Nanotechnology Environmental, Health, and Safety Research

Review the currently used and soon to be used nanoparticles and nano-enabled devices in pharmaceutical and other biomedical industries

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Summary of All Objectives of the Work Project

To produce a comprehensive technology landscape and review of the state-of-the-art and state-of-the-science relevant to nanobiotechnology

- **OBJECTIVE 1.** Review the currently used and soon to be used NPs and nano-enabled devices in pharmaceutical and other biomedical industries
    - Describe the various techniques and tools used to characterize the nanomaterials

- **OBJECTIVE 2.** Assess the *in vitro* and *in vivo* toxicological pathways induced after exposure to NPs and the components of nano-enabled devices

- **OBJECTIVE 3.** Survey the current efforts in the emerging field of predictive toxicology and the regulatory policies that may result from mathematical and statistical based tools in nanotoxicology
    - Examine the gaps in regulation, policies, and standards relevant to the incorporation of NPs in nano-enabled devices

THIS REPORT INCLUDES:

OBJECTIVE 1A. Review of the nanoparticles and nano-enabled devices in pharmaceutical and other biomedical industries

OBJECTIVE 1B. Primer that provides descriptions of the tools and techniques used in research and development of nanoparticles and nano-enabled devices

Objective 1A: Review of the nanomaterials and nano-enabled devices in pharmaceutical and other biomedical industries

The following table includes a comprehensive list of companies who made publicly available their intentions to research, develop, produce, and/or distribute nanomaterials or nano-enabled devices.

The table is sub-divided into the various industries known to be working in nanotechnology. These industries include drug delivery and pharmaceuticals; personal and household care products; other health care products; air and water filtration; fuel additives; membranes; research; materials, engineering, processing, and consumer goods; and fabrics.

Company	Technology
Drug Delivery And Pharmaceuticals	
Advectus Life Sciences Inc.	Polymeric nanoparticles engineered to carry anti-tumor drug across the blood-brain barrier
Alnis Biosciences, Inc.	Biodegradable polymeric nanoparticles for drug delivery
Capsulution NanoScience AG	Layer-by-layer poly-electrolyte coatings, 8–50 nm; coatings to improve solubility of drugs
Eiffel Technologies	Reducing size of the drug particles to 50–100 nm for drug delivery
NanoBio Corp.	Antimicrobal nano-emulsions
NanoCarrier Corp., Ltd.	Micellar nanoparticles for encapsulation of drugs, proteins, DNA
NanoPharm AG	Polybutilcyanoacrylate nanoparticles are coated with drugs and then with surfactant, can go across the blood-brain barrier
NanoMed Pharmaceutical, Inc.	Nanoparticles for drug delivery
Genzyme	Nanotechnology-based tools for delivering oligonucleotide-based therapeutics, development platform that combines Genzyme Pharmaceuticals' LipoBridge ® nanotechnology
Dabur Pharma	Clinical development stage of polymeric nanoparticle delivery systems for cancer treatment
BBI International	Manufacture gold nanoparticles and nanoconjugates for diagnostic purposes
AparnaBio	Developing NanoElectroPlex, a proprietary tissue selective nanoparticle platform technology based on biodegradable macromolecular carriers
Aphios	Developing enabling nanotechnologies for the production of protein nanoparticles, polymer nanospheres and phospholipid nanosomes for the enhanced delivery of drugs
CytImmune	Pegylated colloidal gold tumor-targeting nanotechnology for personalized

Sciences, Inc.	medicines
PSiVida, Ltd.	Exploiting material properties of nanostructured porous silicone for tissue engineering, implants, drugs and gene delivery, bio-filtration
Luna Innovations, Inc.	Nanomedicines based on carbon nanomaterials (fullerenes to counteract inflammation)
Makefield Therapeutics, Inc.	Hydrogel matrix composites for packaging and delivery of a gas (nitric oxide) to target tissues
Nano Science Diagnostics	Diagnostic high efficiency nanometer sized tags serve as probes to tag the target bacteria, virus or protein to be detected
Nanospectra Biosciences, Inc.	Gold nanoshells for selectively destroying tumors
Taiwan Liposome Company (TLC)	Nanoemulsions of polymeric micelle nanoparticles
Traversa Therapeutics, Inc.	RNA delivery technology incorporating cell specific targeting domains and enhanced protein transduction delivery
NanoViricides, Inc.	Nanotechnology-based targeted antiviral therapeutics
Flex Power	Manufacture Flexsomes – nanometer sized liposomes for drug delivery that can penetrate quickly and deeply to provide fast relief
Ablynx	Novel class of antibody-derived therapeutic proteins based on single-domain antibody fragments for a range of serious life-threatening human diseases
Abraxis	Using albumin nanoparticles to deliver drugs into tumors
Access Pharmaceuticals, Inc.	Runs an anticancer program, ProLindac™, that applies the principles of nanoparticulate pro-drugs to enhance the delivery of a platinum-based particle to tumors
Advanced Technologies and Regenerative Medicine, LLC.	Biomaterials and nanotechnology; tissue engineering; local drug delivery systems; bioactives; cell-based technologies
Asklepios BioPharmaceutical, Inc.	Delivering novel protein and cellular based therapies through design of proprietary Biological Nano Particles (BNP)
BIND Biosciences	Using degradable polymers to develop targeted therapeutics (nanoparticles) that deliver high drug concentrations to target cells and tissues with precisely controlled pharmacokinetic and pharmacodynamics properties
Bio Delivery Sciences International	Encapsulating the selected drug in a nanocrystalline structure termed a “cochleate” cylinder
BioCure	Developing amphiphilic self-assembling nanocapsules and planar membranes for drug delivery

