particles or nanomaterials as controls to determine interference and standardization issues for reliable quantitation of cell-specific activities.

Fluoresence-activated cell sorting (FACS) is capable of categorizing and quantifying cells as healthy, dead, apoptotic, or necrotic, and can distinguish alterations in the cell cycle dynamics of a cell population, such as an increased proportion of hypodiploid DNA cells in sub-G0/G1 phases, which is indicative of necrosis or apoptosis [163]. Additionally, FACS may be used to quantify receptor expression by cells to reveal mechanism of cell death, such as the up-regulation of the Fas receptor, an integral component in the formation of the apoptotic, death-inducing signaling complex [57]. FACS has the advantage of automation and parallel processing of multiple cell targets in single multi-color assays. However, multiple antibody-hapten non-specific cross-reactivities and specificities, and assay interference from high specific surface area nanomaterials must be determined in such assays to assert their accuracy and validity with cells.

Other cell viability assays are also pathway-sensitive indicating apoptotic DNA fragmentation and leakage from the cells. These include the enzyme-linked immunosorbent assay (ELISA) to quantify fragmented DNA [132], comet [33], Caspase Glo3/7 [164], Hoechst-DNA [52], and (TdT-mediated dUTP-biotin nick end labeling) TUNEL assays [52, 165, 166]. The comet assay measures fragmented DNA by gel electrophoresis [46, 167], Caspase Glo3/7 quantifies downstream effectors of the mitochondrial apoptotic pathway, and Hoechst-DNA is a fluorescent probe that binds to adenine-thymine-rich regions on double-stranded DNA that has leaked out of the nucleus in cells under stress [131]. TUNEL measures the amount of fragmented DNA in the nucleus to quantify cell apoptosis [52]. Control assays in the presence of nanomaterials should determine assay interference in the presence of culture milieu.

Some quantitative and some more qualitative assays of cell death are accessible by microscopy. Quantitative viability results may be obtained from light microscopy by counting numbers of adherent treated cells or cell colonies relative to control cell adherence [167]. Qualitative light microscopy results range from detection of cellular debris or detached cells to identification of necrotic and apoptotic cells. Visible symptoms of apoptosis are reported to include cell detachment, rounding of cells, cytoplasm retraction, and nuclear shrinkage due to chromatin condensation [33]. This kind of qualitative visual characterization should not be used as a stand-alone viability assay, but may be used to confirm a common pathway of cell death identified in other assays for a variety of materials [56].

3.3. Cell stress assays

These assays seek to identify non-lethal injuries to cells or changes in cellular behavior resulting from environmental insult. These changes/injuries to cells are manifest in their modulation of protein or gene expression, phagocytic ability, and/or inflammation reactions that alter normal phenotype. Hence, metrics for ‘normal, background’ cell phenotype and its retention and stability in untreated cohorts must be convincingly and reliably determined for comparison and control.

3.3.1. Protein/gene expression—Changes in select gene/protein expression patterns may be detected by polymerase chain reaction (PCR), western blotting, and total protein assay (i.e., BCA or Bradford). Quantitative, real-time PCR (qPCR) has been applied to detect toxicity-modulated gene expression in A549 cells exposed to varying dilutions of nano-sized, propane-combustion-generated particles. Specifically, expression modulation of CDKN1A (cell cycle arrest gene), GADD45β (DNA damage-dependent), IL-6 (inflammatory response), NFκBIA (inflammatory response), EGFP (reporter gene), and NF-κB (involved in promoter/enhancer

regions of fibrotic/inflammatory growth factors, cytokines, and adhesion molecules) were assayed. GADD45β and NF-κB expression increased with time and with increasing cell mortality signaling increases in DNA damage and inflammation. Significantly, for cells treated with dilute particle solutions, wherein cell damage, but no appreciable mortality was observed, CDKN1A was up-regulated, arresting cells in G1 phase, possibly to enhance repair of DNA damage. For less dilute particle solutions that caused significant cell mortality, CDKN1A was initially up-regulated, but subsequently down-regulated, possibly indicating apoptotic activity [56]. Thus, changes in gene expression can reveal toxicity-dependent modulation of gene expression and help to elucidate possible mechanisms of toxicity.

