CHAPTER

# 43

# Nanoparticulates

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OUTLINE



## 1. BACKGROUND

### 1.1. Definitions

The National Nanotechnology Initiative (NNI) coordinates federal nanotechnology activities. It defines nanotechnology as "the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling and manipulating matter at this length scale." This definition of nanotechnology introduces the terminology and the important concept that nanoscale products can accomplish many things not previously possible.

Before addressing the potential benefits of nanotechnology, additional definitions may be helpful. The prefix *nano* commonly confers a meaning of very small or a billionth (*Random House Unabridged Dictionary*, Second Edition, 1993). However, within nanotechnology and

*Haschek and Rousseaux's Handbook of Toxicologic Pathology, Third Edition.*
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related disciplines, the prefix *nano* is often used to refer to dimensions from 1 to 100–nm. Thus, nanomedicine is the medical application of nanotechnology, and nanotoxicology is the study of the toxicology of the products of nanotechnology.

The terminology for products of nanotechnology is still evolving, and the definitions may affect how specific products are regulated. Thus, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) recently published a 46-page opinion paper on the definition of the term "nanomaterial." Because of the controversy, we will avoid using the term nanomaterial. In this chapter, the term nanoparticulate (NP) will be used for a particulate with at least ***one*** dimension in the nanoscale range from 1 to 100 nm. The term *nanoparticle* is often defined synonymously, but SCENIHR has proposed that a nanoparticle should be defined as a "discrete entity which has three dimensions of the order of 100 nm or less." As used here, NPs will encompass both solid and liquid NPs.

## 1.2. Historical Perspective

NPs have been present in the human environment for centuries. The National Nanotechnology Initiative website (http://www.nano.gov) maintains a Nanotechnology Timeline (http://www.nano.gov/timeline) that begins with the use of colloidal gold and silver in making dichroic glass in the 4th century. It is the ability to engineer particles in nanoscale dimensions that is a recent phenomenon, and it is this ability that led to the new field known as nanotechnology. This is reflected in the rapid increase in PubMed indexed nanotechnology publications during the past decade. Nanotoxicology and nanomedicine have received increasing attention during the past 5 years (Figure 43.1).

Several earlier key discoveries made nanotechnology possible. Among these discoveries were improved microscopes. In 1986, the Nobel Prize in Physics was shared between Ernst Ruska, and Gerd Binnig and Heinrich Rohrer. Nobel Laureate Ruska was eventually recognized for his role in the design of the electron microscope in the 1930s, which revealed ultrastructural details previously unseen by scientists. Nobel Laureates Gerd Binnig and Heinrich Rohrer were recognized for the design of a particular type of electron microscope, the scanning tunneling microscope, which produced atomic scale maps of the surfaces of biological and inorganic samples and their atomic composition.

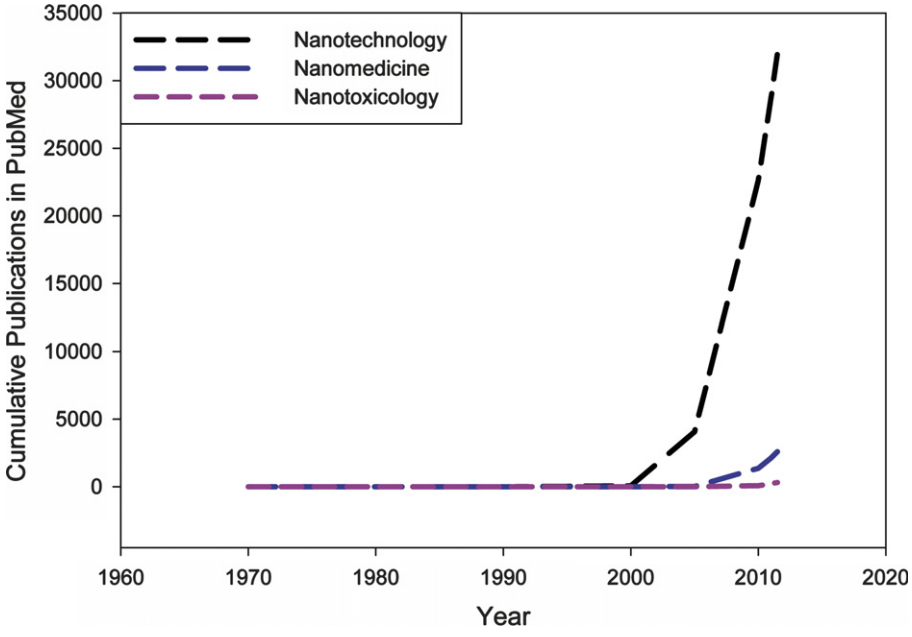


FIGURE 43.1 Publications in nanotechnology (dashed black line) began about a decade prior to publications in nanomedicine (dashed blue line). Nanotoxicology publications (dashed purple line) have lagged behind in both number and time.

In this same time frame, other research groups were working on a new form of carbon that would soon revolutionize nanoscale three-dimensional engineering. In 1981, a group of researchers from Rice University discovered buckminsterfullerene, now also known as the buckyball. As indicated by its chemical formula, $C_{60}$, buckminsterfullerene was comprised solely of carbon. It was shaped like a soccer ball with a sheet of carbon atoms analogous to the leather surface of the soccer ball, so that a cage-like internal structure was formed which was suitable for enclosing specific atoms. The carbon–carbon bonds were the seams of this molecular soccerball, and, like a soccerball, regular hexagons and pentagons contributed to its 3D symmetry. As with other carbon compounds, each carbon molecule in $C_{60}$ formed four bonds with adjacent carbon molecules. This meant that some of the carbon molecules were linked by double bonds. Double bonds were near single bonds and other double bonds so that sites of double bonds between carbons were destabilized; in other word, these were aromatic compounds. Thus, the hexagons and pentagons that comprised $C_{60}$ were actually aromatic; a shell of delocalized $\pi$ electrons surrounded the internal and external surfaces of $C_{60}$ and added stability to the structure (Figure 43.2).

$C_{60}$ was the first characterized member of an amazing new class of carbon compounds devoid of hydrogen and known as the fullerenes. This class of carbon compounds was as different from previously described carbon compounds as the carbon compound coal is from the carbon compound diamond. The fullerenes formed a curved membrane which was one atom thick. That one-atom thick carbon membrane could form 3D structures of varying shapes. Robert F. Curl, Sir Harold Kroto, and Richard E. Smalley received the 1996 Nobel Prize for this discovery.

The group of new carbon compounds soon expanded to include the carbon nanotubes, which formed rolled sheets of carbon hexagons in the shape of single- or multi-walled tubes with a diameter in the nanoscale and a much greater length (Figure 43.2). Like $C_{60,}$ carbon nanotubes have electrons that were used for double bonds and could be aromatic. The chemistry needed to produce the tube-like shape of the carbon nanotubes also used transition metal catalysts, such as iron, which were a variable component of the carbon sheet which comprised the nanotube. Some considered nanotubes to be fullerenes while others considered nanotubes to be a separate group of carbon-based NP. As the understanding of $C_{60}$, carbon nanotubes, and related carbon NPs evolved, so developed the realization that the curved structure and the presence of five- and four-membered carbon rings made some carbon-based NPs more reactive than most aromatic compounds; chemically, many carbon-based NPs behaved more like alkenes than aromatic compounds.

As a group, the carbon-based NPs demonstrated nanoscale dimensions, chemical and physical properties that included durability and an ability to conduct electricity, a diversity of potential shapes which could modify the chemical and physical properties, and many potential commercial applications. However, for toxicologists and toxicologic pathologists, the emergence of a new class of reactive polycyclic carbon compounds devoid of hydrogen, often containing transition metals, and with a 3D shape that could mimic biological molecules, was not yet a major topic of discussion.

## 1.3. Development of Nanotechnology

The earliest nanotechnology products are known as the first-generation products of nanotechnology, and were generally passive structures. The first-generation NPs included $C_{60}$ and the carbon nanotubes, but also included other products that demonstrated the principle of engineering in nanoscale dimensions. These included nanotubes formed from cyclodextrins, nanotubes formed from cyclic polypeptides, DNA stick figures, and DNA arranged in a cube. Additional close relatives of the first-generation products of nanotechnology came later, but included commercially important products such as modified carbon nanotubes (including carbon nanoribbons and functionalized carbon nanotubes) and nanotubes made of additional elements (such as silicon).

By the early 1990s, a vision of nanotechnology emerged within the scientific community. In November of 1991, the journal *Science* included a special section called "Engineering a Small World: From Atomic Manipulation to Microfabrication." Material science, quantum chemistry, and physics had evolved to the point where

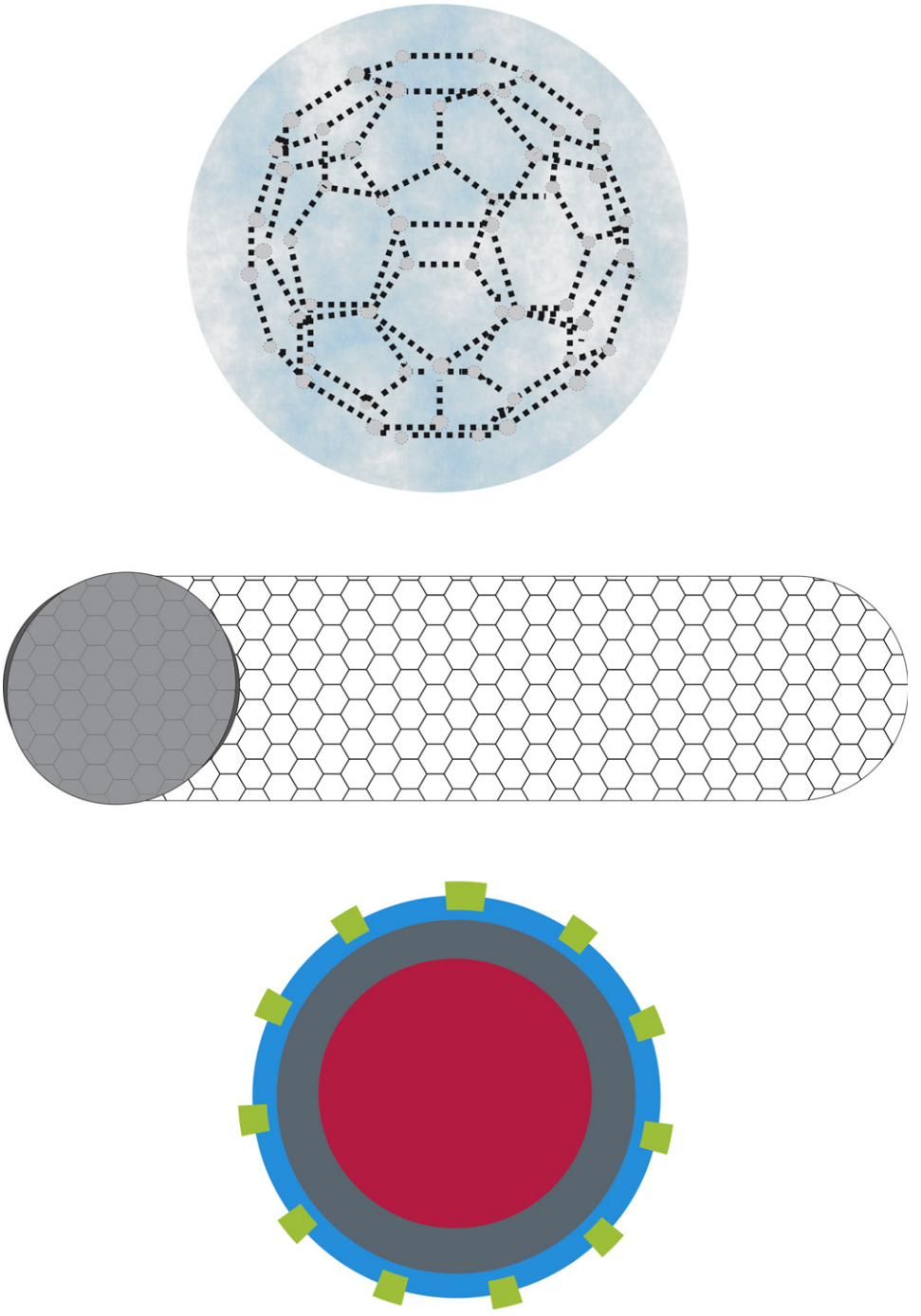

FIGURE 43.2 Nanoparticulates are a virtually infinite group of particulates sharing the common feature of size – at least one dimension in the range of 1–100 nm. The top image is a drawing of one of the early products of nanotechnology, a buckyball, which is comprised solely of carbon arranged into pentagons and hexagons to form a sphere that is surrounded by a cloud of π electrons to illustrate the aromatic nature of the buckyball. The middle image is an image of another early product of nanotechnology, a single-walled carbon nanotube, which is principally comprised of carbon molecules arranged into hexagons but also contains variable amounts of metals used as catalysts during synthesis. Single-walled carbon nanotubes tend to behave as polyalkenes, which allows chemical additions to the carbon wall. The bottom image is a hypothetical NP intended to illustrate the concept that modern products of nanotechnology can be complex structures made from many different compounds such as a drug core (shown in red) surrounded by a protective coating (shown in gray) a tracer for imaging (shown in blue) and a ligand for cellular receptors (shown in green) to target the delivery.

control of atomic arrangements and variety of molecular devices were envisioned. The similarity in size between synthetic nanostructures and biological structures, such as viruses, proteins, nucleic acids, and cellular organelles, was recognized. In addition, the important non-covalent bonds of proteins and nucleic acids were recognized, first for their role in determining the 3D structure and self-assembly of these important natural biological nanoparticulates, and second for their potential role in synthesizing manufactured nanoparticulates. As noted previously, carbon, DNA, and peptides were among the earliest building blocks for nanotechnology products. The biological precedent indeed demonstrated the feasibility of these non-covalent interactions, but also suggested the potential for manufactured nanoparticulates to interact with biological structures.

These early visions of nanotechnology primarily were directed at developing nanodevices. Electronic devices were among the earliest nanodevices. Movement of electrical charge is the basic feature of electrical conductors, and movement of electrons is one way to move electrical charge. As mentioned above, the $\pi$ electrons in $C_{60}$ were destabilized, and it is, with some limitations, an aromatic compound. In single-walled carbon nanotubes (SWCNTs), the mobility of the $\pi$ electrons and the semiconductor properties depend upon the tube diameter and helicity. Thus, SWCNTs could behave as semiconductors or as metallic compounds. This was important because electron flow could be improved by connecting electron-rich with electron-deficient semiconductors. The role of shape in determining semiconductor properties also meant that this important property could be modified. In 1997, by combining some of the greatest scientific advances of the 1980s, the scanning tunneling microscope was used to micromanipulate and measure the electrical properties of SWCNTs to describe an early nanodevice, a SWCNT nanodiode.