Carigent Therapeutics	Attaching high densities of application-specific molecules to the surfaces of biodegradable, polymeric particles ranging in size from tens of nanometers to hundreds of microns
Charlesson	Developing nanoparticle-based gene therapies for eye disease
Cornerstone Pharmaceuticals	Developing nanoemulsion-based drug delivery system that deposits cancer-fighting drugs directly into cancer cells
Ensysce Biosciences	Fullerene carbon nanotubes used in novel therapeutic applications specifically in the area of cancer treatment
GeneSegues	Designing the target nanocapsule technology (e.g. s50 capsules) using a flexible formulation process and can carry large or small molecules, custom target delivery to different organs, tissues and cells
Genetic Immunity	Developing therapeutic immunotherapy by incorporating virus-specific immunogenic DNA into nanoparticles
Nanotherapeutics	Nanoparticles for improving the performance of drug delivery by oral or nasal methods
Tempo Pharmaceuticals	Focused on significantly improving the efficacy and safety profile of existing and new drugs employing advances in nanotechnology
RXi	A discovery-stage biopharmaceutical company pursuing the development and commercialization of proprietary therapeutics based on RNA interference (RNAi) for the treatment of serious human diseases
NanoBioDesign	developing a range of innovative tools based upon its P450 technology
ActiVery	Nanoparticle production method using supercritical fluids (SCF) to create new medicines for pharmaceutical and cosmetic use
Personal and Household Care Products	
BASF	Toothpaste made of hydroxyapatite nanoparticles seems to improve dental surface
EnviroSystems, Inc.	Surface disinfectant in the form of nanoemulsions
Oxonica, Ltd.	Sunscreens doped transparent nanoparticles to effectively absorb harmful UV and convert it into heat
NanoHorizons	Antimicrobial agents made of silver nanoparticles under the trade name Smart Silver
Henkel	R&D in nanotechnology to improve the materials used in consumer products
Philips	Nanosilver coatings on their hair straightener to provide style and shine finish to their product
Samsung	Silver nanoparticle coatings on washing machine and refrigerators interiors to prevent microbial growth
SH Pharma	Toothpaste containing silver nanoparticles was developed to keep strong germ fighting effectiveness of silver longer and it keeps teeth white and healthy

Other Health Care Products	
Biophan Technologies, Inc.	MRI shielding made of nanomagnetic/carbon composite materials to shield medical devices from RF fields Developing nanotechnology drug delivery systems based on novel nanomaterials that provide precise control over location and timing of drug delivery
Nanoplex Technologies, Inc.	Nanomater sized barcodes for bioanalysis and information technology (IT)
Smith & Nephew	Coated bandages made of nanocrystalline silver that is highly toxic to pathogens
Z Medica	These help to clot bood, hence QuikClot Combat Gauze brand, is used in military services as the first-line hemostatic treatment for life-threatening hemorrhage
AcryMed	Manufacturing SilvaGard, a silver nanoparticles antimicrobial surface treatment for medical devices
Biomet 3i	Dental implant combined with a nanometer-scale discrete crystalline deposition of calcium phosphate creates a more complex surface topography
Celsense	Fluorochemical nanoparticles can be developed as MRI contrast agents to monitor the position and quantity of transplanted cells non-invasively in vivo
T2 Biosystems	New technology based on clinically-proven magnetic resonance technology that uses nanoparticles coupled with reagents to quickly detect the presence of specific substances in solution
Nano Bridging Molecules	Advanced medical implant surface modifications designed to render biocompatible
Air and Water Filtration	
Argonide	Nanoporous ceramic materials for endotoxin filtration, orthopaedic and dental implants, DNA and protein separation
KES Science and Technology, Inc.	AiroCide filters made of nano-TiO2 to destroy airborne pathogens
Lehigh Nanotech	Formulation of nanosized iron particles mixed with an additive and a noble metal catalyst in a water suspension for environmental remediation
NanOasis	Membranes consist of a thin, dense polymer film having carbon nanotube pores atop a highly porous support for desalination and water purification
NanoH_2O	Thin-Film Nanocomposite (TFN) membrane technology made of polymer-based membranes with nanostructured material for salt and contaminant rejection for water purification
AI Nano	Application in air purification and as an antimicrobial, antifungal, and antifouling coating
SiREM	Iron nanoparticles to treat groundwater pollutants at variety of Federal and private-sector sites

Fuel Additives	
Energenics	Diesel additive containing cerium oxide nanoparticle catalyst to reduce fuel consumption
Membranes	
Nanoxis	Membrane protein research using nanoscale technology solutions based on soft polymer and fluid-state liquid crystalline materials applicable for production of biochips used in proteomics and genetics and as advanced components for nanofluidics, nanoelectronics and robotics
Research	
Evident Technologies	Luminescent biomarkers made of semiconductor quantum dots with amine or carboxyl groups on the surface, emission from 350 to 2500 nm
Immunicon	Magnetic core surrounded by a polymeric layer coated with antibodies for capturing cells that track and separation of different cell types
Nanoprobes, Inc.	Gold nanoparticle bioconjugates for TEM and/or fluorescent microscopy
Nanosphere, Inc.	DNA barcode attached to nanoprobes for identification purposes Produces gold nanoparticles for applications is diagnostic tests (i.e. ELISA assays)
QuantumDot Corp.	Luminescent biomarkers made of semiconductor quantum dots conjugates to various biomolecules
Agilent	Nanostructured materials used to construct chips and columns for detection of DNA and RNA
Affymetrix	Nanostructured materials used to construct chips for detection of DNA and RNA
RTI International	Non-governmental, non-profit organization involved in nanotechnology research
Sigma Aldrich	Distributes nanometer sized powders (e.g. silver, gold, copper, and palladium)
Carbon Nanotechnologies	Leading world producer of bucky balls and carbon nanotubes (has licensed technology from Rice University)
Nanomaterials Co.	The production of nanomaterials having complex composition and exacting particle size, particle size distribution and tailored surface characteristics
3DM, Inc.	Self-assembling nano-scale scaffolds made of fibers, product name PuraMatrix, for 3D cell culture
Artificial Cell Technologies, Inc.	Using the platform of ultra-thin multilayer nanofilm composed of custom-designed polypeptides to design and produce synthetic capsules or artificial cells that mimic actual living cells
BioForce Nanosciences	Developing the Nano eNabler system, the ultra-miniaturized nanoarray technologies, which is capable of providing nanometer scale, single molecule resolution
BioNanoMatrix	The disposable nanochannel array “chip” is in conjunction with other system components, permits fast and efficient processing, labeling, detection, and analysis of very long segments of DNA and other biomolecules