Western blotting has been used to examine the effects of nanotubes on cell adhesion by assaying adhesion proteins: fibronectin, laminin, p-cadherin, FAK, collagen IV, β-actin, and cyclin D_3 [168]. However, cell protein production is cell-cycle dependent and altered distinctly as a function of applied insult in confluent, contact-inhibited cultures vs. non-confluent (proliferating) or non-contact inhibited cells. So, careful control measurements at similar cell densities and growth stages must be used for comparisons of relative changes. Down-regulation of protein expression from cell stress or oxidative damage results in changes in the extracellular matrix, cytoskeleton and cell morphology as well as displacement of organelles, disturbing normal cell adhesion and spreading [33]. Total cell protein assays are routinely used in combination with protein expression assays to normalize gene/protein expression data (such as luciferin assay data) to monitor up-regulation of genome and cell transcriptional activity [33]. Changes in certain key cellular proteins involved in either toxicity pathways or inflammation (e.g., cytokines, chemokines) in cells under stress are altered compared to controls.

3.3.2. Inflammatory markers—The characteristic *in vivo* response of macrophages to insult includes some form of oxidative (ROS) burst (see section 2.1) and chemical recruitment of additional macrophages and auxiliary cells using cytokine and chemokine chemotactic pathways to the site of disturbance. The primary ROS-independent means of assessing cellular inflammation is through qualitative observation of inflammatory protein expression by immuno-fluorescence [167] or quantification of relevant protein signaling molecules (cytokines) at the protein level (e.g., via ELISA or Western blotting) and up-regulation of the mRNA that codes for their synthesis (using PCR variant assays) [126].

Direct cytokine protein detection has traditionally focused on surface capture immuno-mediated sandwich assays in various permutations, including enzyme-linked immunosorbent assay (ELISA) [48, 49, 54, 126, 156, 160]; or innovations upon the ELISA design that allow for the simultaneous detection of 8 (LINCOplex) [162] or hundreds of cytokines in multiplexed assay formats. Recent permutations of cytokine detection assays are reported. These include multiplexed cytometric bead assays, namely CBA — a distinct application of flow cytometry equipment employed for fluorescence-activated cell sorting, FACS, discussed above), but exploiting libraries of various fluorescent bead-linked antibodies for the simultaneous detection of 30 or more cytokines [169]. Also, microarray -printed immuno-capture multiplex assays such as Quansys Q-Plex™, based on 96-well plates wherein up to 25 different capture antibodies have been printed on each well surface, allow for simultaneous quantification of up to 25 unique analytes from single 5–30 μl samples [170]. Hundreds of unique cytokines have been discovered to-date and classified variously into several families by structure, function or targeted receptor. Cytokines are produced severally by the majority of cell types throughout the body and customarily act in concert, with some cytokines potentiating numerous others [171, 172]. However, very few cytokines have been assayed and identified in nanomaterial-exposed cell cultures, with relevant publications focusing cytokine analysis on only a small list of cytokines (<10) which comprises only inflammatory cytokines (see Table 3). Thus, this present, small beginning of cytokine analysis in cell-based nanomaterial testing is set expand

tremendously as understanding continues to develop toward *in vitro* cellular responses to nanomaterials.