## 1.4. Current and Future Nanotechnology Applications

By 2004, nanotechnology was already in widespread use in the computer and electronics industries. In 2007, the Environmental Protection Agency (EPA) noted that nanotechnology products predominantly fell into four groups:

1. Carbon-based materials such as $C_{60}$ and the carbon nanotubes
2. Metal-based materials, which are nanomaterials principally comprised of metals
3. Dendrimers, which are NPs formed by branched polymers
4. Composites, which contain different NPs or combinations of NPs and larger materials.

In 2005, the Woodrow Wilson International Center for Scholars and the Pew Charitable Trusts established the Project on Emerging Nanotechnologies (http://www.nanotechproject.org/). The Project on Emerging Nanotechnologies includes inventories of products of nanotechnology. The number of nanotechnology products listed in the consumer inventory has increased linearly since 2006. Within the US, federal support for research on nanotechnology was $10.5 billion total from fiscal years 2001 through 2009. In 2011, the greatest number of consumer products in the inventory was in the health and fitness category, followed by home and garden, automotive, and food and beverage products. In contrast, Lux Research, Inc. reported that the automotive industry was the industry with the greatest use of "nano-enabled" products, with the construction, electronics, healthcare, environment and energy sectors also using nanotechnology. The US Patent and Trademark Office has reported a 20% annual growth rate for nanotechnology patents between 1985 and 2005. The total revenue generated by products using nanotechnology is estimated to reach 2.5 trillion dollars in 2015.

Unfortunately, statistics on the use of specific nanotechnology products are incomplete. There are several reasons for this. Under the Toxic Substances Control Act (TSCA), manufacturers of new chemical substances must provide information to Environmental Protection Agency (EPA) before the chemical substances enter commerce, and over 100 such applications were reviewed by the EPA by 2010. However, a chemical is not considered new if it is simply a smaller size or a different shape from a previously existing chemical – and many new engineered NPs are simply smaller versions of existing chemicals. In addition, the majority of the publicly available information is for high production-volume

chemicals. Thus, under the 2011 EPA final rule amending TSCA, reporting is required for companies who for commercial purposes produce or import at least 100 000 pounds/year of compounds on the TSCA inventory. That threshold for reporting will be reduced to 2500 pounds/year over the next few years for chemicals that are targets of TSCA rules. However, in an industry such as nanotechnology, which is rapidly increasing production and where millions of particles can be in a gram of material, existing reporting has undoubtedly missed the majority of activity. Products of nanotechnology can also be regulated as a significant new use of an existing chemical (SNUR), but many producers of NPs are small manufacturers or businesses exempt from those rules. In 2009, the EPA estimated that it only received information on 10% of the commercially available nanotechnology products. The United States Government Accountability Office has noted the challenges for federal agencies and Congress when trying to ensure safety of any rapidly evolving technology. Not surprisingly, the products of nanotechnology are causing a review of current regulations.

Nanomedicine, as mentioned above, is the medical application of nanotechnology. More specifically, it has been defined as "the application of nanoscale technologies to the practice of medicine, namely, for diagnosis, prevention, and treatment of disease and to gain an increased understanding of the complex underlying disease mechanisms. (Bawa et al., 2005)" While a search of PubMed revealed only a handful of publications prior to 2005 (Figure 43.1), a more detailed search for all publications and patents revealed that the earliest nanomedicine publications appeared in the 1990s, and that a sharp increase in nanomedicine patents began about a decade later.

The reason for the interest is obvious: the potential to improve patient outcomes and to make a profit. Nanoengineering of pharmaceuticals can improve solubility and stability, target delivery or decrease drug toxicity (see also see *Pathology in Non-Clinical Safety Assessment*, *Chapter 24*). For example, NPs often have greater solubility than larger particles. Consistent with that greater solubility, the albumin-conjugated nanoparticle form of paclitaxel, Abraxane™ (Abraxis, Los Angeles, CA) has greater solubility than previous paclitaxel formulations and does not require organic solvents that can cause hypersensitivity reactions in some patients. In addition to greater solubility, the albumin in Abraxane may be targeting the caveolae. Caveolae are invaginations of the vascular endothelium that are present in very high numbers, react with specific target molecules (such as albumin), and form trafficking vesicles that move the material through the endothelium and into the target tissue (Figure 43.3). This is just one example of how nanotechnology has been used to overcome toxicity and improve delivery. Other examples include NPs designed to scavenge amyloid-β within the vasculature as a potential Alzheimer's disease therapy. It has recently been suggested that carbon nanotubes may be able to function as scaffolds to guide axonal growth and stimulate nerve repair.

Additional new nanomedical concepts and products are rapidly developing. An entire issue of *Accounts of Chemical Research* was recently devoted to therapeutic and diagnostic ("theranostic") nanomedicine (see Suggested Reading section). The surface and the core of the NP can each be engineered to contain multiple components (Figure 43.2), and each of the components can have important properties. Thus, it is possible to construct NPs that target delivery, are contrast agents, and/or contain a therapeutic payload. The development of NPs designed to cross the blood–brain barrier offers the prospect of the potential to deliver drugs to treat devastating neurologic disorders. However, there are very little data available on how these new nanomedical products will be degraded within cells, such as neurons, which may not have previously been reached by the therapeutic payload or other by-products of nanopharmaceuticals. In other words, drug

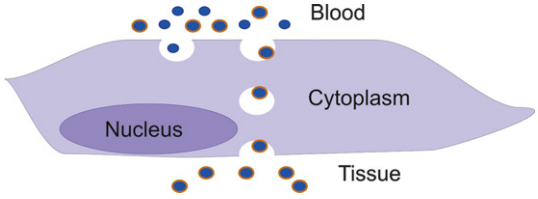


FIGURE 43.3 Caveolae are invaginations of the vascular endothelium that react with target molecules such as albumin and form trafficking vesicles. This permits NPs with surface target molecules (shown here in orange) to move across the vascular endothelium.

delivery to the brain should involve traversing the blood–brain barrier to deliver the drug, *and* safely getting rid of that drug. This is also true of any other target tissue or subcellular organelle uniquely being reached by nanopharmaceuticals.

As has been noted in recent reviews, nanomedicine has the potential to overcome some of the greatest medical challenges of our time. Nanotechnology is already providing innovative new products for a variety of non-medical applications. The challenge is trying to harness this promise as safely as possible. The NPs of today can be made from a virtually infinite number of different compounds and combination of compounds. Part of meeting the challenge for safe development of nanotechnology is understanding how nanosizing alters the toxic effects of particulates. In addition, it is important to understand human exposures to NPs.

## 1.5. Human Exposures

For workers and consumers, inhalation and dermal absorption are the major routes of NP exposure. Inhalation exposures can occur when NPs are aerosolized within the workplace. Some consumer spray products can release NPs into the breathing zone of consumers. Once inhaled, NPs can reach the nervous system via the olfactory nerves, the sensory nerves of the nose and airway, or the vasculature. Additional tissues, such as the spleen and liver, can be exposed to NPs translocated from the lung. However, the magnitude and frequency of parenteral exposure after inhalation remains controversial and may well depend upon the physiochemical characteristics of the NP. The lung itself is a major target of injury from first-generation NPs, including single-walled (SWCNT) and multi-walled carbon nanotubes (MWCNTs). Dermal exposure to engineered NPs can occur during workplace exposure, after environmental contamination, or through topical application of NPs in products such as cosmetics, sun protection lotions, or antibacterial lotions. If NPs are released into the environment they can be incorporated into food and water, and the gastrointestinal tract becomes an additional route of exposure. The eyes can also become a route of nanoparticle exposure. With nanopharmaceuticals, the route of exposure depends upon the route of administration.

NP exposures are difficult to measure. Since the lung is a major target for NP toxicity, particle concentrations in air need to be measured. Standard measurements of particle exposures in air are based upon mass and may then be divided into size classifications. Thus, for environmental exposures, the concentrations usually measured are for total particulates, particulate matter less than 10 μm (PM10), and/or particulate matter less than 2.5 μm (PM2.5). For occupational exposures to aerosolized particulates not otherwise regulated (PNOR), the Occupational Safety and Health Administration distinguishes particles less than 5 μm from total PNOR. For PNOR, for an 8-hour average, the permissible exposure limit (PEL) for total particles is 15 $mg/m^3$ and for particles less than 5 μm the PEL is 5 $mg/m^3$. Thus, occupational and environmental exposure limits for particulates are based upon mass and historically have been used for particles in a size range of 1–5 μm or greater. NPs are a *log* or more smaller, with at least one dimension less than 0.1 μm (100 nm). With most studies of ambient workplace particulates, there is no way of knowing what percentage of the particles were NPs. However, in the NP size range, collecting the particles to measure them by mass, surface area, particle number, or any other measure becomes a challenge. It has recently been noted that the existing measuring devices are too large to be worn in the actual breathing zone, which means that NP personal exposures cannot be measured with existing technology. However, current technology does include some bulky equipment, such as cascade impactors, condensation nucleus counters, and diffusion chargers, that allow workplace area measurements of NP mass, number, and surface area. One potential approach to measuring and distinguishing NP exposure from larger particle exposures in workplace air is the use of a preseparator so that the number of size-specific NPs can be measured in air. Thus, a major research goal is to improve knowledge of human NP exposures and improve techniques for measuring them.

Carbon nanotubes (CNTs) and carbon nanofibers (CNFs) can be released into the air (aerosolized) within workplaces if sufficiently agitated (see Suggested Reading). Activities causing

aerosolization of MWCNTs include oven-opening, preparation, weighing, transferring, blending, spraying, and sonication. Diameters of the MWCNTs aerosolized into workplace air depend on the manufacturing process, with mode diameters ranging from 20–30 nm for catalyst preparation and 120–300 nm for ultrasonic dispersion. CNFs can be aerosolized during weighing, mixing, handling, transfer, and bagging of dry CNFs as well as during wet sawing of CNF composites. Additional NPs have also recently been demonstrated to produce aerosols under workplace conditions. Local exhaust ventilation has been reported to be effective in controlling nanoparticle exposures in at least one MWCNT laboratory (see Suggested Reading).

## 2. EXPERIMENTAL TOXICOLOGIC PATHOLOGY OF NANOPARTICULATES

In 2005, the ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group identified the key elements for toxicity screening of NPs as "Physicochemical Characteristics, *In Vitro* Assays (cellular and non-cellular), and *In Vivo* Assays" (Oberdorster *et al.*, 2005a). Determining the physicochemical characteristics of nanoparticles is essential if the study is to produce data that can be interpreted for risk assessment purposes. The chemical composition, size and size distribution, shape, agglomeration, surface properties, porosity, and a biologically relevant measure of exposure dose are each important to understanding the relevance of NP toxicology studies. Some common features of NPs that can influence toxicologic pathology are surface area, solubility, quantum chemistry, and size.

### 2.1. Enhanced Toxicity of Nanoscale Particulates

#### *Surface Area*

Several studies have compared the bioactivity of fine vs ultrafine carbon black, or fine vs ultrafine $TiO_2$, after pulmonary exposure. On an equal mass basis, ultrafine carbon black or $TiO_2$ were found to be more inflammatory than fine particles of the same chemical composition. However, when dose was converted to total particulate surface area delivered to the lung, the bioactivities of fine vs ultrafine carbon black or $TiO_2$ were similar. NPs have the tendency to agglomerate. If particulate surface area influences pulmonary response, then the agglomeration state of NPs should have a significant effect on bioactivity. Indeed, Shvedova and colleagues have shown that well-dispersed nano carbon black (dispersed in diluted lung lining fluid) was more inflammatory after intratracheal instillation in rats than an equal mass of poorly-dispersed nano carbon black (suspended in phosphate-buffered saline). The influence of dispersion was confirmed in a more extensive study which reported that, on an equal mass basis, well-dispersed ultrafine carbon black was 65-fold more inflammatory and cytotoxic in the lung than fine carbon black. Similarly, Sager and colleagues reported that well-dispersed ultrafine $TiO_2$ was 42-fold more inflammatory and cytotoxic than fine $TiO_2$. When exposure doses were equalized on a basis of total particulate surface area instilled into the lung, no significant difference in potency of fine vs nano carbon black or $TiO_2$ was noted.

#### *Solubility*

For metals, particle surface area is a major determination of solubility. As noted above, for a given mass of particulates, the surface area goes up as particle size goes down. Thus, the log solubility of dilute solutions of organic-coated silver NPs increases on a mass basis as particle diameter decreases. This is important because dissolution of certain nano metallic particles and the formation of toxic metal ions has been proposed as an important mechanism determining bioactivity. For example, nano ZnO has been shown to exert toxicity in a cell culture system via the formation of $Zn^{2+}$ ions and the resultant generation of reactive oxygen species. Doping of ZnO with iron (10%) has been shown to decrease dissolution by 93%. This decrease in $Zn^{2+}$ formation was associated with a striking reduction in pulmonary inflammation and lung damage in a rat model after intratracheal instillation of nano ZnO. Dissolution of nanoparticles would also affect the translocation of metals from the lung to systemic organs. Indeed, the

lung burden of Zn rapidly declines 24 hours after exposure to ZnO, with a concomitant rise in Zn levels in systemic organs over this time.

### *Quantum Chemistry*

NPs can have different chemical and physical properties than larger particles with the same chemical properties. This difference is attributed to changes that occur in the nanoscale where quantum phenomena predominate, particularly in the size range of 10–50 nm. These effects are described in quantum theory in physics, and occur when particle size becomes similar in size to physical and chemical phenomena such as wavelengths. Even thermodynamic properties can be different in the nanoscale as opposed to bulk materials of the same composition. Further, different nanoparticles with the same chemical composition can differ in their thermodynamic properties because particle volume within the nanoscale size range influences quantum mechanical behavior. Thus, quantum phenomena are very important in the nanoscale and can markedly alter the properties of compounds that are relatively inert when larger.

Fortunately, pathologists do not need to understand quantum theory to understand nanotoxicology. However, it is important that toxicologic pathologists understand that fundamental properties of compounds can change in the nanoscale, and that the fundamental properties of compounds clearly can change toxicity. For an in-depth understanding of the quantum realm and altered properties in NPs, collaborators in other scientific fields are particularly important members of many nanotoxicology research teams.

### *Size*

Size affects the properties of surface area, solubility, and quantum chemistry. However, size itself influences the ability of a particulate to translocate within the body, enter cells, and interact with subcellular structures. Even the ability of a pathologist to find the particulate in a tissue section is dependent upon particulate size.