Particular	Produces nanoparticles using a new innovative method in liquid
Nanophase	Develops, manufactures, and sells an integrated family of nanomaterials (mainly metal oxide)
Nanopartz	Innovator and a quality supplier of gold nanoparticle based products including spherical gold nanoparticles, gold nanorods, micron sized gold, and gold nanowires suited for sensors, solar cells, liquid crystals, non-linear optics, polarizers, negative refractive index materials, standards, catalytics, and as robust subcomponents in electronics
American Elements	Produces nanometer and submicron scale materials specializing in ultrafine and fine particulate matter and metals
SkySpring Nanomaterials	Offers nanoparticles, nanopowders, micron powders, and CNTs (carbon nanotubes) in small quantity for researchers and in bulk order for industry groups
Invitrogen	Produce nanosized beads and spheres for research applications in nucleic acid purification & analysis, nucleic acid amplification & expression, protein expression & isolation, epigenetics & gene regulation, cell & tissue analysis
Acceler8 Technology	Developer of innovative materials and instrumentation for advanced applications in medical diagnostics, basic research, drug discovery, and bio-detection - a new diagnostic platform based on its proprietary surface coatings, assay processing, and detection technologies
Qiagen	A provider of innovative technologies and products for sample preparation and molecular diagnostics solutions that uses Resonance Light Scattering (RLS), a technology based on the optical light scattering properties of nano-sized metal colloid particles
NanoLight Technology (USA)	Developing broad applications of natural, light-emitting marine proteins, or marine bioluminescence
Keystone Nano	An exclusive license to Penn State patented technology that allows the creation of stable, non-toxic, 5 to 50 nm-composite particles that they call Molecular Dots (MDs) that can encapsulate drugs and/or fluorescent molecules
dermaCM	Using lipid-based particle system provides the ability to control the size of the final particle by controlling the number of lipid-based particles used to assemble the finished particle
Materials, Engineering, Processing, and Consumer Goods	
InMat, Inc.	Aqueous suspensions of dispersed nanometer sized silicates in a polymer matrix applied via coating processes to polyester films providing gas barrier
Yonex Co., Ltd.	Fullerenes inserted in the frames of racquets and other sporting goods to improve internal strength and enhance stiffness
Applied Science, Inc.	Development efforts on CNT nanocomposites including enhanced epoxies, nylons, carbon fiber and glass fibers and panels
Bayer	Carbon nanotubes to be used as additives in materials such as plastics, metals

Hewlett Packard	Nanoelectronics to develop defect-tolerant architectures, nanometer scale imprint lithography methods and tools as well as new nanoscale electronic switches for both memory and logic circuits
DuPont, Co.	Research material properties of bucky balls and nanoscale materials science and engineering
Inframat, Corp.	Nanopowders for medical and industrial applications, they manufacture Al2O3,MgO,TiO2,ZrO2,Fe2O3 powders
IBM	Research on manipulating shapes (e.g. preparing them in ring form) and altering mechanical properties
Saint Gobain	Nanosilica coated glass for low e reflecting, commercially available in different colors
Motorola	Uses carbon nanotubes and a breakthrough technique that could create large, flat panel displays with superior quality, longer lifetimes, and lower costs than current offerings
Shrink Nanotech	Manufactures Nano Shrink - an advanced plastic material that is commercially available
Foster	Developing a family of nanocomposite materials designed in increase mechanical performace in medical applications, or manufacturing the nanocomposites according to customers' requirements
Applied Nanotech Holdings, Inc.	Produces nanometer sized ink particles
Antaria	Zinc oxide nanoparticles used in coatings to reduce UV exposure
Nanoledge	Green-based epoxy resins strengthened with nanoparticles
Nanosolar	Solar cells manufactured in a low temperature process using semiconductor nanoparticles
Fabrics	
Nanotex	Fabric enhancers based on nanotechnology
Odegon Technologies	DeOdour Tags is a product containing iron particles on fabric for odor control utilize a nano-porous material that traps odor molecules
BASF	Fabric enhanced with nanoparticles

OBJECTIVE 1B: Primer that provides descriptions of the tools and techniques used in research and development of nanoparticles and nano-enabled devices

THE PHYSICOCHEMICAL PROPERTIES COVERED IN THIS OBJECTIVE

- The Size and Shape
  - Dynamic Light Scattering (DLS)
  - Electron Microscopy (EM)
  - Nanoparticle Tracking Analysis (NTA)
  - Diffraction Patterns

- The Surface
  - Specific Surface Area (SSA)
  - Zeta potential as a measure of surface charge
  - Reactivity via chemical reaction
  - Water solubility
  - Iso-Electric Point (IEP)

- The Chemical Composition
  - Atomic Emission Spectroscopy (AES)
  - Energy Dispersive X-ray Spectroscopy (EDS

INTRODUCTION

Engineered nanoparticles are synthesized with various physicochemical properties in an effort to yield specific structural or functional features. However, to date, it still remains a possibility that the same physicochemical properties which are beneficial to product development, may also elicit a negative biological response, in either *in vitro* or *in vivo* test systems. Therefore, it is necessary to determine what physicochemical characteristics of an individual nanoparticle or a mixture of nanoparticles may be important for potential, unintentional, adverse effects in a biological system.