Cytokine detection assays, like many other bioassays, are notoriously fraught with user- and milieu-dependent variation requiring meticulous attention to multiple-step preparations and good standard curves *performed in the specific media being assayed*. Assay answers are often qualitative in nature or only indirectly and relatively quantitative compared to a standard curve that, cannot reflect accurate answers unless run under identical media conditions (i.e., spiked standards). Moreover, serum-modulated cytokine adsorption to nanoparticles has been demonstrated, particularly for the case of carbonaceous nanomaterials, such as road tunnel, wood smoke, and diesel exhaust particulate samples, as well as ultrafine carbon black. All were shown to bind cytokines in solution and modify cytokine concentrations to varying degrees. Conversely, quartz mineral particles did not alter detectable cytokine concentrations for the specific cytokines tested, including human variants of TNF-α, IL-1β, IL-6 and IL-8 [173]. Because transient nucleic acid messaging in cells during gene up-regulation and as quantified by the various PCR techniques does not necessarily get expressed as protein or correlate with the amount of its protein product, protein assays (e.g., ELISAs, Western blots) are often encouraged to ensure such correlations. Therefore, quantification of soluble cytokines should be corroborated by concomitant quantitation of cytokine-specific mRNA message expression obtained by reverse transcriptase polymerase chain reaction (RT-PCR) of the lysates of treated cells [156, 160, 174] or analysis of up/down regulation of inflammation-related genes by a microarray gene expression technique such as the Affymetrix mouse genome gene chip oligonucleotide array or other appropriate gene chip modality [54]

3.3.3. Cell visualization—phagocytic activity, internalization, and organelle interaction—Cellular uptake of colloidal materials, particles, and nanomaterials is typically divided among four common routes of cell internalization, including phagocytosis, clathrin-mediated endocytosis, macropinocytosis, and non-clathrin-mediated endocytosis, though these four categories may fit under the two original internalization pathways defined by the work of Silverstein and Steinman [52]. Phagocytosis (typically from macrophages, monocytes and neutrophils) is the uptake of particles larger than 500nm, often triggered by particle opsonization (i.e., protein-particle adsorption) and subsequent receptor-mediated activation of F actin-driven pseudopods that engulf the particle in a cytoplasmic phagosome. Alternately, phagocytosis may be blocked by application of cytochalasin D [175], which blocks the polymerization of actin [176], inhibiting pseudopodial action. While macrophages, monocytes, and neutrophils are normally termed “professional phagocytes”, epithelial cells, fibroblasts, and other cells also can uptake particles [177], likely by pinocytosis — a process common to nearly all cells [178]. Pinocytosis usually occurs at the sites of clathrin-coated pits (present in most animal cells) and involves the passage of particles smaller than 200nm in an inadvertent sort of particle uptake along with cell-surrounding fluid in cell drinking. Macropinocytosis (a type of fluid-phase endocytosis) is the formation of vacuoles up to 1–5 micrometers by considerable ruffling of the cell membrane that may be seen frequently in certain cell types (i.e., macrophages, dendritic cells, and fibroblasts) and has also been suggested as the uptake mechanism responsible for the concentration of polymers and macromolecules seen in tumor cells manifesting enhanced permeability and retention (EPR) [179]. Caveolae-mediated uptake, or potocytosis, also occurs in most cells and is best detected after deactivation of the clathrin-dependent internalization pathways. Rather than mediating material sequestration in lysosomes or endosomes, caveolae are thought to mediate transport of material across endothelial cells (transcytosis) [180].

Cell-particle internalization may be monitored directly in culture to reflect relative cellular uptake activity, phenotype and health [181, 182]. Phagocytic ability, measured by cellular uptake of colloidal 2-micron diameter gold latex beads or other fluorescent tracking particles, has been

assayed as an indicator of nanomaterial toxicity in alveolar macrophage cultures. Following a 6-hour incubation with a sample array of test materials (one low and one high sample concentration for each material), cells are transferred to gold latex bead-containing media and phagocytic ability is assessed as the relative (compared to untreated controls) proportion of cells capable of ingesting 2-micron diameter gold latex beads during a 16-hour bead-in-media incubation [50]. However, uptake of a reference bead may vary significantly from the uptake experimental particles, particularly in the case of disparate sizes or surface chemistries. In some macrophages, cellular activation is prerequisite to phagocytic activity, as shown in glial cells [183] and THP1 human macrophages [184]. Hence non-activated, non-phagocytosing healthy cells must be distinguished from injured/dying cells whose phagocytic ability has been retarded by toxic insult. Particle dose-cell uptake response relationships should be first controlled to ascertain whether the colorimetric reference particle might by itself exert an influence on the overall cell response alone, and whether there is a reliable correlate in its uptake with nanomaterial exposure (e.g., dose response or saturable response changes in phagocytic activity. Variations of this experiment using naive phagocytes exposed to conditioned media only (centrifuged away from cells and nanoparticles in pre-exposed cultures) or as transwell-insert co-cultures in various configurations exposed to nanomaterials in different ways also offer new possibilities to assay cell response to these materials in the presence of other cells, reporters or media influences.