## 3. VISUALIZING NANOPARTICULATES IN TISSUE

Some NPs can be very similar to normal subcellular components (Figure 43.4). The general principles of identifying NPs in light microscopic and ultrastructural tissue sections include familiarity with (1) the appearance of the NP, (2) the appearance of normal and diseased tissue, and (3) a means for clearly distinguishing between the NPs and changes that may be associated with the NPs. For example, eosinophil granules contain tubular structures within their granules, and eosinophils can be a component of an inflammatory response. However, the eosinophil granules are not engineered nanoparticles (Figure 43.4). Thus, structures in NP-exposed animals will *not necessarily* be the NP, even when similar in appearance to the test article and absent in controls. It is essential that the evaluation of tissues from NP-exposed animals for intracellular distribution (1) is conducted by someone familiar with the spectrum of pathologic responses in the exposed tissues, (2) includes a means for clear distinction between the NP and cellular responses to the NP, and (3) is conducted with an understanding of the dilution effect and detection limits for NPs in tissue. Failure to identify NPs in an organelle is more likely evidence that the technique is not sensitive enough to detect them, than evidence that they are not there.

In cultured cells and in tissue sections, NPs may be imaged by using the intrinsic optical properties of the nanoparticle or by labeling. For example, many nanoparticles block light, which allows them to be seen in standard H&E stained sections (Figure 43.5).

### 3.1. Factors that Limit the Ability to Identify NPs in Tissue Sections

When preparing to examine NPs in tissue, it is critical that the NPs occur frequently enough for the sampling strategy. The frequency of occurrence must be sufficiently high that NPs are likely to be in each field of view. Given the high number of NPs present in even a microgram of NP material, visualization of NPs might, at first, appear to be a routine microscopy exercise. For instance, a 1-μg lung burden of well-dispersed MWCNTs in the mouse lung could easily distribute into 200 million or more nanotubes throughout the lungs. Given that the mouse lung has in the order of 4 million alveoli, this would, on average, yield approximately 50 or more nanotubes per alveolus. For light

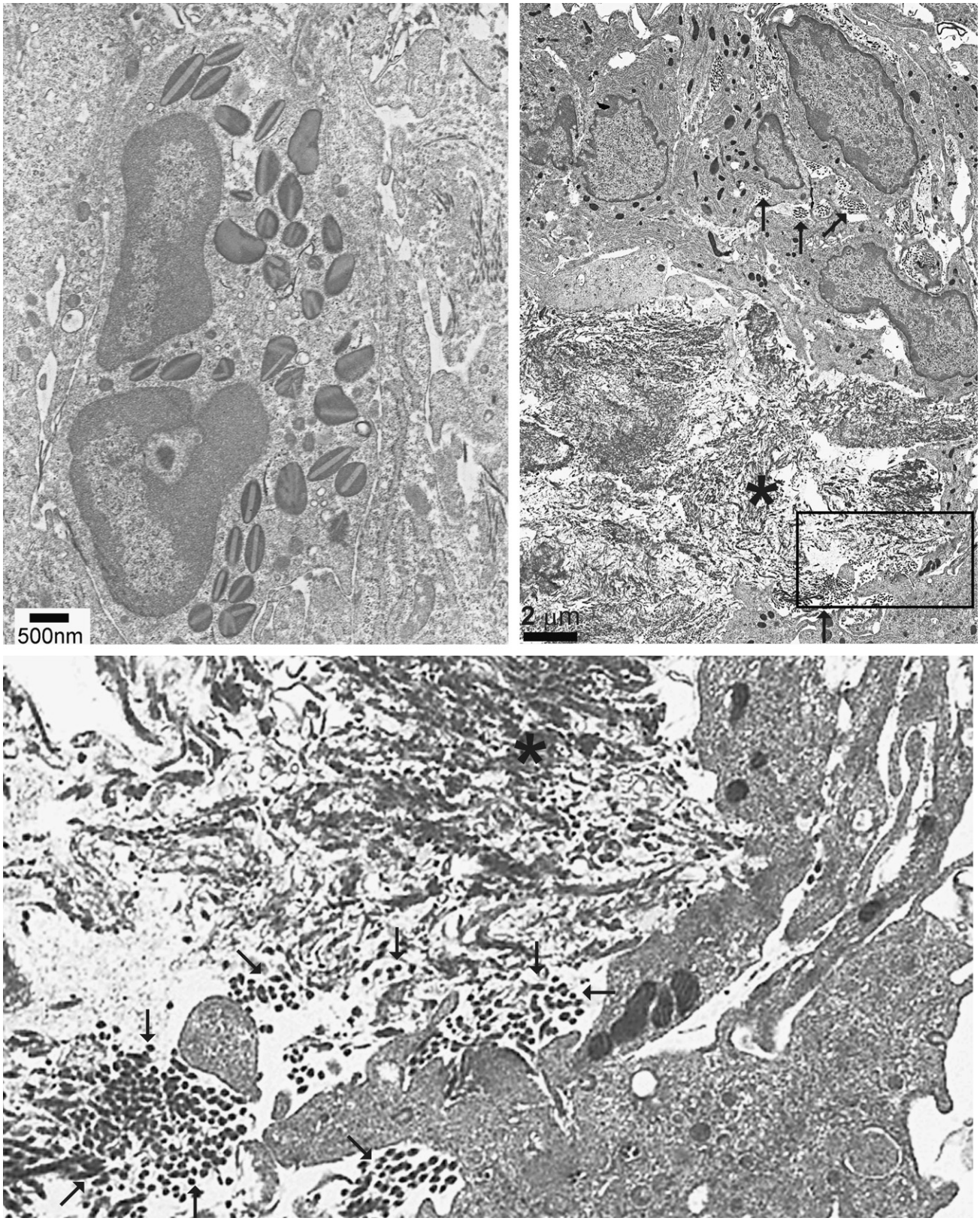


**FIGURE 43.4** The image in the upper left is a normal eosinophil in the lung of a SWCNT-exposed mouse. The eosinophil granules (in the cytoplasm) contain normal variations in staining intensity that can give the appearance

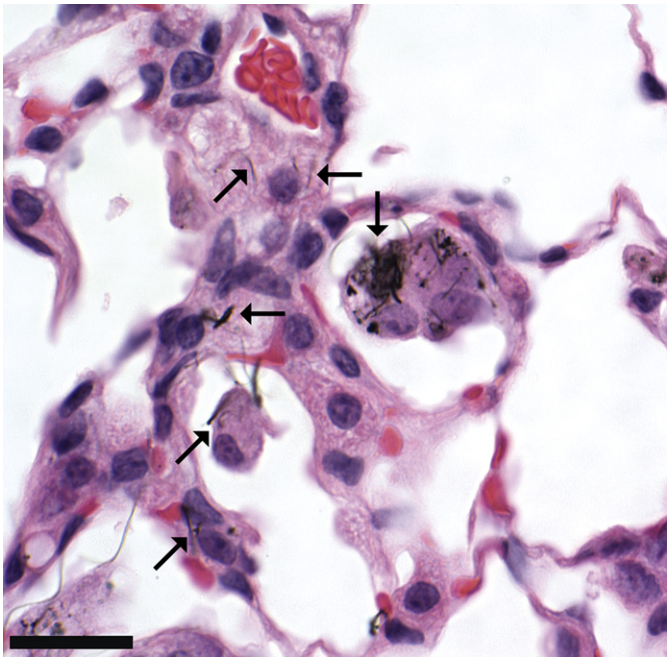

FIGURE 43.5 In this H&E stained section from a MWCNT-exposed mouse, transmitted light is blocked by the MWCNTs which show up as black structures (arrows) in macrophages, giant cells, and alveolar epithelial cells. Bar = 20 μm.

microscopy of paraffin sections, this would yield 10 nanotubes in a typical alveolar profile (a 5-μm section would be approximately one-fifth of the 25-μm alveolar diameter in the mouse). Thus, the frequency of occurrence is sufficiently high that NPs would likely be in the field of view. However, given the limited resolution of a conventional light microscope (0.2 μm), NPs may not be detectable in conventional light microscopy. An alternative would be to consider examination with TEM. However, examining the same tissues/burden under TEM would be prohibitively difficult at this dose. A typical TEM section might contain 20 alveolar profiles but would be only 60 nm thick; thus, on average, it would be necessary to examine the entire TEM section to find one or two NPs.

The above example illustrates that both the inherent visibility and the frequency of occurrence of NPs must be considered in order to successfully evaluate NPs in tissue sections. Numerous other factors work in concert to reduce the possibility of detecting NPs in section. These include the following.

1. *Lack of adequate dispersion.* Although NPs are characterized by the dimensions of single particles, the individual NPs often agglomerate to form functionally larger particulates, particularly when suspended in aqueous solutions. Failure to adequately disperse NPs is one of the major problems in preparation, administration, and detection of NPs. Many of the NPs that are of interest for health-risk evaluations have a high self-affinity and require treatment with special dispersants to prevent agglomeration into micrometer dimensions. In the absence of such treatment, as much as 80% of SWCNTs may remain agglomerated into micrometer-sized clumps.
2. *Limitations in visualization due to narrow depth of field.* Due to their small dimensions, examination of well-dispersed NPs requires high objective powers which typically also have a limited depth of field. Because of the limitations of the depth of field at high numerical aperture, only a fraction of the particles in the section will be in focus at one time. For example, a 100× high numerical aperture lens may have a depth of field of only 0.2 μm; thus, only 1/25 of the thickness of a 5-μm section would be in optimal focus at one time. For larger fibers such as asbestos this does not pose a significant problem, as the out-of-focus regions of the large fibers are still detectable.
3. *The small fraction of the section area covered by the NPs.* In order to be detectable in a microscope section, the nanomaterial must cover

of fiber-like particles, but these are normal structures that should not be confused with SWCNTs even when absent in controls. The image on the upper right is a granuloma containing SWCNTs (asterisk) that are being walled off by epithelioid macrophages. The SWCNTs are very similar in size and shape to the collagen fibers (solid arrows) within the granuloma. The rectangle is a region that was photographed at higher resolution as shown in the lower panel. At this higher magnification, the SWCNTs (asterisk) can be distinguished from the collagen fibers, which have a more distinct fiber shape. At even higher magnification, the gold label that had been attached to the SWCNTs confirmed that these were the SWCNTs.

a sufficient area of the section to alter the light path. In a 0.5-$cm^2$ tissue section of a mouse lung exposed to 50 μg of MWCNTs, there may be as many as 2 million MWCNTs each being 50 nm in diameter by approximately 5 μm long. Even if these fibers were maximally aligned side to side into a sheet parallel to the section, the fibers would cover less than 1% of the 0.5-$cm^2$ tissue section. This, combined with the lack of contrast, makes individual and small clumps of NPs difficult to detect in microscopic sections.

4. *Lack of contrast between the biologic tissue and the nanomaterial.* Many NPs, such as carbon nanotubes, were developed as structural materials, and as such are relatively unreactive to conventional biologic stains. Furthermore, the dimensions of the NPs are frequently less than the visible wavelengths of light, which further diminishes the likelihood of detection. Carbon nanotubes only give the appearance of being differentiated in the sections because there is sufficient mass of nanomaterial in the light path to block light. Detection of these difficult nanomaterials requires one or more of the special techniques for detection described in subsequent sections.

These and other factors frequently limit the ability to detect and identify NPs in tissue sections. Specialized instruments and techniques have been developed to overcome these problems. The techniques include labeling of the NPs, use of FESEM in thick sections, and enhanced darkfield microscopy.

## 3.2. Labeled NPs

Labeling of NPs with a fluorescent indicator or some conveniently detected particle such as colloidal gold is one possible solution to make NPs easily visible in sections. Functionalization such as the oxidation of the carbon–carbon bonds may be used to label the carbon nanotubes with colloidal gold. Labeling with colloidal gold allows the application of a variety of established techniques developed for immunohistochemistry and other fields. These techniques can be used to allow detection in microscopic sections by silver enhancement, and to aid in identification in TEM/FESEM observation.

## 3.3. High-Resolution FESEM

Conventional scanning electron microscopes (SEMs) that are used for biologic specimens have been applied with great success to imaging of micrometer-dimensioned inhaled particles which were studied prior to the advent of nanomaterials. For NPs, the conventional SEM does not have the sub-micrometer resolution necessary to resolve or identify NPs. Difficulties in imaging NPs with an SEM are further complicated by the fact that many NPs, such as carbon nanotubes, have no significant difference in secondary electron or backscatter emissions from the organic carbon in which they are immersed.

Introduction of the Field Emission Scanning Electron Microscope (FESEM) has significantly improved the resolution and applicability of the SEM to examination of NPs in tissue. The unique "cold" cathode design of the FESEM produces high-quality, low-voltage images with significantly lower electrical charging that can be used to identify NPs in tissues at levels of resolution not previously available with the conventional SEM. The high-resolution capability of the FESEM greatly facilitates the imaging of MWCNT interactions with cells and tissues of the lung. The FESEM images in Figure 43.6 show the penetrating nature of MWCNTs in an alveolar macrophage 28 days after exposure.

To image NPs at high magnification with the FESEM, some consideration of the methods of specimen preparation is necessary to obtain a stable image. Use of thin sections from paraffin-embedded tissue has been found to be preferable to large, unevenly cut blocks, because it provides a uniform thickness of organic material on a conductive carbon planchet. At 5–8 μm of thickness, paraffin sections are thick enough to convey 3D information and less likely to charge or undergo physical shifts when examined at the high magnifications necessary to study nanomaterials.