There has been great effort in the nanotoxicology field to establish a structure-activity relationship specifically for all classes of nanomaterials. This relationship would be generated from gathering, compiling, and normalizing both nanomaterial characterization and nanotoxicological data sets in an effort to predict fate, transformation, distribution, and adverse health effects through the use of *in silico* techniques (Sayes and Ivanov 2010). While the list of the recommended physicochemical properties needed to produce these structure-activity relationships is constantly being updated, the most basic nanoparticle properties of chemical composition, particle size, surface charge, crystallinity (or lack thereof), and surface chemistry/reactivity are generally agreed by the majority of scientists and engineered as the properties that play the most critical role in the development of toxicological conditions.

OBJECTIVE 1B. The Size and Shape. Dynamic Light Scattering (DLS)

Dynamic light scattering is also known as photon correlation spectroscopy or quasi-elastic light scattering. It is a particle sizing technique which can be used to determine the size distribution profile of nanoparticles in suspension or polymers in solution (Berne, B.J. and Pecora, R. Dynamic Light Scattering; Wiley: New York, 1976). Light scatters in all directions, referred to as Rayleigh scattering, when it hits nanoparticles. The light source is usually a monochromatic and coherent laser. The detector measured fluctuations in the scattered laser light

In actuality, DLS measures Brownian motion of the nanoparticles. Brownian motion is the random movement of suspended particles due to bombardment of the particulate by solvent molecules. As solvent molecules collide with the nanoparticles, they exert a force which is capable of moving the particles in a random direction. This random movement will be much more exaggerated in a small particle that it will in a large particle of the same composition over the same time period. This movement, otherwise known as the translational diffusion coefficient (D), can then be related to the particle's hydrodynamic radius via the Einstein-Stokes Equation:

$$d(H)=kT/3\pi nD$$

Combining D and Boltzmann's constant, k, with the physical parameters of the system including a stable temperature T, and the viscosity of the solvent n, one might deduce the hydrodynamic radius of the nanoparticle of interest.

DLS is a common instrument in the nanotoxicology laboratory. It provides the researcher with a quick, cost effective means of generating a size profile of nanomaterials in suspension. DLS output, on a machine such as the Zetasizer NanoZS, displays intensity on the dependent axis and size on the independent. In addition, DLS may also be used to measure biological samples such as proteins when in a biological buffer

While DLS is widely used among laboratories, one must constantly be aware of its limitations. For example, in a polydisperse sample, the larger particles will scatter more light than smaller particles. More specifically, the intensity of scattered light is proportional to the diameter of the particle to the 6th power. Therefore a 20 nm particle would scatter 64X as much light as a 10 nm particle (64,000,000 U vs. 1,000,000 U). This property is called Rayleigh Scattering. It is difficult to measure single particles using light scattering when aggregates or agglomerates are present in the focal suspension; thus, a major limitation of the instrument. Therefore, a polydisperse sample mixture may read as if it were void of the smaller fraction of nanoparticles, or simply be unsuitable for analysis via DLS. Secondly, many nanoparticles tend to agglomerate once in solution. This agglomeration state may yield particulates in the micrometer (10-6) range. This in turn would make it hard to distinguish a particle size on the order of 3 orders of magnitude lower. Thirdly, DLS measurements should be taken in pristine solutions. For example, cell culture medium with the addition of fetal bovine serum contains many proteins which interact with the nanoparticle surface. While this alteration in the surface chemistry by the addition of FBS is known to play an important role in toxicology, this protein

adsorption would make the nanoparticle size larger due to the increased hydrodynamic radius. In situations where biomolecules are utilized, it is important that control samples be run without the nanoparticle to determine the sizing profile of components in the suspension medium.

OBJECTIVE 1B. The Size and Shape. Electron Microscopy (EM)

The use of transmission electron microscopy (TEM) has become a necessary characterization tool in the fields of nanotechnology and nanotoxicology. TEM takes advantage of the extremely small wavelength of electrons (λ=0.2 nm) to increase the resolution and magnification of nano-scale particulates and cellular organelles. In transmission electron microscopy, the specimen is exposed to an electron beam and subsequently the transmitted electrons are viewed on a phosphorescent screen, a CCD camera, or film. Depending on nanomaterial characteristics, the TEM can provide information such as size, shape, agglomeration state, and crystalline structure of nanoparticles. In addition TEM may be used post-exposure both in vitro and in vivo to determine the intracellular localization of nanoparticles as well as any cellular structural changes.

- Some critical EM requirements are:
  - Nanoparticles must be supported to be analyzed (carbon film or copper grid)
  - Particles < 10 nm analysis require field-emission EM
  - Increased magnification requires high specimen stability

- The most common sample preparation procedure is:
  - Using a micropipette, put a few drops of sample on light side of carbon grid.
  - Wick away excess with filter paper.
  - Let sample dry for several hours.
  - View sample in TEM and take photos for later analysis.

While TEM provides a plethora of information surrounding nanomaterial characterization, there are some limitations to this technique. Due to the high energy of the electron beam (i.e. 60-300 keV), biological specimens are rapidly damaged upon exposure. Therefore, proper biological specimen preparation determines the quality of the specimen. Proper preparation is a multistep process consisting of fixation, dehydration, embedding, and sectioning. Further explanation on this process can be found in Electron Microscopy by Bazzola and Russel as well as Biological Specimen Preparation by Glauert and Lewis. Subsequently, post-staining with a combination of uranyl acetate and lead citrate will enhance contrast and allow better visualization of subcellular structure [1].