Receptor-mediated internalization pathways are thought to be temperature dependent and are thus assayed by examining the variation of analyte uptake with temperature, with decreased or discontinued analyte uptake at lower temperatures (4°C) being indicative of receptor-mediated uptake [185]. Clathrin-mediated pathways may be inhibited by application of chlorpromazine or by over-expression of Eps15 and thus identified. Macropinocytosis-dependent internalization may be inhibited and identified by application of 5-(*N*,*N*-diethyl) amyloidal hydrochloride, a macropinocytosis inhibitor [186]. Presence/absence of caveolae-mediated internalization pathways for a given particle may be demonstrated by BODIPY-labeled LacCer, a sphingolipid that is caveolae uptake dependent, applied in combination with filipin or genestein, inhibitors of caveolae-mediated uptake, to demonstrate caveolae dependence [186]. Cellular uptake of optically active particles may also be assessed and quantified by flow cytometry or by various microscopies. Significantly, flow cytometry is capable of sorting free particles, particle aggregates, cell-associated particles, and particle-free cells, allowing for the assessment of fractions of cells that associate with particles if the particles are intrinsically fluorescent or can be tagged post-facto with fluorescence [186].

Aside from cell-particle uptake assays, microscopy provides the added advantage of revealing particle localization and trafficking within the cell. High-resolution inspection of chemically fixed and carefully desiccated cell samples by TEM—its high-resolution (HR-TEM) and energy-filtering (EF-TEM) embodiments—can reveal organelle-nanomaterial interactions that may help to elucidate nanomaterial-specific mechanisms of cellular toxicity [48, 49, 52–54, 187]. Cell-nanomaterial interactions observed by TEM include differentiation of lysosomal aggregates from membrane-bound aggregates [50], nucleus migration toward membrane-bound clusters of single-walled nanotubes [55], and disordering of the cytoskeletal actin networks [33], all of which are indications of nanomaterial-modulated cell activity. Additionally, Electron Energy Loss Spectroscopy (EELS) [33] and confocal Laser Scanning Microscopy (LSM) fluorescence microscopy have also been used to image particle uptake and internalization within cell organelles (e.g., endosomes, lysosomes) or nuclear penetration [49]. Moreover, LSM offers accessibility of pseudo-3D images through image reconstruction algorithms combining several axial and lateral images [49, 187].

The complexity of extra- and intra-cellular environments presents a variety of obstacles and interactions to particles that require elucidation in order to fully understand cellular reactions

and processing. Multiple particle tracking (MPT) assays offer a method to observe and understand particle interactions with various cell components in real-time using video - interfaced phase microscopy to track particle movement through the various environments encountered in cellular trafficking. Tracking of individual particles can reveal information on pore sizes, particle adhesion, intracellular particle transport mechanism, and barriers to particle transport by observation of particle movement characteristics or immobilization behavior and localization [50]. Additionally, two-point micro-rheology, a variation of MPT, utilizes automated computer-based analysis of thousands of micrographs, including particle sizes and motions over time, to observe particle Brownian motion yielding information concerning viscosity and shear moduli in various parts of the cell [188]. MPT allows for the interrogation of cell-particle interactions not accessible by any other means. That this method is readily extended to nanoparticles in cellular systems remains to be reported.