## 3.4. Enhanced Darkfield Microscopy

Traditionally, darkfield microscopy has been used to examine larger fine sized particles in tissue sections. Darkfield microscopy suffered from lack of resolution, in part due to the fact that transmitted light is not blocked from

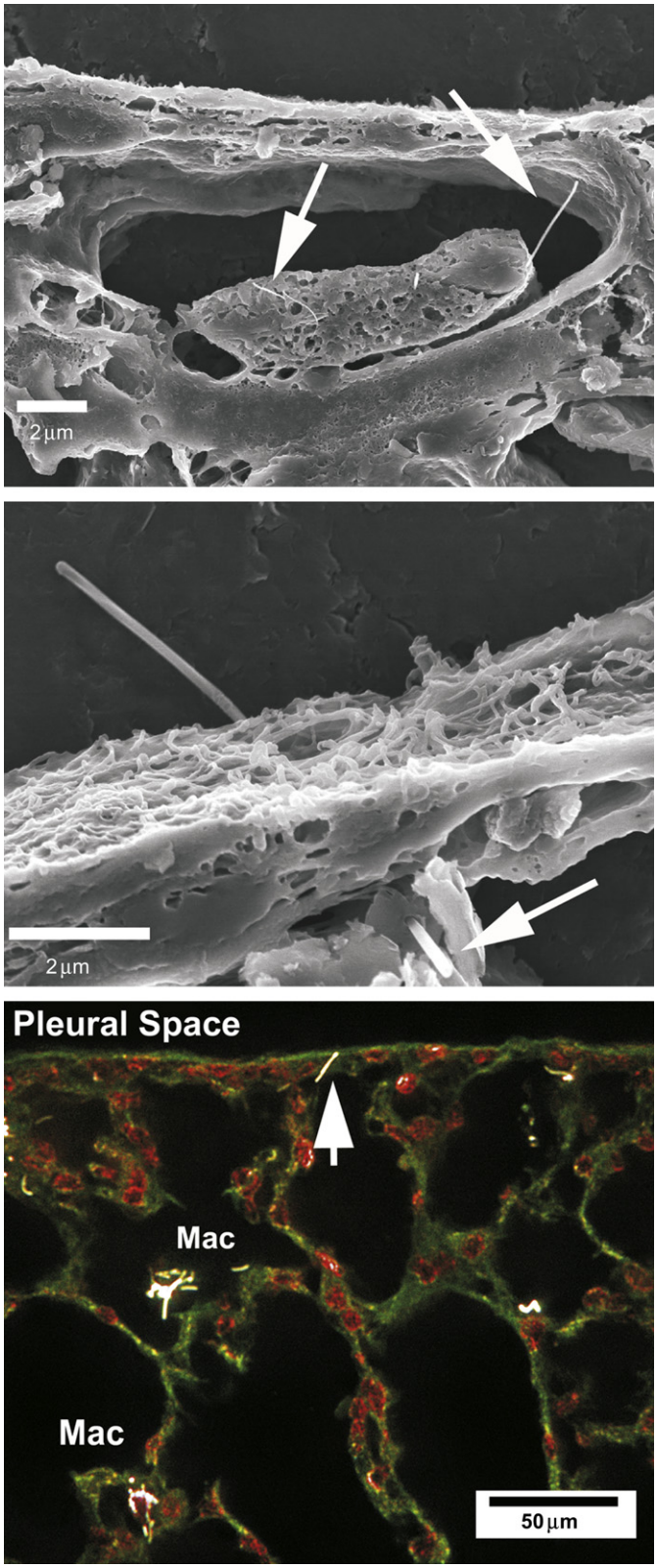


FIGURE 43.6 The top image demonstrates MWCNTs within a subpleural lymphatic using FESEM. In the middle image, FESEM is used to demonstrate penetration of the visceral pleura by MWCNTs. In the lower image, enhanced darkfield imaging demonstrates MWCNTs within macrophages (Mac), the interstitium and pleura (arrow). *Figures reproduced from Mercer* et al. *(2010) Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes,* Particle and Fibre Toxicology *7, 28; and Mercer* et al. *(2011) Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes,* Particle and Fibre Toxicology, *8, 21, with permission.*

the image path. Newer, enhanced darkfield systems have modified optics that virtually eliminate transmitted light from the image. In the newer systems, a specialized illuminator is used to generate a highly angled and focused illumination light source that, unlike previous darkfield systems, completely eliminates transmitted light from the system. This greatly enhances the contrast between sectioned tissue (a poor source of scattered light) and NPs, as can be seen in the enhanced darkfield image of Figure 43.6.

The value of enhanced darkfield microscopy is based on the fact that the vast majority of nanomaterials efficiently scatter light while normal tissue sections do not. Nanomaterials, such as carbon nanotubes, have many of the characteristics which produce Rayleigh scattering of light. These include dimensions less than the wavelength of light, having close and ordered alignment of atoms, and typically having a refractive index significantly different from that of biologic tissues and/or the mounting medium. Normal preparation of mounted tissue is designed to minimize scattered light. For instance, the refractive indices of glass, mounting medium, and tissue are all closely matched (1.47–1.55). The refractive indices of nanomaterials are much higher, being 2.2 for cerium oxide (a nanometer-sized diesel fuel catalyst), 2.6 for zinc sulfide (a component of quantum dot) and 3.6 for crystalline silica (nanosilica). Together, these characteristics produce significantly greater scattering of light by nanoparticles than by the surrounding tissues. The enhanced darkfield optical system images light scattered in the section, and thus nanomaterials in the section stand out from the surrounding tissues with high contrast. Using this method of imaging, large areas can easily be scanned at relatively low magnification to identify NPs that would

not be detected by other means. Although enhanced darkfield technology is relatively new, in our laboratory we have found the technique useful to detect a wide variety of NPs in tissue sections. These have included cerium oxide, titanium oxide, diesel exhaust, welding fumes, SWCNTs, MWCNTs, silver nanowires, silicon nanowires, nanosilica, quantum dots, colloidal gold, and others.

## 4. CYTOPATHOLOGY

### 4.1. Cytoplasmic Membrane Damage

NPs can be produced in almost any shape. The *aspect ratio* of a particle is the ratio of its longest dimension to its shortest dimension (Figure 43.7A). Fibers are the classic particulates with a high aspect ratio. Asbestos fibers are naturally occurring carcinogenic mineral fibers, and some asbestos fibers have diameters in nanoscale dimensions. In 1981, Mearl Stanton and colleagues noted that experimental pathology studies indicated that "The probability of pleural sarcoma correlated best with the number of fibers that measured 0.25 μm or less in diameter and more than 8 μm in length …" Some asbestos fibers, and by definition all nanotubes, have diameters less than 0.25 μm; therefore, a great deal of concern has been expressed regarding the similarities between asbestos fibers and some of the nanotubes. The similarities in some cases include a high aspect ratio, durability, surface reactivity, inflammation in the exposed lung, an ability to translocate through the pleura, and incomplete phagocytosis. It is not just nanotubes that can have these properties; NPs now include nanofibers, nanowires, nanobelts, and many other high aspect ratio particulates with nanoscale dimensions.

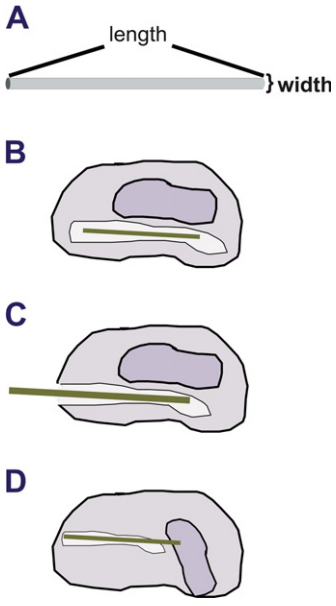


FIGURE 43.7 High aspect ratio particles. (A) A high aspect ratio particle is a particle with a much greater length than width. (B) Complete phagocytosis. (C) Incomplete phagocytosis. (D) Destructive phagocytosis with membrane penetration.

Discussions of the potential carcinogenicity of biologically persistent particles with high aspect ratios often focus on incomplete phagocytosis. In normal particle phagocytosis, macrophages and neutrophils phagocytize particles and are then carried out of the lung via mucociliary clearance or by the lymphatics. In some cases, high aspect ratio particles appear to undergo phagocytosis and fusion with the lysosome to form a phagolysosome (Figure 43.7B). Incomplete phagocytosis is the failure to completely internalize a fibrous particulate within the cytoplasm of a phagocytic cell. Classically, phagocytosis is incomplete when the length of the fibrous particle exceeds the dimensions of the phagocytic cells. When phagocytosis is incomplete, the phagocytic vacuole may still be open to the exterior of the cell when it fuses with the lysosome to form the phagolysosome (Figure 43.7C). Lysosomal enzymes include enzymes that produce free radicals for microbial killing, and enzymes capable of digesting cells. Release of those enzymes outside of the phagolysosome is thus able to cause cell injury through damage to the cytoplasmic membrane and inflammation.

Human macrophages are larger than rodent macrophages. Human macrophages often show less fiber-induced incomplete phagocytosis and less cytotoxicity than rodent macrophages – a finding that is consistent with a role for incomplete phagocytosis in the pathogenesis of fiber-induced lung disease. However, the asbestos fibers most associated with pleural sarcomas are longer than 8 μm, while 8 μm is less than the length of an average macrophage and certainly less than the length of a giant cell. This suggests that incomplete phagocytosis is not the only pathogenic mechanism for

fiber-induced cell injury. NPs are providing data that may help pathologists understand the pathogenesis of diseases, such as mesothelioma and asbestosis, that have long been associated with durable particles with a high aspect ratio. Some of these NP-induced changes are also manifested as cytoplasmic membrane damage.

MWCNTs are NPs with a high aspect ratio, and can be engineered within narrow dimensional ranges that may permit a greater understanding of fiber toxicology. MWCNTs block transmitted light, which makes them relatively easily identified by light microscopy where they are seen as black rod-like structures within the light-transmitting cytoplasm. In the lungs of mice exposed to MWCNTs (median length of 3.86 μm and a median width of 49 nm), incomplete phagocytosis is observed and would be anticipated to release lysosomal enzymes and cause cell membrane damage. Importantly, incomplete phagocytosis or partial engulfment is seen with tangled mats of MWCNTs as well as with long MWCNTs. In addition, the MWCNTs appear to migrate within the lung much like a nanoscale version of a splinter might migrate through tissue. Thus, MWCNTs are seen penetrating the visceral pleura of the lung, extending from alveolar septa, and within lymphatics (Figure 43.6). In addition, MWCNTs can penetrate nuclei of macrophages (Figure 43.7D). Within the cytoplasm, MWCNTs are frequently outside of vacuoles, suggesting either that they enter cells by means other than phagocytosis or that they do not stay in the phagolysosome. Functionalized MWCNTs have recently been demonstrated to enter cells without phagocytic capabilities and to escape the phagolysosomes, suggesting that both mechanisms play a role in the location of MWCNTs within cells. Since the cytoplasmic membrane is basically a protein-containing lipid bilayer, the ability of a thin tube with high tensile strength to migrate through the membrane(s) of mobile cells such as macrophages and into additional cells is not surprising. Nor is it surprising that the migration may continue through additional cells in a tissue such as the lung, which moves and undergoes pressure changes with every breath. In addition, the tips of nanotubes with caps on their ends and the tips of crocidolite asbestos fibers appear to be recognized by cellular receptors which mediate a tip-first entry into the cell. Once initiated, the internalization process appears to continue irrespective of the length of the particle and can lead to incomplete phagocytosis. Thus, data support penetration of the cytoplasmic and nuclear membranes by NPs with high aspect ratios, and this may be explained by three potential processes: (1) classic incomplete phagocytosis of a high aspect ratio particle that exceeds the length of the cell; (2) migration out of the phagolysosome and/or through the cytoplasmic membrane; and (3) receptor-initiated tip-first phagocytosis which continues irrespective of the length of the NP and has the potential to puncture the nuclear and cytoplasmic membranes.

## 4.2. Mitotic Spindle Interactions

Toxicologic anatomic pathologists rarely evaluate changes involving the mitotic spindle. However, it is very important that toxicologic pathologists recognize changes in histopathology that are outside of the spectrum of possible changes in normal tissue sections. During the evaluation of histopathology in the lungs of mice inhaling SWCNTs, the toxicologic pathologist noted what appeared to be present in a single dividing cell: SWCNTs were gathered at the site of the spindle pole, chromatin appeared to be streaming from one chromatin bundle towards the other, SWCNTs appeared to be attached to the streaming chromatin, and the other chromatin bundle was unusually condensed (Figure 43.8). This strongly suggested that SWCNTs could interfere with the specialized system responsible for sending the correct genetic material to the daughter cells during cell division: the mitotic spindle. A summary of some of the more critical information on the mitotic spindle and the genotoxicity of NPs is included below. The Further Reading section lists publications that contributed to that information and are highly recommended reading for those interested in the genotoxicity of NPs.

### *Overview of the Mitotic Spindle*

The mitotic spindle is a structure that forms during cell division and separates duplicated chromosomes. In eukaryotic cells, the mitotic apparatus is composed of two centrosomes and spindle microtubules (Figure 43.9). The centrosome is 1–2 μm in diameter. Each centrosome is composed of two centrioles of approximately

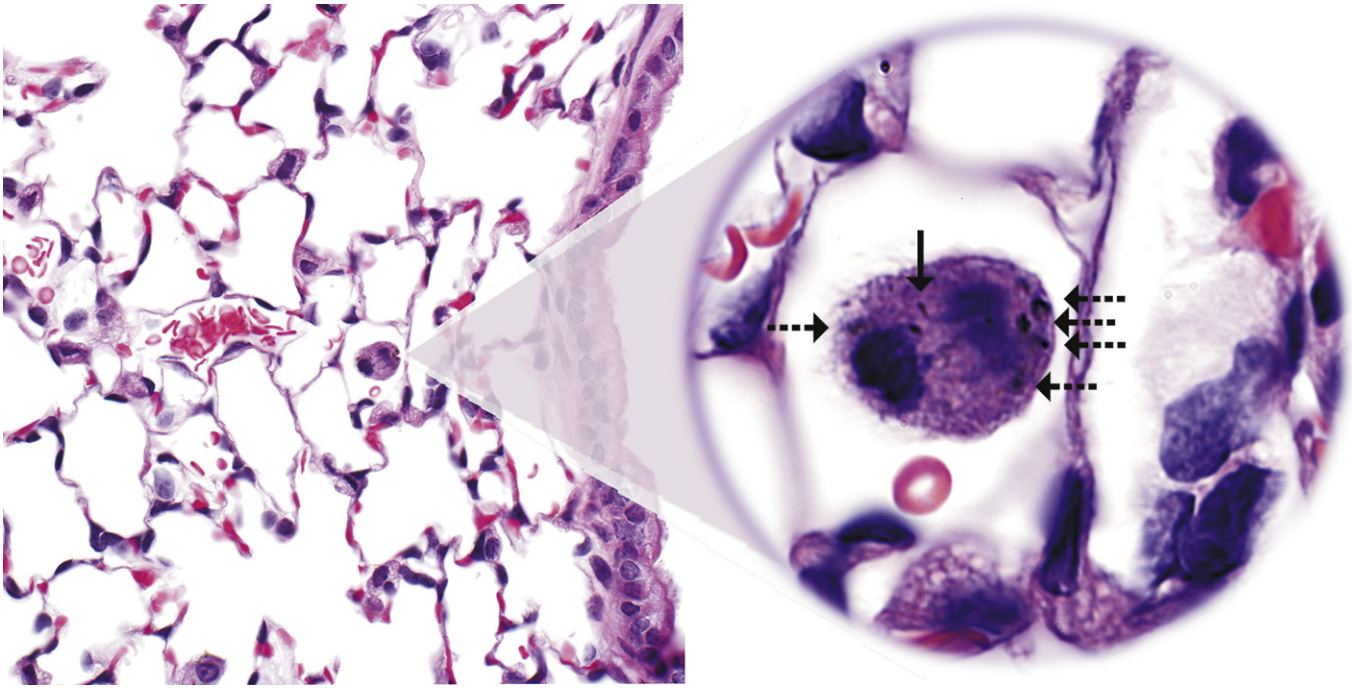

FIGURE 43.8 On the left is an actual 60× oil field examined by the pathologist and on the right is a high-resolution enlarged digital image from a 100× objective showing material consistent with SWCNTs near the spindle poles (dashed arrows) and attached to streaming chromatin (solid arrow). Identifying this unanticipated change required a detailed histopathologic evaluation and the use of high magnification. Photographing the interaction took a high-resolution digital camera and cropping of the image. Changes such as these are at the limits of resolution of light microscopy. Subsequent studies of SWCNT-exposed cells detected high levels of aneuploidy as well as interactions between SWCNTs and the mitotic spindle.