As previously mentioned, specimen preparation plays a major role in the quality of data retained from an electron microscope. Scientists must take exceptional precautionary measures when drawing conclusions based solely on TEM images. For example, post-fixation with osmium tetroxide (OsO4) may introduce small (1-2 nm) nanoparticles into the specimen. In addition, if samples are post stained, one must be assured that visualized nanoparticles are not artifacts from the protocol itself. Careful analytical techniques, such as those described in the next section, when combined with image analysis are the undefined standards in nanotechnology.

While TEM may itself be useful as a characterization tool, combinations of TEM with alternative methodologies such as energy dispersive spectroscopy (EDS), electron diffraction (ED), and High Resolution Transmission Electron Microscopy may be useful for determining additional characterization parameters such as chemical composition and speciation. Electron diffraction is a tool used by materials scientists to determine the crystal structure of a solid particle. When excited electrons pass through the nanoparticles the electrons produce a signature diffraction pattern. Once this pattern has been attained, it may be compared with that of a standard material. This technique may come into play when a particle with a single composition (i.e. TiO2) has multiple crystalline states which induce differential amounts of toxicity [2]. Similarly, high resolution transmission electron microscopy (HRTEM) may also be used to study the crystallographic structure at the atomic scale.

OBJECTIVE 1B. The Size and Shape. Nanoparticle Tracking Analysis

The technique of Nanoparticle Tracking Analysis (NTA) counts, sizes and visualizes nanoparticles in liquid suspension. Similar to Dynamic Light Scattering (DLS) relates the rate of Brownian motion to particle size. The rate of movement is related only to the viscosity and temperature of the liquid, it is not influenced by particle density or refractive index. The light scattered by the particles is captured using a camera and the motion of each particle is tracked from frame to frame. This rate of particle movement is related to a sphere equivalent hydrodynamic radius as calculated through the Stokes–Einstein equation.

As with many analytical chemistry techniques, there are some limitations associated with NTA. For instance, the particles of interest have to be between 10 and 2,000 nm in primary particle size. However, there is an exception to this rule: if particles are less than 10 nm, it is possible to measure their size if the particle has a high refractive index, such as gold and silver. Particles greater than 2,000 nm move slowly and, therefore, the accuracy of sizing via NTA diminishes. Lastly, the viscosity of the solvent also influences the movement of particles, therefore, careful consideration must be taken into account regarding the suspension matrix of the particle system.

NanoSight, Ltd. currently produces products using nanoparticle tracking analysis and ultramicroscopy. Instruments are comprised of a camera, microscope, and sample viewing unit in conjunction with computer control. Their model LM20 is proven with most nanoparticle classes dispersed in a wide range of solvents, including ceramic & metallic particles, pigments & sunscreens, pharmaceuticals, liposomes, viruses, carbon nanotubes, colloids & polymer particles, cosmetics & foods, and fuels & oils.

OBJECTIVE 1B. The Size and Shape. Diffraction Patterns

Nanoparticles may be synthesized as either crystalline or amorphous in form. Moreover, different crystalline forms exist for nanoparticles of the same composition. For example, while the most common forms of titanium dioxide (i.e. anatase, rutile, and brookite) are the most studied in the nanotoxicology literature, other forms of TiO_2 do exist. Many toxicological comparisons among anatase TiO_2 and rutile TiO_2 have demonstrated that there are stark differences in cytotoxicity,

inflammation, and oxidant production among crystalline phases. For example, in acellular assays utilizing oxidant reactive dyes, anatase has been shown to generate greater oxidant production that anatase/rutile mixtures or rutile alone (Jiang et al. 2008a; Sayes et al. 2006). Sayes et al. (2006) then concluded that the anatase crystal phase was 100 times more toxic than TiO_2 in the rutile crystal phase (Sayes et al. 2006). On the other hand, Bradydich-Stolle and others (2009) suggest that crystalline state is only one mediator of TiO_2 toxicity identifying different cellular responses including necrosis and apoptosis for the anatase and rutile crystal phases respectively (Braydich-Stolle et al. 2009). In the animal model, mixtures of 80% anatase: 20% rutile have been reported as more inflammogenic than exposure to rutile alone (Warheit et al. 2007a; Warheit et al. 2007b).

Similarly, amorphous forms of traditionally inert material may become biologically active at the nanometer size scale. For example, both amorphous TiO_2 and amorphous SiO_2 are able to generate both oxidant production and subsequent oxidative stress in both acellular and cellular environments at the nanometer size scale (Jiang et al. 2008a; Lin et al. 2006; Chang et al. 2007). This increased activity in the amorphous form may be due to the increased surface defect density at the surface which increases the ability to interact with the external environment (Carp et al. 2004). Furthermore, an increase in the surface density of functional groups may contribute to the development of oxidants. Thus, it is considered that changes in crystalline form may alter the surface chemistry of the particle thereby leading to altered toxicological effects.

OBJECTIVE 1B. The Surface. Specific Surface Area (SSA)

Nanoparticle size is reported in a variety of ways including primary particle size, aerodynamic diameter, hydrodynamic diameter, and agglomerate size (Hwang et al. 2008; Wuelfing et al. 1999; Zhang et al. 2005; Pauluhn 2009). Although each of these measurements assumes that nanoparticles are spherical in diameter, each may lend itself to a different reported diameter. Primary particle size is often reported through the use of electron microscopy or calculated through specific surface area analysis using the Brunauer, Emmett and Teller (BET) method (Brunauer et al. 1938).

Specific surface area (SSA) is performed on a dry powder. The most common method is the BET method (S. Brunauer, P. H. Emmett and E. Teller, *J. Am. Chem. Soc.*, 1938, 60, 309.). BET theory aims to explain the physical adsorption of gas molecules on a solid surface and serves as the basis for an important analysis technique for the measurement of the specific surface area of a material. The result is often reported as the aerodynamic diameter.