As an alternative to determining cell reactivity to particles using mammalian cell-based assays, phenotypic behavior of other cell cultures to nanophase materials can also be used to provide cell toxicity information. For example, the single-celled ciliated protozoa, *Tetrahymena thermophila*, (grown in the absence of mammalian cells in supplemented or unsupplemented salt solutions, protease peptone yeast extract (PPYE) and Osterhout's solution, respectively) have also been used as a model of cellular stress in the presence of test materials. Intrinsic protozoan bacterivory activity (ingestion of green fluorescent protein labeled-*E. coli* bacteria) is monitored by video-enabled, phase-contrast microscopy over a period of 1–2 hours in real-time cultures. Correlative symptoms of cell aggregation, matrix accumulation, diminished mobility, and death in the presence of culture stresses (chemicals, pollutants) are used to corroborate the bacteriovory result. These protozoa have relevance to aquatic toxicity and specific relevance to wastewater treatment [189]. Analogous methods might also be adapted and employed to assay toxicity to nanomaterials for prokaryotes and other non-mammalian cells.

3.4. High-throughput screening methods

Recent advances in cell-based assays allow for toxicity and/or efficacy screening of multiple nanomaterials at multiple concentrations with multiple cell lines, simultaneously. This expansion of experimental design is practically enabled through the miniaturization and multiplexing of the experimental apparatus and method by utilization of either ultra-small 384-well cell culture plates or nanodrop sample chambers on a chip. The nanodrop assay setup allows for different assays with suitable detection features (e.g., fluorescence, luminescence) to be performed in a fraction of the volume without the the cell-activation or photometric effects of the culture plate since the cell culture is performed in a self-contained drop [190]. However, since cells are typically microns in size, nanodrops do not necessarily capture cells themselves, only fluidic cellular exudate for assay and analysis. By assaying numerous material types/functionalizations and material concentrations on numerous cell types, all in parallel, complex interactions between materials and cells may be ascertained through complex data analysis that correlates phenotypes with multi-well plates, cell culture, detection schemes, and recognition schemes [191, 192].

4. Conclusions

The multi-disciplinary nature of the nanotechnology field that brings it strength in innovation also presents significant challenges in the interpretation, validation and correlation of cell and tissue toxicity data collected for nanomaterials. As others have indicated, needed advances in nanotoxicology will come from developing a valid set of reliable toxicity tests [192–194] and nanomaterial characterization protocols for application to the overwhelming variety of nanomaterials that have been produced and the even greater variety that is yet to come.

Standardization against materials reference standards [195] and validation by corroboration and comparison will eventually produce some trends and increased confidence in newly developed methods that do not currently exist. The community is searching now for such reliable methods, reference materials, standard protocols and validations. Without these, the efforts have little scientific credibility and the community must be very careful about conclusions drawn from assays *in vitro*, particularly in their relevance to *in vivo* systems and organismal toxicology. The unique challenges in nanotoxicity assessments lie in addressing the current lack of appropriate tools to directly observe and interrogate nanomaterials in complex biological systems [196]. Specifically, materials aggregation, physical, and chemical reactivity are nearly impossible to understand currently. Their individual and collective effects on dynamic living systems are even more difficult to accurately assess, predict and model. Significantly, pharmacological dose-response relationships are complicated by time- and condition-dependent nanomaterial chemical and physical states. Acute versus chronic nanomaterial exposure effects and hazards are therefore difficult to monitor.

Hence, multiple different measurement techniques must be adapted, carefully assessed for validity, and applied to complex nanomaterial systems. Similar to the blind man describing the elephant, decades of surface analytical chemistry of biomaterials has shown that one can assert most any conclusion from a single analytical measurement. No single analysis can provide sufficient information on the biomaterials surface to correlate biological response. Truths (or approaches to truths) pertaining to complex nanosystems necessitate 1) compilation of multiple experiments focused on common aspects of the same system, 2) corroboration of these results, 3) synthesis of supportive trends in the data, 4) careful exclusion of artifacts and 5) use of proper controls. Specifically, nanomaterial toxicities in biological systems present unique and complex problems. Investigators should be wary of forming conclusions based on single biological assays, isolated cell lines, or protein-free media, and strengthen observations by correlating measurements from multiple different assays.

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Table 1

Select cell-based *in vitro* assays to study particle internalization in cultures.