20 nm in diameter surrounded by a pericentriolar matrix. The matrix surrounding the centrosome is composed of a variety of proteins, including motor proteins and cell cycle control genes. The centrosome determines the shape of the cell as well as the mitotic spindle apparatus. In eukaryotic cells, polymerization of microtubules from alpha and beta tubulin is initiated at the centrosome to form the mitotic spindle and the structure for cytokinesis. During cell division the microtubules continue to polymerize and the mitotic apparatus elongates (Figure 43.10A). The chromosomes are separated by the mitotic apparatus as it elongates (Figure 43.10B). At the end of mitosis, a furrow is formed between the two dividing daughter cells (Figure 43.10C). The furrow between the dividing cells (midbody) contains microtubules from each pole of the mitosis. Disruption of centrosome number or structure, or of the microtubule assembly, results in aberrant mitotic spindles, failure of cell separation, and errors in chromosome number (aneuploidy). Disruption of centrosome number and structure is common in most cancers.

### *Centrosomal Interactions*

Single-walled carbon nanotubes (SWCNTs) have been shown to fragment the centrosome, resulting in multipolar mitotic spindles and dramatic aneuploidy. SWCNTs were strongly associated with the centrosome. Three-dimensional reconstructions of mitotic figures from SWCNT-dosed respiratory epithelial cells have shown SWCNTs located inside the centrosome structure. SWCNTs have also been shown incorporated into the microtubules of mitotic cells.

### *Microtubule Interactions*

SWCNTs have been observed within the nucleus and in association with cellular and mitotic tubulin, in the bridge separating dividing daughter cells (midbody), as well as in the DNA, potentially disrupting the normal mitotic process (Figure 43.11). The basis of the incorporation of

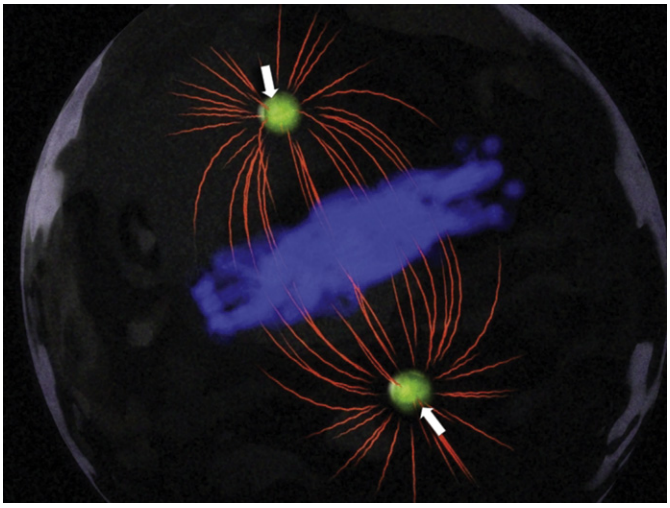

FIGURE 43.9 Drawing of a normal mitotic spindle apparatus. The green-stained centrosomes are indicated by white arrows, the microtubules are in red and the DNA is in blue. The cell is in metaphase stage of cell division with the chromosomes lined up in the middle of the mitotic spindle. *Figure reproduced from Sargent* et al. *(2012) Single-walled carbon nanotube-induced mitotic disruption,* Mutation Research, *745, 28–37, with permission.*

carbon nanotubes into the mitotic apparatus may be due to a number of mechanisms. In recent laboratory studies, carbon nanotubes have been shown to form functional hybrid molecules with tubulin. The carbon nanotube/microtubule hybrid molecules were transported by the spindle motor kinesin, which is essential for normal cell division; however, the hybrids were transported with less efficiency than the cellular microtubules. In addition, spherical nanoparticles less than 40 nm in diameter inhibit the activity of the kinesin motor further, indicating the potential for the disruption of mitosis by nanomaterials. Inhibition of kinesin motor activity has been shown to result in mitotic spindle disruption. Carbon nanotubes and microtubules have many physical properties in common, including high tensile strength. Carbon nanotubes are five times stronger than steel. Microtubules are 100 times stronger than any other cellular cytoskeletal fibers; however, their strength is 100 times less than that of carbon nanotubes. Although they have many physical properties in common, there are also some distinct chemical differences between microtubules and carbon nanotubes. Carbon nanotubes are composed of covalently-bound carbon molecules rolled into a tube, while microtubules are polymers of alpha- and beta-tubulin subunits that are bound by non-covalent hydrogen bonds. The microtubules are dynamic structures that polymerize and de-polymerize within the cell during cell division. Once they are synthesized, individual carbon nanotubes are static in size. The similarity in size and shape of the carbon nanotubes and microtubules may make it possible for the nanotubes to displace microtubules at critical cellular targets, including the centrosome (Figure 43.12). Alternatively, the nanotubes have also been shown to be incorporated into the microtubules as well as the centrosome. Incorporation of the strong carbon nanotubes into the cellular structures may be responsible for the fragmenting of the centrosome during cell division. Fragmented centrosomes have been shown in other systems to result in multipolar mitotic spindles.

### *Chromosomal Interactions*

Carbon nanotubes have a high affinity for DNA. SWCNTs have the highest affinity for DNA of G–C-rich DNA sequences in the chromosomes, and have been shown to bind to the G–C-rich regions of the chromosome ends (telomeric DNA). The DNA intercalation of the nanotubes results in a conformational change which can be stabilized by carboxyl modification of the SWCNTs by acid treatment. Intercalating agents can induce chromosome breakage and instability. The damaging effects of carbon nanotubes may be induced by a variety of mechanisms linked in part to the physical and chemical properties of nanotubes. DNA damage and increases in multinucleated cells have been observed following *in vitro* exposure to SWCNTs. In addition, in some (but not all) studies of SWCNT-exposed cells there is evidence of lactate dehydrogenase leakage from cells as well as depletion of the oxidant protective enzymes (glutathione and superoxide dismutase) indicating reactive oxygen species generation. The generation of reactive oxygen species can damage cell membranes, proteins, and DNA. Oxidant-induced DNA damage has been reported *in vivo* in both mice and rats following exposure to iron-contaminated MWCNTs and SWCNTs.

Genomic instability can result from damage to the DNA or damage to the mitotic spindle

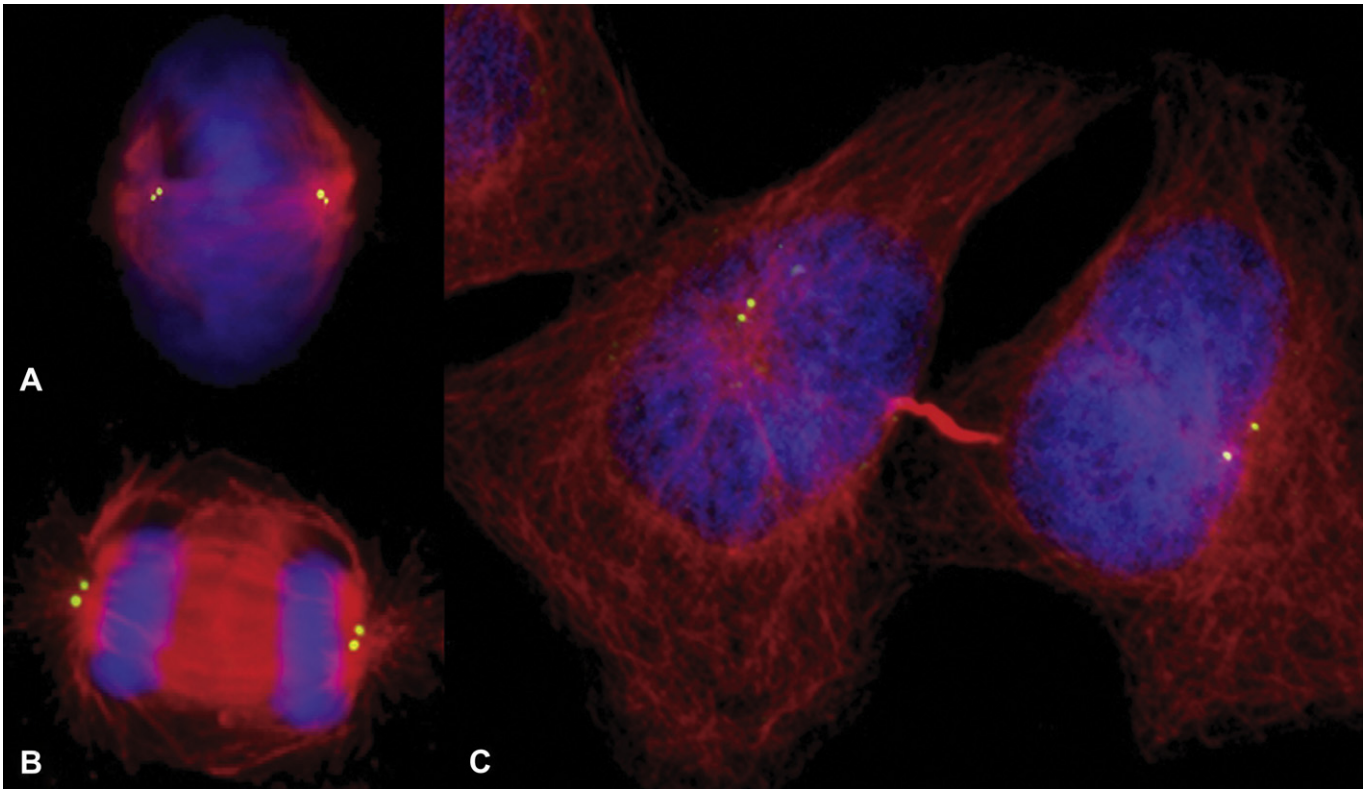


FIGURE 43.10 The figure is a composite of mitotic figures. (A) Cell in metaphase. The duplicated centrosomes have formed two mitotic spindle poles. In (B), the mitotic spindle apparatus has elongated separating the chromosomes. In (C), the cell has progressed through mitosis and a bridge of cytokinesis or mid-body separates the cells. *High-resolution confocal images of dividing cells courtesy of Jeffrey L. Salisbury, Department of Biochemistry and Molecular Biology, Tumor Biology Program, Mayo Clinic, Rochester, MN.*

apparatus. The loss of whole chromosomes has been reported in established cancer cell lines, indicating a disruption of the mitotic spindle. Exposures of rodents to MWCNTs have demonstrated micronuclei in primary mouse Type II epithelial cells 3 days following intratracheal administration of 1 mg/kg. Micronuclei indicate either a high level of chromosomal breakage or mitotic spindle disruption. Two recent *in vitro* investigations have shown dramatic errors in chromosome number after treatment of primary small-airway epithelial cells and immortalized bronchial epithelial cells with 0.024- to 96-μg/cm$^2$ SWCNTs. As indicated previously, the chromosome errors were attributed to disruption of the mitotic spindle. The SWCNTs were observed within the nucleus, in the DNA, in association with cellular and mitotic tubulin, and in the bridge separating dividing daughter cells (mid-body). The association of the nanotubes disrupted the normal mitotic process (see Figure 43.11).

## 4.3. Additional Cytopathologic Interactions

The findings noted above demonstrate that some NPs cause important alterations in cytoplasmic membranes and the mitotic spindle. These are among the best-described cytopathologic effects of NP exposure. However, the size range of NPs (1–100 nm) is similar to that of many subcellular organelles and structures, making interactions with any of these a potential concern. As a general group, NPs can have an almost infinite spectrum of one or more chemical constituents, so the designation of something as a NP only refers to a size range – not its chemical composition.

There is similar diversity in the intracellular environments that may interact with NPs. The *in vivo* environment contains an enormous spectrum of different cell types. The diversity of those cells, their ability to internalize NPs, their ability to respond to injury, and their ability to

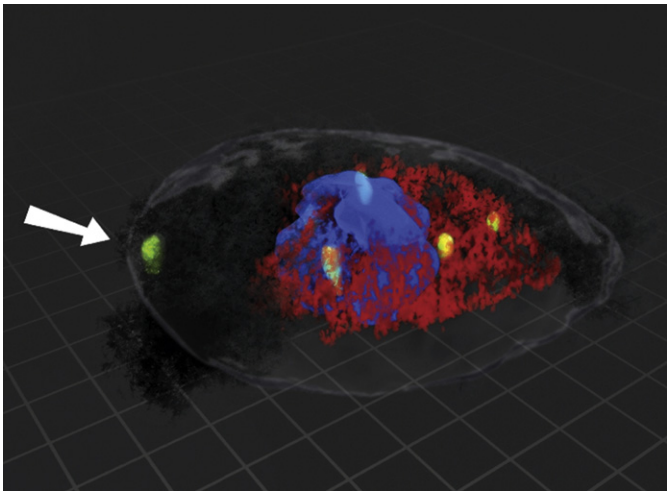

FIGURE 43.11 Three-dimensional reconstruction of a mitotic spindle with three mitotic spindle poles (tripolar mitosis). The mitosis was isolated from a cell exposed to SWCNTs for 24 hours. The DNA was detected with DAPI and is blue. The tubulin and centrosomes were detected using immunohistochemical methods. The tubulin is red and the centrosomes are green. Differential interference contrast imaging images the nanotubes. The nanotubes block the light and produce a black image. The cell was imaged using confocal microscopy. Serial optical sections of 0.1 μm in depth were used to construct a three-dimensional reconstructed image of the tripolar mitosis. The reconstructed image shows nanotubes inside the cell in association with each centrosome fragment. The white arrow indicates association with one of the centrosome fragments. Nanotubes are also integrated with the microtubules and the DNA.