The aerodynamic diameter is often utilized to measure the size of an aerosolized nanoparticle powder. This aerodynamic size is important in inhalational studies as particles size in this form defines their impaction and travel within the lower levels of the respiratory tract. If individual nanoparticles were to reach the lower level of the respiratory tract (i.e. not ciliated), particle clearance may be hindered as nanoparticles of various composition have been reported to decrease alveolar clearance mechanisms (Renwick et al. 2001).

In contrast, the hydrodynamic diameter, measured via dynamic light scattering in a suspension (described above), gives a measure of the nanoparticle size at the nanoparticle slipping plane. The nanoparticle slipping plane is comprised of the nanoparticle core with the surrounding layer of water and adsorbed ions (Murdock et al. 2008). Both the aerodynamic and hydrodynamic measurements are subject to alteration through a process termed nanoparticle agglomeration. Agglomerates of nanoparticles occur when individual primary particles are held together by weak intermolecular forces including salvation forces, van der Waals forces, electrostatic attractions, and hydrophobic interactions (Hakim et al. 2007; Min et al. 2008; Fichthorn and Qin 2008).

OBJECTIVE 1B. The Surface. Zeta Potential as a Measure of Surface Charge

The zeta potential of a nanoparticle represents the charge on the surface in addition to the adsorbed layer of counter ions. The adsorbed layer, hereafter known as the electric double layer, is generated from oriented solute molecules and ions surrounding the nanoparticle surface. The electric double layer is sensitive to various changes in pH and ionic strength. All nanoparticles in suspension carry a net charge. This charge, be it negative, positive, or neutral, is dependent upon the micro environmental conditions of the suspension media. For example, all nanoparticles have an isoelectric point (IEP). This isoelectric point occurs when a particles zeta potential has zero net charge (IEP: Z=0 mV). When this occurs in the Nanosizer ZS system, the particle experiences no movement in response to alternating polarity in the test cuvette. Recent work in our lab has shown that the IEP may play a role in the toxicity of nanomaterials [3]. One hypothesis that has arisen is the fact that the charge at physiological pH may direct certain protein modification of the nanoparticle surface. This modification may then mediate cellular response.

Zeta potential is one of the most common tools to characterize nanomaterials. Surface charge is most commonly measured by Zeta potential. Zeta potential is the boundary of the diffuse layer of ions within which the particle acts as a single entity.

What properties influence this measurement?

- Surface functionalization
- Surfactant Ions
- pH of suspension medium
- Ionic strength of suspension medium

The zeta potential is also used as a measurement of colloidal stability. Zeta Potential value above ±30 mV is considered a stable system. Zeta potential values with an absolute value less than 30 mV are considered an unstable system and are prone to agglomeration. This agglomeration is due to the lack of charge-charge repulsion between individual nanoparticles.

OBJECTIVE 1B. The Surface. Reactivity via Chemical Reaction

While many components are expected to contribute to nanoparticle toxicity, decreasing nanoparticle diameter leads to exponential increases in particle surface molecules (Oberdorster et al. 2005). These exponential increases in the surface area make the nanoparticle unique from that of the bulk material. In fact, it has been suggested that the particle surface characteristics may be more important than particle size when determining nanoparticle cytotoxicity (Warheit et al. 2007a; Warheit et al. 2007b). For example, increases in surface area provide greater ability for contaminants to bind albeit organics, transition metals or even biomolecules (Donaldson et al. 2001). For example, nanoparticles in biological medium (i.e. serum) bind biological macromolecules on the surface which may further dictate their uptake or distribution *in vitro* or *in vivo* (Lundqvist et al. 2008). While unintentional macromolecule surface modification may lead to unwanted distribution, intentionally coating nanoparticles with various polymers including polyethylene glycol (PEG) for medicinal techniques may increase their residence time and help avoid macrophage clearance (McNeil 2005).

Surface chemistry can be determined using a variety of techniques including X-ray photoelectron spectroscopy (XPS) or Fourier transform infrared spectroscopy (FTIR). XPS, otherwise as electron spectroscopy for chemical analysis (ESCA), utilizes a focused X-ray beam to emit electrons from the surface (~15 nm) of the materials which may then be analyzed for elemental composition (Murdock et al. 2008). Furthermore, XPS may be utilized to detect surface labeling of nanoparticles using fluorescent dyes (Hens et al. 2008). In addition to XPS measurements, FTIR may be used to verify the presence of surface functional groups.

While XPS and FTIR are analytical tools that provide elemental and functional composition at the nanoparticle surface, the Vitamin C assay may be utilized to determine surface reactivity. The Vitamin C yellowing test has been previously developed as a means to correlate the surface reactivity of the nanoparticle to its chemical stability. Available evidence indicates that the color change mechanism is the formation of a charge transfer complex between ascorbic acid-6-palmitate and the "active sites" on the nanoparticle surface (Warheit et al. 2007b; Rajh et al. 1999).

OBJECTIVE 1B. The Surface. Water Solubility

Knowing the solubility of a particle in the aqueous phase is an important physicochemical properties in nanotoxicology. Nanoparticles are either hydrophobic (lipophilic) or hydrophilic (lipophobic). Most metal colloids, metal oxide, and carbonaceous nanomaterials are hydrophobic in their pristine, newly synthesized state (i.e. not surface modified). In an effort to suspend nanoparticles in the aqueous phase, the surface of these particles must be functionalized or modified. Nanoparticles can be surfaced-modified by:

- Surrounding core in surfactant molecules to form a micelle
- Bonding polyethylene glycol (or other polymer) onto the core's surface (aka PEGylation)
- Altering the zeta potential to either highly negative or highly positive surface charge
- Changing the pH of the suspension media

OBJECTIVE 1B. The Surface. Iso-Electric Point (IEP)