Cell Line/Name	Particle	Functionalization	Other details	Reference
Human umbilical vein endothelial cells	$C_{60}(OH)_{24}$ hydroxyl fullerenes		*d*=7.1±2.4 nm	Yamawaki, 2006[197]
Human dermal fibroblasts HDF; human liver carcinoma HepG2; neuronal human astroctytes, NHA	C_{60}	COOH, OH, Na^+	*d*=100 nm	Sayes, 2005[198]
Rat alveolar macrophage cell line NR8383; human alveolar epithelial cell line A549	SWNT(Single Walled Carbon Nanotubes); CB14; crystalline quartz particles (DQ12)		SWNT: *d*=1–2 nm, *l* = up to 100 μm; CB14: size =14 nm; DQ12: size < 5 μm;	Pulskamp, 2007[199]
Human 3T6; murine 3T3	SWNT (coating FITC(fluorescein isothiocyanate))		*d*=1 nm, *l*=300–1000 nm	Pantarotto, 2004[200]
Human lung-tumor cell lines, H596, H446, and Calu-1	MWNT; CNF; CB	MWNT: CdO, COOH, OH CNF: CdO, COOH, OH	MWNT: *d*=20 nm, AR = 80–90; CNF: *d*=150 nm, AR = 30 – 40; CB: *d*=submicrometer AR =1;	Magrez, 2006[201]
T lymphocytes; Jurkat T leukemia cells	MWNT; CB	MWNT: COOH, OH	MWNT: *d*=20–40 nm, *l*= 1–5 μm	Bottini, 2006[56]
RAW264.7 macrophage cells	Gold nanoparticles Au(0)	Lys, Poly-L-Lysine, FITC	*d*=3 – 8 nm	Shukla, 2005[202]
Vero cells	Au^3Cu^1 (gold nanoshell)	Polyelectrolytes polyethylenimine (PEI), poly(acrylic acid) (PAA)	*d*= 48.9 ± 19.1 nm, shell thickness = 5.8 ± 1.8 nm	Su, 2007[203]
Hela cells	Gold Nanorods	Phosphatidylcholine [PC]	*w*=11±1nm, *l*=65±5 nm, AR = 5.9	Takahashi, 2006[204]
Rat pheochromocytoma cell line PC12M	Magnetic nanoparticles (MNPs)Fe_2O_3	DMSA	*d*= 5–12 nm	Pisanic, 2007[205]
Human monocyte-macrophages (HMMs)	Fe_3O_4	Dextran	*d*=30 nm	Muller, 2007[206]
Human breast cancer cells (SK-BR-3); human dermal fibroblast cells	Fe_3O_4	PMAO-PEG	*d*=9.6 nm	Yu, 2006[207]
Primary human epidermal keratinocytes (HEKs)	QD 565; QD 655	PEG, PEG-amine, polyacrylic acid	565: *d*=4.6 nm 655: *w*=6 nm, *h*=12 nm	Ryman-Rasmussen, 2007[208]
Human lymphoblastoid WTK-1	CdSe QD	MUA (COOH), cysteamine (NH^2), thioglycerol (OH)	*d*=9–48 nm	Hoshino, 2004[209]
Human Dermal Fibroblasts and Human Lung Epithelial Cells	TiO_2 (anatase and rutile)		*d*=3–10 nm	Sayes, 2006 [131]
Mouse J6456 lymphoma cells (J6456-FR), human head and neck KB cancer cells (KB-FR)	lipodot (CdSe QD + lipid coat)	QD: 1,2 dipalmitoyl-sn-glycero-3 phosphocholine, mPEG-DSPE	QD: 2.6, 2.9 nm lipodot: 100 nm	Schroeder, 2007[210]
HepG2 cell	Micellar-like core shell nanoparticles (2 types used)	Type 1: MePEG–PCL Type 2: PCL–PEG–PCL	10 to 200 nm	Hu, 2007[211]