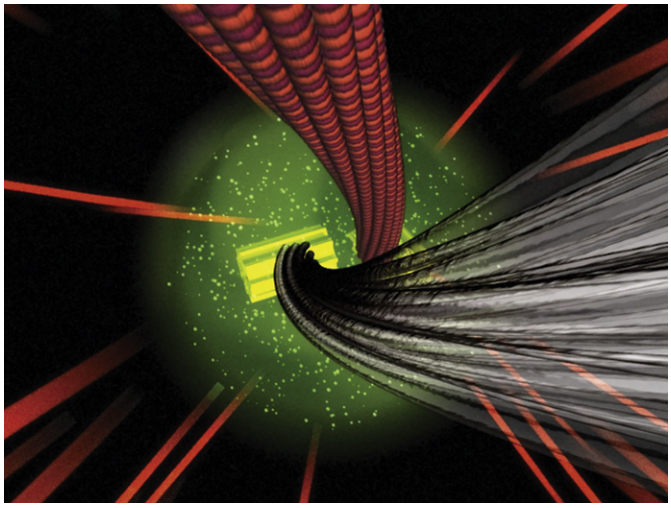

FIGURE 43.12 The drawing demonstrates a proposed model for the interaction of carbon nanotubes with subcellular structures. The carbon nanotubes in this drawing are attached to the centrosome and displace microtubules at the centrosome.

metabolize or detoxify NPs and their products must each be considered in evaluating the cytopathology of NPs. NPs can be modified to permit entry into specific tissues to target drug delivery, with the classic example of that kind of targeting being crossing the blood–brain barrier. Some NPs, particularly cationic NPs, that can cross the blood–brain barrier can increase the permeability of the barrier. Obviously, cytopathologic effects in the blood–brain barrier are a concern, and these are discussed in more detail later in this chapter in Section 5.2 on the neurotoxicity/neuropathology of NPs.

Another concern is that exogenous chemicals in the body are usually metabolized into compounds that are more easily eliminated. This process, known as xenobiotic metabolism, uses enzymes. Many of these enzymes come in a number of different forms, known as isoforms. The different isoforms have different capabilities for metabolizing different compounds. The foreign compounds, which are often drugs, are the substrate for the enzymatic reactions. Exposure to substrates can markedly increase (induce) some types of xenobiotic metabolism. The products of xenobiotic metabolism are sometimes less toxic and sometimes more toxic than the substrate itself. That depends upon the substrate and the metabolic pathway.

Cytochrome P450 is essential to many xenobiotic metabolizing reactions. There are numerous isoforms of cytochrome P450, and each tends to metabolize different substrates. The expression of the different cytochrome P450 isoforms is tissue-specific, and within tissues can be highly cell-specific. In addition, expression of some cytochrome P450 isoforms is affected by particle exposure, inflammation, and/or disruption of microtubules. In the brain the cytochrome P450s are often regulated differently than in other tissues, and some of the brain, cytochrome P450s have a role in regulating critical endogenous hormones and signaling molecules. Within cells, the cytochrome P450s tend to be expressed in specific intracellular sites such the endoplasmic reticulum, mitochondrial membranes, and the cytoplasmic membrane.

Cell-specific and organelle-specific NP-targeted drug delivery may deliver drugs to cells

and to subcellular structures that have not previously metabolized the drug. The xenobiotic metabolizing systems of the target sites may be unable to metabolize the drug, may produce unanticipated metabolites, or may be induced to greater activity. Thus, it is very important that the cytopathologic effects of NPs be evaluated with an understanding that NP-mediated passage of drugs to tissues such as the brain or subcellular structures that are not traditionally exposed to those substances can potentially alter xenobiotic metabolizing pathways. In addition, drugs that are easily detoxified by cells of traditional exposure portals in the liver and lung may not necessarily be detoxified in the brain. Xenobiotic metabolizing pathways may also be affected by side effects of NP exposure, such as inflammation.

A major technical challenge is localizing NPs within subcellular compartments. This technical challenge affects the evaluation of NP cytopathology in many different cell types. In evaluating NP-induced cytopathologic damage in tissue sections, it is extremely important to recognize the limitations of techniques that detect subcellular distribution of NPs. Thus, co-localizing a fluorescent NP with a mitochondrial marker could mean that the NP is in the mitochondria or it could mean that it is located on the surface of the mitochondria.

NPs enter cells in several potentially different ways that are affected by the normal function of the cells. Importantly, NPs can use pathways for entering the cell and trafficking mediators within the cell that use endocytic pathways which are not believed to operate for larger particulates. However, some NPs can use pathways that are used for micron-sized particulates. For example, the phagocytic cells that respond to micron-sized particles can sometimes recognize NPs. As mentioned earlier in this chapter, when recognized by a phagocytic cell, the NPs are first engulfed into a phagosome, which then fuses with a lysosome to form a phagolysosome – a system generally designed to degrade potential pathogens. The phagolysosome is an acidic environment with abundant enzymes that digest pathogens and other complex biological material. Within the phagolysosome, the fate of the NP is determined by the composition of the NP, which may or may not be digested. In some phagocytic cells, the digested contents of the phagolysosome may translocate to the cell surface and be presented to the immune system. Therefore, the potential to appropriately or inappropriately stimulate an immune response is a consideration when NPs are recognized by phagocytic cells. NPs that cannot be digested in the phagolysosome can also adversely affect some cells.

Some phagocytized NPs may be cytotoxic. For example, unmodified and negatively charged polystyrene particles are much less inflammatory in the lung than positively charged (cationic) polystyrene NPs. Polystyrene NPs with surface $NH_2$ molecules and a positive charge have been used as a model of cationic NPs in phagocytic cells. When labeled with FITC to create green fluorescence that allows intracellular tracking, the cationic NPs are seen first in the phagolysosome and are then released into the cytosol due to lysosomal rupture, which is believed to be mediated by pumping of chloride ions. As with incomplete phagocytosis, lysosomal rupture following phagocytosis of cationic NPs is associated with cytotoxicity.

NPs can also enter cells by pinocytosis or macropinocytosis, and can utilize endocytosis pathways that are distinct from phagocytosis and are particularly effective for intracellular delivery of NPs. Endocytic pathways exist in all cell types, including neurons. Macropinocytosis creates relatively large endocytic vacuoles, incorporates extracellular material into the cell, and can internalize particulates that are less than a micron in diameter. The endocytic processes which function to transport particles in the 100-nm or smaller size range can be highly selective. They provide a pathway to enter cells that are unlikely to be targets of larger particles. In addition, the intracellular pathways can be different when a NP enters a cell by pinocytosis than when the NP enters the cell by phagocytosis. Trafficking for endolysosomal degradation is only one of many potential fates for the cargo of endosomes.

Endocytosis that principally operates in the nanoscale can broadly be divided into three groups: (1) caveolin-mediated endocytosis in caveolae; (2) clathrin-mediated endocytosis; and (3) endocytosis through processes that are independent of these mediators. Entry into the cell through these endocytic pathways plays a critical role in cytopathology, and is dependent upon the cell type and the NP composition. Caveolae were mentioned earlier in this chapter for their role in

shuttling NPs and proteins across the endothelium. However, caveolae also play in important role in endocytosis with delivery into the cell. Thus, cationic NP entry and cytotoxicity in a bronchial epithelial cell line is dependent upon caveolae. Similarly, cerium oxide NP uptake by keratinocytes is dependent upon caveolae. In the previous example of cytotoxicity of cationic polystyrene NPs, the cationic NP cytotoxicity is dependent upon the pathway of internalization, and is higher in cells with caveolin-mediated or phagocytic pathways of NP internalization than in cells without those pathways.

Clathrin-mediated endocytosis involves binding of molecules on the surface of the transported protein or NP (the cargo) to cellular receptors. These receptors are sequestered by adapter proteins that interact with clathrin at the cell membrane to form a clathrin-coated pit. These endocytic adapter proteins play a critical role in determining whether a given cell will endocytose a NP or protein on the cell membrane, and the number of described endocytic adapters is rapidly increasing. The clathrin-coated pit matures and eventually forms an intracellular vesicle. The clathrin-coated vesicle then delivers the cargo into the cell, usually through fusion with early endosomes. The cargo is then sorted within the endosomal network and trafficked to designated sites.

Clathrin- and caveolin-independent endocytosis (CIE) is the least understood form of pinocytosis. Recent studies suggest that there are multiple types of CIE. At least one of these types of CIE is the major endocytosis pathway in migrating fibroblasts. In neural cells, CEI has also recently been shown to be the method for endocytosis of polyethylenimine (PEI)-decorated polymer nanospheres designed for nanomedical applications. PEI is used as a cationic NP carrier of gene therapy and, like the cationic NPs mentioned earlier, causes swelling of the enclosing membrane-bound structure. In the case of an endosome carrying PEI–DNA vectors, this leads to rupture, release into the cytosol. and improved gene transfection, but also cytotoxicity.

NPs can enter the nucleus of a cell through passive diffusion or active pathways. Very small NPs less than the diameter of the pores in the nuclear membrane can diffuse into the nucleus if located free in the cytosol. However, the nuclear membrane dissolves during cytokinesis, allowing nuclear access for larger NPs that are free in the cytosol. An example of an active pathway is the use of nucleolin to transport NPs. Nucleolin is a shuttling protein, microtubule-dependent, and able to transport DNA NPs, hyperbranched polylysine NPs, and F3-peptide conjugated NPs from the cell membrane or cytoplasm into the nucleus.

Since NPs can be similar in size to subcellular organelles, or even smaller, interactions with subcellular structures can also be produced intentionally rather than as a toxic side-effect. Thus, surface functionalized NPs can be conjugated with antibodies and transfected into living cells for fluorescent visualization of cellular organelles such as the mitochondria. As mentioned earlier, important new NPs include imaging agents, pharmaceuticals, and transporters of gene therapy. Understanding of the routes used to enter and exit cells is critical to targeting these new products.

## 5. ORGAN AND TISSUE TARGETS

Less than a decade ago, the scientific community realized that nanoscale manipulation of matter could produce products with adverse health effects not anticipated for larger particles of similar chemical composition. The earliest *in vivo* nanotoxicology studies included studies demonstrating the neurotoxic effects of buckyballs in fish and pulmonary inflammation and fibrosis in mice aspirating single-walled carbon nanotubes. The lung and the brain are among the best investigated of the target tissues for nanotoxicology studies today. Inhaled respirable particles have long been associated with increased cardiovascular mortality, and studies of NP effects on the cardiovascular system are providing insights into the cardiovascular effects of the smallest particles. The lymphatics are also increasingly recognized both as a means for transporting NPs and as a potential target tissue.

### 5.1. Pulmonary Pathology

Because many NPs are easily aerosolized, the lung is potentially exposed to NPs through inhalation. While particles 1–5 μm in diameter are classically thought to have optimal alveolar

deposition, recent inhalation studies clearly demonstrate significant pulmonary deposition of NPs. In addition, particles less than 1 μm in diameter are more likely to cross epithelial and endothelial barriers. Clearly, this is important in occupational and environmental toxicology. In the pharmaceutical industry, the inhalation of NPs is a highly effective route for drug delivery. The lung can also be exposed to NPs that are not inhaled but are instead delivered via the vasculature, and surface modification of NPs can affect lung delivery. Surface modification of NPs is one strategy used to alter toxicity, increase transport across cell barriers, and prolong the half-life of nanopharmaceuticals. Coating NPs with poly(-ethylene glycol) is a common modification for pharmaceuticals because it decreases uptake by phagocytes and increases half-life in circulation. However, a recent study of mesoporous silica NP biodistribution demonstrates increased lung deposition in NPs coated with poly(ethylene glycol). Thus, the lung is an important potential target for evaluation by toxicologic pathologists in the pharmaceutical industry, as well as those concerned with environmental and occupational exposures.

*Hypertrophy and hyperplasia* of the bronchiolar epithelium can be observed following inhalation or aspiration of SWCNTs or MWCNTs. Hypertrophy and hyperplasia were also observed by histopathologic evaluation of bronchi and bronchioles in high-dose (50 $mg/m^3$) rats after short-term inhalation of $TiO_2$ with a mean primary diameter of 25.1 nm. However, labeling of dividing cells with BrdU provides a directly quantitative measure of cell proliferation which identified hyperplasia in bronchi and bronchioles of rats inhaling much lower doses (2 $mg/m^3$) of nanoscale $TiO_2$. Thus, histopathology plays an essential role in identifying tissue responses to NPs, but cellular labeling may be more sensitive and a better quantitative measure for cellular proliferation than histopathology.

*Inflammation* with rapid development of fibrosis was a sentinel finding in one of the earliest studies of the pulmonary toxicity of NPs, a study of aspirated SWCNTs in the mouse lung. Inflammation is frequently described following acute or subchronic inhalation exposure to several different NPs, including metallic gold, ZnO, $TiO_2$, SWCNTs, and MWCNTs. However, different mechanisms may trigger inflammation with different NPs. Thus, nano $TiO_2$ and nano $SiO_2$, but not nano ZnO, trigger IL-1α and IL-1β release from macrophages in an Nlrp3 inflammasome-dependent process. In addition, an acute inhalation study using amorphous silica nanoparticles (exposures as high as 86 $mg/m^3$, 6 h/day for up to 3 days) did not detect significant pulmonary inflammation, suggesting that not all insoluble nanoparticles cause acute inflammation.

*Fibrosis* can develop rapidly in the alveolar septa and within granulomas following a single exposure to aspirated SWCNTs (10 μg) or MWCNTs (80 μg). The apparently greater fibrogenicity of dispersed SWCNTs versus MWCNTs may be due to the higher interstitial dose of SWCNTs. Pulmonary fibrosis was a component of the lung disease seen in young women exposed to nanoparticulate polyacrylate. In addition, fibrosis was observed in lung biopsies from responders to the World Trade Center events of September 2011 who subsequently developed severe interstitial lung disease. Although the exposures of World Trade Center responders were clearly complex, SWCNTs were observed in the biopsies from three of the four patients with the most severe interstitial disease and are presumed to have been produced as a combustion product.

*Pleural penetration* has been described in the lungs of mice exposed to MWCNTs by aspiration. This is a concern because MWCNTs have a high aspect ratio, carcinogenic asbestos fibers also have a high aspect ratio, and mesothelial cells lining the pleura are the target cells for the development of mesothelioma.

*Lymphatic dilation* can also occur in the pulmonary lymphatics following nanoparticle exposure. Lymphatic toxicity and the role of the lymphatics in translocation of NPs are discussed in Section 5.4, on lymphatic pathology.

## 5.2. Neurotoxicity/Neuropathology of Nanoparticulates

Interest in the neurotoxicity of NPs arises from studies that demonstrated extrapulmonary translocation and deposition of ultrafine particles in the brain. Subsequent studies demonstrated ultrastructural nasal pathology in children exposed to air pollutants; DNA damage, neuroinflammation, and neurodegeneration in

nasal and brain tissues of canines exposed to air pollutants; and chronic brain inflammation, blood–brain barrier disruption, and Alzheimer's disease (AD)-like pathological changes in humans exposed to fine and ultrafine particulate matter from air pollution. High levels of cyclooxygenase 2 (COX2) mRNA, an index of inflammation, were detected in the frontal cortex and hippocampus areas of autopsied brains from human subjects who had prolonged exposure to severe air pollution. Concurrent accumulation of β-amyloid was also observed in these brain regions. Increased production of β-amyloid is thought to precede the formation of plaques and tangles, and is known to cause microglial activation and neuroinflammation in the brain. Elevated β-amyloid has also been linked to cognitive decline. Based on the findings of Calderon-Garciduenas and colleagues, it is likely that inhalation of fine and/or ultrafine particulates present in the polluted air may be the underpinning for such abnormal AD-like pathology. As extracellular deposition of aggregated β-amyloid into senile plaques and intracellular accumulation of hyper-phosphorylated aggregated Tau as neurofibrillary tangles, respectively, are pathological hallmarks of AD, there is emerging concern that similar exposures to NPs might potentially cause neurotoxicity and neurodegeneration-like changes. In the workplace environment, workers may be at risk for exposure via inhalation of fine and/or ultrafine NPs, either incidental or engineered. Ingestion or dermal penetration may also be potential routes of exposure, albeit to a lesser extent. Moreover, injection (systemic administration) of NP-based drug delivery vectors, an area of intense research in drug delivery, may result in distribution of such NPs into specific brain targets. However, exposure to NP-based vectors may be limited to select populations, compared to what individuals may be exposed to via inhalation at the workplace. Unfortunately, at this time, there is limited understanding of the neurotoxicological potential of exposure to NPs, particularly engineered nanomaterials.