Nanoparticle surface charge, often measured as zeta potential, is a dynamic characteristic often dependent upon the microenvironmental conditions in which the particle is suspended. The zeta potential of the nanoparticle represents the charge of the nanoparticle at the slipping plane of the electric double layer. This double layer is influenced by the surrounding ions in solution (Malvern 2008). Nanoparticle zeta potential is measured using dynamic light scattering combined with phase analysis light scattering (Berne and Pecora 1976). Because the zeta potential measurement is influenced by the surrounding ions in solution, the characteristics of the suspension medium such as pH, ionic strength, and/or presence of surfactants may all influence the zeta potential (Tiyaboonchai and Limpeanchob 2007; Mayer et al. 2009; Berg et al. 2009; Handy et al. 2008). Alterations in the zeta potential will, in effect, lead to modification of other physicochemical characteristics such as changes in the agglomeration state (Berg et al. 2009). The largest nanoparticle agglomeration state is theoretically present at the nanoparticles isoelectric point. The isoelectric point is defined as the pH at which the net zeta potential is equal to 0 mV. In fact, zeta potential values greater than +30 mV or less than -30 mV allows increased nanoparticle stabilization following deaggregation using sonication techniques (Murdock et al. 2008). However, sonication alone does not alter the nanoparticle zeta potential.

Alterations in the zeta potential of a nanoparticle will lead to different biological effects providing that the remaining physicochemical properties remain the same. It has been suggested that surface protein adsorption is significant to the toxicological characteristics of the particle (Lundqvist et al. 2008). This protein adsorption is often mediated by the surface parameters of the individual particles including surface charge. Beyond protein modification, surface charge has been shown to play a role in internalization and distribution of nanoparticles. For example, nanoparticle zeta potential plays an important role in delivery across the blood brain barrier (Lockman et al. 2004; Fenart et al. 1999). Similarly, alteration in the zeta potential may mediate the route of cellular internalization (Dausend et al. 2008; Harush-Frenkel et al. 2008). For example, when using an ameloride inhibitor of macropinocytosis, Dausend et al. (2008) noted that only positively charged particles were blocked from cellular entrance while negatively charged particles were unaffected (Dausend et al. 2008). As nanoparticle zeta potential represents an area that is currently under investigation, it is suggested that, when possible, nanotoxicology studies incorporate this measurement into the battery of characterization techniques.

While these brief proceedings on nanoparticle physicochemical characteristics is not by any means conclusive, it is suggested that they are provided as a base set of physicochemical parameters accompanying current nanotoxicological data. The hope is that future work may utilize these characteristics as a base set of properties from which to develop the basis of mathematical quantitative structure activity relationship (QSAR) studies relating these physicochemical properties to biological response (Sayes and Ivanov 2010). However, while use of physicochemical properties and *in vitro* cytotoxicity tests are useful, it is evident that *in vitro* tests will need to be further developed prior to the replacement of *in vivo* testing.

OBJECTIVE 1B. The Chemical Composition. Atomic Emission Spectroscopy (AES)

Atomic emission spectroscopy (AES) is one of the few quantitative methods in particle physicochemical characterization. It is primarily used for metal-based nanoparticles, such as colloids, oxides, and bimetallic semiconductors. The most common atomization method is inductively coupled plasma (ICP). AES can gives information on total ion concentration, presence and concentrations of impurities, the oxidation state of the metal, and presence of the nanoparticle inside of a cell (via analyses exposed cell lysate.

There are many advantages of the technique:

- Determination of 68 metals
- Ability to make ppb determinations on major components of a sample
- Precision of measurements by flame are better
- Analysis is subject to little interference
- Most interference that occurs have been well studied and documented
- Sample preparation is simple (often involving only dissolution in an acid)
- Instrument easy to tune and operate

New research efforts in analyzing individual nanoparticles using AES, as opposed to dissolving particles completely into ions, is currently underway.

OBJECTIVE 1B. The Chemical Composition. Energy Dispersive X-ray Spectroscopy (EDS)

Energy dispersive spectroscopy as a micro-analytical tool can yield both quantitative and qualitative results when utilized by the trained personnel. When emitted electrons strike the atoms of interest in the sample, inelastic reactions occur which generate emitted electrons and X-rays subsequently detected via spectroscopy. With the advent of powerful computers, these detected signals may then be processed for exact elemental composition. When preparing a specimen for EDS analysis, one must be careful that no additional elements have been added to the specimen that may interfere with the spectral analysis of the element of interest.

An alternative method of electron microscopy has recently been used to identify nanoparticle composition in a heterogeneous exposure. Scanning Transmission Electron microscopy (STEM) (field emission gun) utilizes a very small electron beam to scan the specimen of interest. STEM emission may be combined with subsequent high angle annular dark field (HAADF) analysis to provide a reverse contrast image with brightness dependent of atomic number squared. The use of STEM-HAADF as a characterization tool provides the toxicologist with a novel method to determine the composition of individual nanoparticles in a complex matrix.