Cell Line/Name	Particle	Functionalization	Other details	Reference
Macrophages and foam cells	Dexamethasone-liposomes (in 3 sizes)		518.7 ± 49.5 nm (L500) 202.2 ± 23.1 nm (L200) 68.6 ± 6.5 nm (L70)	Chono, 2006[212]
Human lung fibroblasts (ATCC, MRC-9)	CeO_2 Cerium Oxide Nanoparticles		*d*= 25–50 (diffusion) up to 250–500 (sedimentation) nm	Limbach, 2005[213]
Dendritic cells (DC)	Fluoresbrite™ carboxylated yellow-green microspheres; yellow-green fluorescent (505/515) carboxylate-modified FluoSpheres	FITC, (TT, PS, PA, PLL, WGA for 0.1 – 1 μm only)	Fluorebrite: 0.1, 0.5, 1.0 and 4.5 μm Fluospheres: 0.04, 10 and 15 μm	Foged, 2005[214]
Hamster kidney cell line (BHK-21)	25-kDa polyethylenimine (bPEI); beta-cyclodextrin-containing polymer (βCDP)	PEG (adamantane-PEG_{5000} conjugate)	100 nm	Mishra, 2004[215]
Langerhans cells (LC), DC	Human papillomavirus-like particles (HPV 6bL1 VLP)	Carboxifluorescein Diacetate (CFDA) (label)		Yan, 2004[216]
Caco-2; MTX-E12 (mucus)	Polystyrene NP, chitosan NP and PLA-PEG NP; Chitosan NP	PLA-PEG & Chitosan: labeled with FITC-BSA	PS: 213 ± 8 nm Chitosan: 290 ± 7 nm PLA-PEG: 196 ± 20 nm	Behrens, 2002[217]
Human alveolar epithelial cell line A549; human monocytic leukemia cell line THP-1; human monocytic leukemia cell line Mono Mac 6	Hematite ((-Fe2O3); silicasol particles (SiO2, amorphous silica) DQ-12 (reference)		Hematite : 50± 90nm (mean 70nm) silicasol S100: 80±110 nm (mean 100 nm) silicasol S60: (mean 60 nm) DQ12: < 5 μm	Wottrich, 2004[218]
Tissues from liver, spleen, kidney, heart, lung, brain	Iron oxide MNP	Oleic acid (OA) and Pluronic	Core *d* =11 ± 2 Nm	Jain, 2007[219]
3T3 fibroblast cells	Silver nanoparticle	Phosphorylcholine (PC), phosphorylethanolamine (PE)	*d*=3.8 nm	Chung, 2008[220]
Endothelial and Kupffer cells	Liposome particle (PC, Chol, PS)		200±38 nm	Rothkopf, 2004[221]
Kupffer cells	PS microspheres; polystyrene-polyethylene oxide (PS-PEO) copolymer microspheres		PS: 1 μm PS-PEO: 1 μm	Harper, 2003[222]
Mouse Peritoneal Macrophages (lavage)	Polystyrene Nanospheres and Microspheres	Poloxamer 338 and 188, Egg Lecithin, Secretory immunoglobulin A, & ^{131}I surface (label)	*d*= 60 nm and 5.25 μm; 160 nm (uncoated only)	Illum, 1986[223]
Mouse Peritoneal Macrophages (lavage)	Fluorescent polystyrene microspheres	Various surfactants and protein Isothiocyanate fluorescein (label)	*d*= 0.995 μm	Naon, 1995[103]
Rat glioma cell lines: 9L gliosarcoma (GS-9L); RG-2; F-98; Caco-2 (control)	Doxorubicin–loaded nanoparticles (Dox-PBCA-NP); Empty NP (PBCA NP)	Poloxamine 908, polysorbate 80, poloxamer 188	Dox-PBCA-NP: 270± 20nm PBCA NP: 250±30 nm	De Juan, 2006[104]
MG63 osteoblast-like cells; Primary human osteoblast-like cells	UHMWPE; commercially pure titanium (cpTi); Ti–6Al–4V (Ti-A); cobalt-chrome (CoCr)		UHMWPE: *d*= 1.0±0.96 μm; cpTi: *d*= 0.84±0.12 μm; Ti-A: *d*= 1.35±0.09 μm;	Lohmann, 2000[224]