Inhalation of NPs can result in their deposition in olfactory and/or pulmonary regions. Translocation of NPs to the brain (Figure 43.13), either directly from the olfactory regions or indirectly via the systemic circulation, can potentially cause damage to the brain. The limited

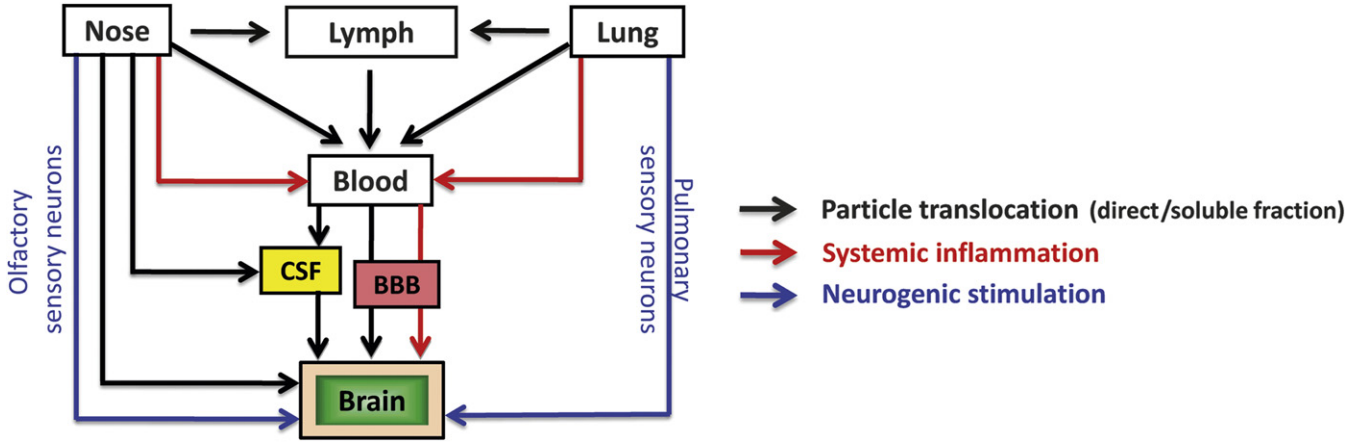


FIGURE 43.13 Potential routes of NP translocation to the brain or NP-mediated stimulation of the brain. Direct translocation of NPs can occur via retrograde transport through olfactory or pulmonary sensory neurons. Indirect translocation of NPs deposited in olfactory or pulmonary targets can occur via systemic circulation of the particle *per se* or of its soluble fraction (likely in the case of metal-based NPs). In the absence of particle translocation, either through direct or indirect mechanisms as described above, inflammatory mediators released into systemic circulation from other affected organs (primarily lung) can cause disruption of the blood–brain barrier and activation of glial cells and/or neuronal population in discrete brain areas. Besides, perturbation of the olfactory or pulmonary sensory neurons can itself suffice to elicit neurogenic inflammation and subsequent activation of specific brain areas. These may include neuronal populations associated with or controlling olfactory or respiratory function, and which are in the synaptic pathway of the sensory neurons. Repeated or sustained activation of such pathways may eventually contribute to neuronal dysfunction and glial activation.

regenerative capability of the nervous system, its cellular heterogeneity, and high lipid content may further render it vulnerable to toxic insults.

The olfactory sensory neurons occupy nearly two-thirds of the sensory or olfactory epithelium. Because of their widespread distribution in the olfactory epithelia, they experience direct access to odorant molecules, as well as allergens, airborne pollutants, toxic chemicals, and microorganisms. Ultrafine NPs inhaled through the nose and air passages have been shown to translocate to the brain via transport across olfactory neurons, and accumulate in deeper brain areas (Table 43.1). Following pulmonary deposition, ultrafine particles can permeate the lung–blood barrier by endocytosis, transcytosis, or stochastic transport and enter the systemic circulation. NPs deposited in the lungs have been shown to translocate to the circulatory system and eventually to organs like lung, liver, kidneys, and brain. Translocation mechanisms that have been demonstrated include the rapid diffusion of $^{99m}$Technetium-labeled colloidal albumin NPs from lung into systemic circulation, and passage of ultrafine carbon black across the air–blood barrier through large gaps formed between the cytoplasmic processes of alveolar epithelial cells. These findings indicate that translocation of NPs from the lung into the systemic circulation and potentially into the brain is possible. When administered orally, water-miscible $^{14}$C-fullerene has been shown to penetrate the blood–brain barrier. Similarly, oral administration of ultrafine and fine titanium dioxide to mice caused hippocampal neuronal damage. It has also been shown that systemic (intraperitoneal) administration of 50-nm silica-overcoated magnetic NPs results in migration of these particles across the blood–brain barrier and subsequent deposition in the brain. Intravenous administration of gold NPs (10–50 nm) to mice or rats resulted in biodistribution of these particles in the brain. Regardless of the route of exposure, once in systemic circulation, NPs may interact with the blood–brain barrier or can translocate to specific neuronal or glial cell populations and elicit a series of intracellular events, including neuroinflammation, oxidative stress, mitochondrial dysfunction, glial activation, and disruption of neuronal function.

On the other hand, in the absence of translocation of NPs to the brain, the release of inflammatory mediators into systemic circulation due to pulmonary exposure may suffice to stimulate blood–brain barrier changes and elicit neurotoxicity (Figure 43.13). Additionally, stimulation of olfactory or pulmonary sensory neurons may likely cause perturbations in specific brain nuclei that can elicit neuroinflammation and abnormal neural changes (Figure 43.13). Indeed, olfactory signals have been shown to be relayed from the olfactory bulb to olfactory cortical areas (including piriform cortex) to the entorhinal cortex, which is a major afferent to the hippocampal dentate gyrus. The close anatomical association between the olfactory and hippocampal regions, and the critical role played by the hippocampus in odor memory, suggests that stimulation and/or disruption of olfactory sensory neurons may contribute, at least in part, to abnormal hippocampal function. Regardless of how NPs stimulate neuronal responses (directly by translocation, or indirectly via systemic or neurogenic effects), the nervous system appears to be a potential target for NP toxicity. Finally, a variety of underlying health conditions (e.g. hypertension, diabetes, infection) can predispose an individual to NP exposure by altering the permeability of the blood–brain barrier and thus compromising its normal functioning. Such a breach in the blood–brain barrier will facilitate rapid translocation of these particles or inflammatory mediators into the brain, thereby increasing the risk of a neurotoxic exposure. In the light of these observations, it is critical to evaluate the effects of NP exposure on blood–brain barrier integrity and CNS function.

Translocation of NPs can be highly influenced by their physicochemical characteristics. Particle size, shape, and surface chemistry may further influence their retrograde translocation across olfactory sensory neurons, transport across the lung–blood barrier, or circulation in blood. Gold NPs of 10–50 nm in diameter accumulated in the brain following intravenous administration; however, administration of larger (100- to 250-nm) gold NPs did not result in biodistribution to the brain. Similarly, subcutaneous injections of silver NPs resulted in their translocation to brain, whereas silver microparticles failed to reach the brain. These findings suggest that particle size is a critical factor for brain delivery of NPs. Besides particle size, the surface

**TABLE 43.1** Evidence for Translocation of NPs to the Brain

| NP | NP size | Animal model | Exposure route | Exposure dose | Experimental observations | Reference (Suggested reading) |
|---|---|---|---|---|---|---|
| $^{13}$C-NP (from graphite rods) | 36 nm; GSD = 1.66 | Rat | Whole-body inhalation | 160 μg/m$^3$ for 6 h | Persistent increases in olfactory bulb; transient increases in cerebrum and cerebellum | Oberdorster *et al.* 2004 |
| Ag NP | 52–70 nm (average 60 nm) | Rats | Oral | 30, 300, 1000 mg/kg daily for 28 d | Dose-dependent increase of elemental Ag in brain | Kim *et al.*, 2008 |
| Cu NP | 23.5 nm | Mice | Intranasal instillation | 1, 10, 40 mg/kg body weight, alternate days for 14 and 20 d | Dose-dependent increase of elemental Cu in olfactory bulb | Zhang *et al.*, 2011 |
| Fluorescent magnetic-NP | 50 nm | Mice | Nose-only inhalation | *Low dose*: ~4.9 × 10$^5$ particles/cm$^3$ *High dose*: ~9.3 × 10$^5$ particles/cm$^3$, 4 h/d, 5 d/wk for 4 wks | Brain MRI revealed $T_2$-weighted spin-echo suggestive of particle translocation | Kwon *et al.*, 2008 |
| Magnetic (Co-Fe)-NP ($SiO_2$-RITC coated) | 50 nm | Mice | Intraperitoneal administration | 25, 50, 100 mg/kg body weight, single dose | Increased RITC fluorescence in brain suggestive of translocation across blood–brain barrier and accumulation in brain parenchyma | Kim *et al.*, 2006 |
| MnO | 30 nm | Rat | Whole-body inhalation | 18 × 10$^6$ particles/cm$^3$ (~500 μg/m$^3$), 6 h/d, 5 d/wk for 12 d | Increased Mn distribution in olfactory bulb, striatum, frontal cortex, and cerebellum | Elder *et al.*, 2006 |
| PbO | 20 nm | Rat | Intratracheal instillation | 2, 4 mg/kg body weight, 5 d/wk for 3 or 6 wks | Increased elemental Pb content of whole brain | Oszlanczi *et al.*, 2011 |
| Quantum dots | 21 nm | Mice | Intravenous (tail vein) | 5 nmol/mouse, single dose | Accumulation in whole brain, but in low amounts | Z. Chen *et al.*, 2008 |
| $TiO_2$ | 25 nm, 80 nm | Mice | Gastrointestinal administration | 5 g/kg body weight, single dose | Small increase in Ti content of whole brain | J. Wang *et al.*, 2007 |
| $TiO_2$ (rutile) | 80 nm | Mice | Intranasal instillation | ~500 μg/mouse, alternate days for 2, 10, 20, 30 d | Increased Ti levels in olfactory bulb and hippocampus | J. Wang *et al.*, 2008 |
| $TiO_2$ | 5 nm | Mice | Abdominal injection | Varying doses, daily for 14 d | Small increase in Ti content of whole brain | Ma *et al.*, 2010 |

chemistry of the NP can also influence its translocation and/or permeability across the blood–brain barrier. Cationic and anionic emulsified wax NPs have been shown to be taken up into the brain more efficiently than neutral NPs. Further, cationic emulsified wax NPs have been shown to disrupt blood–brain barrier and exert toxicity. Thus, understanding the toxicological influence of the physicochemical aspects of NPs may have significant impact on nanomaterial development considering their projected huge market potential in biomedicine, particularly for CNS drug delivery, diagnostics and therapeutics.

While there is tremendous enthusiasm for developing and manufacturing metal or metal oxide-based NPs like gold, silver, copper, and titanium dioxide for various industrial or biomedical uses, evaluation of their toxicological potential is necessary to avert any adverse health effects. To this end, investigations on the toxicological potential of NP have gradually garnered active research interest (Table 43.2). Neuronal damage can be mediated by several mechanisms, including inflammation, mitochondrial dysfunction, oxidative damage, and excitotoxicity. Microglia, the macrophages of the brain, play a crucial role in brain inflammatory responses. Pro-inflammatory cytokines and chemokines elicited by microglia can initiate mitochondrial impairment and oxidative stress. The presence of high levels of polyunsaturated fatty acids in the brain renders this organ vulnerable to reactive oxygen and nitrogen radical attack, due to the presence of double bonds within the membrane. In addition, the presence of high levels of iron in the brain and its accumulation in specific areas following neuronal injury, suggests loss of homeostatic mechanisms responsible for its regulation. As a result, the brain becomes susceptible to oxidative stress. Thus, neuroinflammatory and oxidative stress responses following NP exposure may serve as the basis for neurotoxicity. Both neuroinflammation and oxidative stress can cause mitochondrial dysfunction and elicit neuronal injury (Figure 43.14).

Indeed, a few studies have investigated the neurotoxic effects of carbon-based NPs, indicating the involvement of neuroinflammatory and oxidative stress events. Intranasal instillation of ultrafine carbon black induced the expression of pro-inflammatory cytokines and chemokines in the olfactory bulb. When administered orally, water-miscible $^{14}C_{60}$-fullerene has been shown to penetrate the blood–brain barrier. $C_{60}$-fullerenes have also been shown to induce oxidative stress in the brains of largemouth bass. These findings suggest that NPs can pose a risk for targets sensitive to oxidative stress, like the brain, due to its high iron and lipid content. Thus, carbon-based nanomaterials can pose significant neurological risk following exposure. Indeed, we have observed brain-region specific mRNA expression of several pro-inflammatory chemokines, cytokines, selectins, and markers of cellular stress in mice exposed to a single dose of MWCNTs (10–80 μg) by pharyngeal aspiration. Further, MWCNTs decreased the expression of certain blood–brain barrier-related markers, suggestive of altered blood–brain barrier integrity.

Intraperitoneal administration of 25-nm Ag NPs caused alterations in the expression of genes associated with oxidative stress in specific brain areas that resulted in apoptosis and neurotoxicity. Subcutaneous administration of Ag NPs was shown to cause blood–brain barrier changes, neuronal injury and astrocytic swelling. Ag and Cu NPs (50–60 nm) administered via intravenous, intraperitoneal, or intracerebral routes caused disruption of blood–brain barrier function and induced brain edema in rats. Inhalation of ultrafine manganese oxide resulted in translocation of these particles to the CNS. Nano-sized $TiO_2$ (Degussa P25™) has been shown to trigger oxidative stress response and interfere with mitochondrial function in brain BV2 microglial cells. Administration of 80-nm $TiO_2$ NPs to mice produced subtle brain lesions and vacuolation in hippocampal neurons. Neuroinflammation in discrete brain areas was observed 24 hours following pharyngeal aspiration of titanium dioxide nanowires but not titanium dioxide nanospheres.