Summary Table of Tools and Techniques Used in Nanomaterial Characterization

Property	Technique	Description and Reference	Theory and Practice
The Size and Shape	Dynamic Light Scattering (DLS) (also known as photon correlation spectroscopy or quasi-elastic light scattering)	DLS is a particle sizing technique which can be used to determine the size distribution profile of nanoparticles in suspension or polymers in solution. *(Berne, B.J. and Pecora, R. (1976). Dynamic Light Scattering; Wiley: New York.)*	Light scatters in all directions, referred to as *Rayleigh scattering*, when it hits nanoparticles. The detector measured fluctuations in the scattered laser light; fluctuations are due to *Brownian motion.*
	Electron Microscopy (EM)	As with any other microscopy method, electron micrographs are a two dimensional representation of a three dimensional object. *(Powers, K.W. (2006) et al. Toxicological Sciences 90(2):296.)*	Nanoparticles must be supported to be analyzed (carbon film or copper grid); particles < 10 nm analysis require field-emission EM; increased magnification requires high specimen stability
	Nanoparticle Tracking Analysis (NTA)	NTA counts, sizes and visualizes nanoparticles in liquid suspension. It relates the rate of Brownian motion to particle size. The rate of movement is related only to the viscosity and temperature of the liquid, it is not influenced by particle density or refractive index. *(Filipe, V. (2010) et al. Pharmaceutical Research 27(5):796.)*	Particles have to be between 10 and 2,000 nm; particles < 10 nm is only possible for particles with a high refractive index, such as gold and silver; particles > 2,000 nm move slowly and accuracy of sizing diminishes; and the viscosity of the solvent influences the movement of particles
	Diffraction Patterns	Diffraction from a three dimensional periodic structure such as atoms in a crystal is called Bragg diffraction. *(Sun, S. et al. (2004). JACS 126(1):273.)*	There are 2 common methods to acquire a diffraction pattern: Electron diffraction and X-ray diffraction. Bragg diffraction is a consequence of interference between waves reflecting from different crystal planes.
The Surface	Specific Surface Area (SSA)	SSA is performed on a dry powder. Most common method is the BET method. *(S. Brunauer, P. H. Emmett and E. Teller, (1938). JACS 60:309.)*	BET theory aims to explain the physical adsorption of gas molecules on a solid surface and serves as the basis for an important analysis technique for the measurement of the specific surface area of a material.
	Zeta Potential as a Measure of Surface Charge	One of the most common tools to characterize the nanomaterials is taking a Zeta potential measurement. Surface charge is most commonly interpreted via Zeta potential. *(Berg, J.M. (2009). Nanotoxicology 3(4): 276.)*	Zeta potential is the boundary of the diffuse layer of ions within which the particle acts as a single entity.
	Reactivity via Chemical Reaction	Stimuli responsive materials can be manipulated to enable reversible change in their	Most chemical reactions involving nanoparticles occur on the surface of the particle.

		physicochemical characteristics in response to changes in their environmental surroundings. *(Kapoor S. (1998). Langmuir 14(5):1021.)*	Redox chemistry on the nanoparticle surface is the most common chemical reaction.
	Water Solubility	Nanoparticles are either hydrophobic (lipophilic) or hydrophilic (lipophobic). Most metal colloids, metal oxide, and carbonaceous nanomaterials are hydrophobic in their pristine state, because they are not surface modified. *(Franklin N.M. (2007). ES&T 41(24):848.)*	These nanoparticles can be surfaced-modified by: surrounding core in surfactant molecules to form a micelle, bonding polyethylene glycol (or other polymer) onto the core's surface (aka PEGylation), altering the zeta potential to either highly negative or highly positive surface charge, or changing the pH of the suspension media.
The Chemical Composition	Atomic Emission Spectroscopy (AES)	It is one of the few quantitative methods in particle physicochemical characterization. Most common atomization method is inductively coupled plasma (ICP). *(Murray C.B. (2000). Materials Research 30:545.)*	AES can gives information on: total ion concentration, impurities, REDOX state, and presence of nanoparticle inside of a cell. Primarily used for metal-based nanoparticles, such as: colloids, oxides, or bimetallic semiconductors.
	Energy Dispersive X-ray Spectroscopy (EDS)	Energy dispersive spectroscopy as a micro-analytical tool can yield both quantitative and qualitative results. These results are typically spectra with signature peaks identifying atomic species preset in the sample. *(Wang, Z.L. (2001). VCH Verlag GmbH, Weinheim, FRG.)*	When emitted electrons strike the atoms of interest in the sample, inelastic reactions occur which generate emitted electrons and X-rays subsequently detected via spectroscopy. These detected signals may then be processed for exact elemental composition When preparing a specimen for EDS analysis, one must be careful that no additional elements have been added to the specimen that may interfere with the spectral analysis of the element of interest.
The Suspension Matrix	pH and Iso-Electric Point (IEP)	The zeta potential plane is measured as the primary indicator of surface charge Surface charge is altered when the pH is increased or decreased. The downstream effect of altered zeta potential is a change in agglomeration state, which influences the cytotoxicity. *(Berg, J.M. (2009). Nanotoxicology 3(4): 276.)*	In a model nanoparticle system, the largest aggregate size would be observed at its isoelectric point (zeta potential=0 mV). The farther the zeta potential deviates from 0 mV, the smaller the particle agglomerate due to increasing repulsive forces.

Author's Short Biography

Dr. Christie M. Sayes is the Program Manager for Nanotoxicology & Nanopharmacology in the Center for Aerosols and Nanomaterials Engineering at RTI International. She was formerly a professor of toxicology at Texas A&M University. Dr. Sayes maintains her adjunct faculty appointment at Texas A&M in the Department of Biomedical Engineering and the Interdisciplinary Program in Toxicology. She has more than a decade of experience in the fields of nanotechnology and nanotoxicology. She has authored numerous publications, including original research, invited reviews and book chapters. She is a member of the Society of Toxicology, the American Chemical Society, and the Society of Environmental Toxicology and Chemistry. She serves on the Scientific Advisory Board for the EPA's FIFRA Program and on the Editorial Board of the journals *Nanotoxicology* and *Toxicology Letters*. Recently, she was elected onto the Executive Committee of the North Carolina Chapter Society of Toxicology. Dr. Sayes has proven abilities in providing technical guidance and leadership to students, technicians, and colleagues; a high aptitude for development of complex particle toxicological and biocompatibility basic and applied research projects in cell culture based and animal based models; substantial training in nanomaterial & nanotoxicology research techniques & instruments; significant experience working independently & collaborating across disciplines and organizations; excellent communication and interpersonal skills with colleagues in science and engineering, senior management, and new and existing clients and other funding sources.