Cell Line/Name	Particle	Functionalization	Other details	Reference
			CoCr: *d*= 1.21±0.16 μm	

Abbreviations: AR = aspect ratio, BSA= bovine serum albumin, C+ = positive control, C- = negative control, CB = carbon black, Chol = Cholesterol, CNF = carbon nanofibers, DMSA = Dimercaptosuccinic acid, mPEG-DSPE = methoxy–polyethylene–glycol–distearoyl–phosphatidyl–ethanolamine, MePEG–PCL=di-block copolymer of methoxy poly(ethylene glycol)–polycaprolactone, MUA= 11-mercaptoundecanoic acid, PA= poly-d-l-alanine, PBCA= polybutylcyanoacrylate, PCL–PEG–PCL= polycaprolactone–poly(ethylene glycol)–polycaprolactone, PEG = poly(ethylene glycol), PLA= poly (lactic acid), PLL= poly-L-lysine, PMAO = poly(maleic anhydride-alt-1-octadecene) PS= protamine sulphate, QD = quantum dot, TT= tetanus toxoid, WGA= lectin wheat germ agglutinin

Table 2

Techniques to detect ROS and RNS *in vitro* (adapted from Ref.33)

Assay	ROS detected	Advantages	Disadvantages
Chemiluminescence	Oxygen radicals	Quantitative	Specificity
Salicylate	·OH and $ONOO^-$	Quantitative	Limited to ·OH and $ONOO^-$ detection only
Cytochrome C	ROS and RNS	Quantitative and Simple	Only *in vitro*, no information about ROS species
DCDHF	ROS	Both $intra^-$ and $extra^-$ cellular ROS can be detected, visualized	Autocatalytic degradation, no information about ROS
Product analysis	ROS	Employs well established analytical techniques	Does not provide unequivocal evidence
Total GSH depletion	ROS	Simple	Does not provide GSH:GSSG ratio
ESR/EPR	Free radicals	Uses both *in vitro* and *in vivo*, Quantitative, structural information	Not possible to calibrate *in vivo*
Inhibition by SOD	Superoxide	Simple, highly specific	Only applicable to superoxide
Inhibition by antioxidants	ROS	Simple	Little information about radical species

Table 3

Inflammatory and healing cytokines related to nanotoxicity[†]

Cytokine	Receptor	Source	Targets	Major Function
Interleukin (IL)-1α; IL-1β	IL1RI and IL1R-AcP	Macrophages, many others	Macrophages, thymocytes, CNS, others	Inflammatory; promotes activation, costimulation, and secretion of cytokines and other acute-phase proteins; pyrogenic
IL-6	IL6Rα and gp130	Macrophages, T cells, fibroblasts, and others	Wide variety of cells: B cells, T cells, thymocytes, myeloid cells, osteoclasts	Inflammatory and costimulatory action; induces proliferation and differentiation; synergizes with TGFβ to drive Th17
IL-8	ILa and ILb	Macrophages, endothelial, keratinocyte	leukocytes	Proinflammatory
MCP-1	CCR2, CCR12	Macrophages	Macrophages, Glial cells	Proinflammatory
MIP-2	CCR1	Macrophages	T lymphocytes, T cells	Proinflammatory, chemokine
Prostaglandin E_2	PTGER2: EP1, EP2, EP3, EP4 subtypes	Macrophages, Fibroblasts	Osteoblasts, endothelial, dendritic, and carcinoma cells, various others	Inflammatory, anti-apoptotic, and neuromodulator
TNFα	Murine: TNFR,p55; TNFR,p75 Human: TNFR,p60; TNFR,p80	Macrophages, monocytes, T cells, others	Neutrophils, macrophages, monocytes, endothelial cells	Inflammatory; promotes activation and production of acute-phase proteins

[†]adapted from Ref. [225–228]

(those reported in response to particle exposure are bolded: to date, only inflammatory cytokines have been reported)