Although studies on the neurotoxic potential of NPs are limited, there is sufficient evidence to suggest that the brain is a vulnerable target for NPs. Therefore, it warrants more extensive and detailed investigations to determine if persistent adverse brain changes, reminiscent of neurodegenerative disorders, can result from chronic inhalation exposures to NPs. Such efforts are critical for neurological risk assessment of NPs and for determining the safety efficacy of NPs generated either at the workplace or, following attrition, into the environment. Many

**TABLE 43.2** NP-Mediated Neurotoxicity/Neuropathology

| NP | NP size | Animal model | Exposure route | Neurotoxic or neuropathological outcome | Reference (Suggested reading) |
|---|---|---|---|---|---|
| Ag NPs | 25 nm | Mice | Intraperitoneal | Oxidative stress in cortex, hippocampus and striatum | Rahman *et al.*, 2009 |
| Ag NPs | 50–60 nm | Mice | Intravenous, Intraperitoneal | Decreased cerebral blood flow, blood–brain barrier leakage and edema; loss of myelinated fibers; glial activation. | Sharma *et al.*, 2009 |
| Ag NPs | 50–60 nm | Mice | Intravenous, Intraperitoneal | Blood–brain barrier disruption and edema in frontal cortex and cerebellum | Sharma *et al.*, 2010 |
| Cu NPs | 50–60 nm | Mice | Intravenous, Intraperitoneal | Blood–brain barrier disruption and edema in frontal cortex and cerebellum | Sharma and Sharma, 2007 |
| MnO | 30 nm | Rat | Whole-body inhalation | Robust neuroinflammation in olfactory bulb; subtle neuroinflammation in striatum, frontal cortex and cerebellum; astrogliosis in olfactory bulb | Elder *et al.*, 2006 |
| PbO | 20 nm | Rat | Intratracheal instillation | Altered neurobehavioral and neurophysiological function; increased horizontal motor activity, but decreased vertical motor activity; increased somatosensory cortical evoked potential latency | Oszlanczi *et al.*, 2011 |
| $TiO_2$ | 25 nm, 80 nm | Mice | Gastrointestinal administration | Brain lesions; vacuolation and fatty degeneration in hippocampal neurons | Wang *et al.*, 2007 |
| $TiO_2$ | 5 nm | Mice | Abdominal injection | Oxidative stress and lipid peroxidation | Ma *et al.*, 2010 |
| $TiO_2$ (rutile) | 80 nm | Mice | Intranasal instillation | Oxidative stress and lipid peroxidation in olfactory bulb and hippocampus; increased neuronal number and irregular arrangement of neurons in olfactory nerve layer; enlarged and elongated pyramidal cell soma, and decreased Nissl bodies in CA1 area of hippocampus | Wang *et al.*, 2008 |
| Ultrafine Carbon Black | 14 nm | Mice | Intranasal | Inflammation in olfactory bulb | Tin Tin Win *et al.*, 2006 |

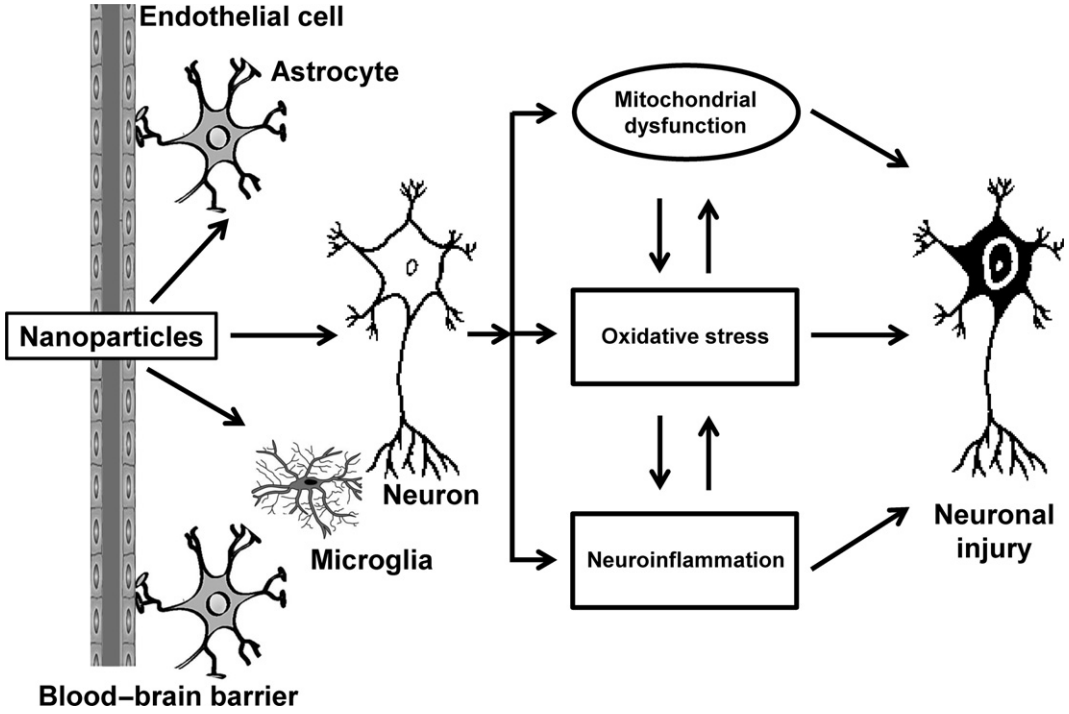


FIGURE 43.14 Potential mechanisms of NP-mediated neuronal injury. Direct translocation of NPs into neural cells (neurons, astrocytes or microglia) can elicit oxidative stress, neuroinflammation, or mitochondrial dysfunction. Indirectly, inflammatory mediators released into systemic circulation can potentially activate neural cells. Particularly, activation of microglia, the macrophages of the brain, can result in inflammatory and oxidative burst that can contribute to neuronal injury and astrogliosis.

of the existing studies on NP neurotoxicity are summarized above, and these studies are listed in the Further Reading section.

## 5.3. Cardiovascular Pathology

Inhalation of nano $TiO_2$ results in significant inhibition of the ability of systemic or coronary arterioles to respond to dilators. This microvascular dysfunction has been associated with generation of reactive oxygen species and the scavenging of dilator-induced endothelial nitric oxide. This cardiovascular response to pulmonary exposure to particles appears mediated in part by potentiation of blood neutrophils, adherence to microvessel walls, and release of reactive oxygen species. In addition, a neurogenic mechanism, involving stimulation of pulmonary sensory neurons by particle exposure and resultant activation of sympathetic input to microvessels, appears to be involved.

Furthermore, inhalation of MWCNTs ($26\,mg/m^3$ for 5 h; calculated lung burden of 22 μg) resulted in a depression of the responsiveness of coronary arterioles to dilators 24 h post-exposure. This MWCNT-induced microvascular dysfunction may involve a neurogenic mechanism, similar to that described above with nano $TiO_2$, since pulmonary exposure to CNT increases baroreflex function by two-fold. In addition, multiple aspirations of SWCNTs (20 μg/mouse every 2 weeks, for 2 months) in Apo E −/− mice caused an increase in aortic plaques.

## 5.4. Lymphatic Pathology

Lymphatics are difficult to see in standard histopathologic sections. In conducting NP studies, it is important to remember that the lymphatic vasculature is a circulatory system which plays a key role in fluid homeostasis, particle clearance, cellular transport, metastasis, and the immune system. The lymphatics are increasingly recognized as being dynamic structures with unique molecular signaling essential for their role in maintaining interstitial and blood capillary homeostasis. The endothelium of the

lymphatic capillaries plays an active role in movement of cells into the lymphatics. Physically, the lymphatic endothelium is attenuated, the intercellular spaces between lymphatic endothelial cells are sufficient for movement of cells, and the basement membrane of the lymphatic capillaries is discontinuous. These features provide NPs with remarkable access to the lymphatic circulation. In addition, colloidal carbon and ferritin particles have been reported within vesicles in the lymphatic endothelium and alveolar Type I cells. This implies that transcellular transport may occur through the lymphatics and alveolar Type I cells of the lung as well as the vascular endothelium, and does not always require phagocytosis. In addition, instilled carbon and ferritin particles have been observed in the gaps between lymphatic endothelial cells in the pulmonary lymphatics, suggesting intercellular movement of particulates. Once within the lymphatic lumen, the lymphatic contents filter through lymph nodes and eventually empty into the vasculature at the thoracic duct. This means that the lymphatics are a potential route for delivery of NPs and inflammatory mediators to the blood. Recently, lung-deposited NPs less than 30 nm in diameter were demonstrated to first reach the draining lymph nodes and then reach the blood after a time lag not observed with low molecular weight molecules. This suggests that lymphatic drainage into the vasculature may indeed be important in vascular dissemination of some NPs.

Particles can enter the lymphatics when carried by phagocytic cells, and this appears to be the major route for transport of fine particles in the micron size range. Fine particles injected into the footpad or deposited in the alveolar region do not reach the draining lymph node as free particles. Instead, they are phagocytized by macrophages and neutrophils, which can enter the lymphatics and are transported to the draining lymph nodes within phagocytic cells. However, extracellular routes of particle transport may be important with some NPs. Small NPs (20 nm) that are injected into the footpad rapidly translocate to the draining lymph node and can be seen in the subcapsular sinuses and in the antigen-presenting cells of the lymph node, including dendritic cells and plasmacytoid dendritic cells. Recent studies demonstrate that many NPs are rapidly and widely transported through the lymphatics. For lung-deposited NPs, rapid translocation by the lymphatics is highly influenced by surface coatings and charge, but, importantly, is size-limited, with a threshold estimated to be at a functional diameter of between 34 and 48 nm. A previous study of radiolabelled iridium and carbon NPs indicated that 20-nm diameter particles, which are below this threshold, translocated from the lung to secondary target tissues more effectively than 80-nm diameter particles, which are above this threshold. Extracellular lymphatic transport of small NPs is also suggested by recent studies of NPs used in lymphangiography.

This feature of some NPs can help to map the lymphatics and lymph nodes draining critical sites, such as tumors. In addition, chemotherapeutic agents can be adsorbed onto the NP surface, carried into the lymphatics, and from there distributed to the lymph node. This suggests the potential use of NP-conjugated chemotherapeutic agents in therapy targeted to the lymphatics, which play such an important role in metastatic spread of cancer.

One important implication of the tropism of NPs for the lymphatics is that the lymphatics represent a pathway for transport of NPs from an exposed tissue to distant tissues and organs. While this feature can be used for therapeutic purpose in some situations, it is a feature that needs to be considered when toxicologic pathologists evaluate studies. Certainly, the lymph nodes play an enormous role in immune function and receive the contents from the lymphatics. Therefore, lymphatics should be considered a potential route for delivering immunotoxic and antigenic compounds to the immune system. In addition, alterations in the lymphatics themselves can play an important role in disease pathogenesis. Recently, lymphangiectasia (lymphatic dilation) was noted in the lymphatics of mice aspirating MWCNTs. This suggests that the lymphatics may not just transport NPs; they may also be damaged by them. Thus, pathologists need to recognize that the lymphatics are potential targets in NP studies and that this can have potential functional consequences. In addition, the lymphatics need to be considered as a potential route for translocation of NPs, a feature which distinguishes NPs from traditional organic pharmaceuticals. The lymphatics and the lymph nodes are, therefore,

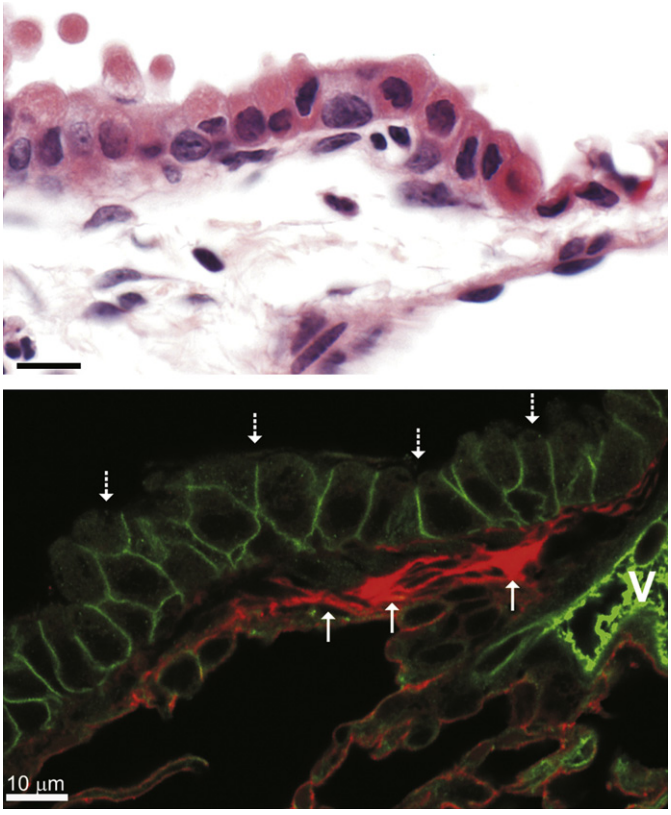


FIGURE 43.15 Lymphatics are difficult to identify in H&E sections (upper panel). In the lower panel, indirect immunofluorescence for podoplanin allows the clear demonstration of peribronchiolar lymphatics in red (solid arrows) while indirect immunofluorescence for e-cadherin permits visualization of the airway epithelium (dashed arrows) so that the tissue location of the lymphatics is also demonstrated. Low level staining for both e-cadherin and podoplanin causes the alveolar type I cells to stain orange. Serum retained in the blood vasculature (V) stains green because the green secondary antibody recognizes IgG in normal mouse serum.

particularly important for pathologists studying NPs. Fortunately, lymphatic endothelial markers can be used to help pathologists visualize the lymphatics in tissue sections (Figure 43.15).

## 6. HUMAN RELEVANCE OF EXPERIMENTAL STUDIES IN ANIMALS

*Dose considerations* are important in designing NP toxicologic pathology studies. For risk assessment purposes, the exposures should include a range of exposures which, at a minimum, includes exposures producing no or little effect, and exposures comparable to those anticipated in the people who receive the highest exposures. Exposures in workers or in patients receiving intentionally administered NPs are potentially quite high relative to exposures received by the general public. For example, in a study of coal miners, the lung-deposited dust burden ranged from 2.6 to 36 g/lung. An additional consideration in toxicologic pathology studies is obtaining a tissue-deposited dose that will allow study of particle interactions with critical target cells *in vivo*. As has previously been noted, a 50-μg dose of 50 nm × 5 μm MWCNTs in a mouse lung would affect less than 1% of a tissue section evaluated by a pathologist. Thus, the highest exposure dose should be high enough to allow detection of critical interactions that may occur in people, such as pleural penetration by high aspect ratio NPs. However, exposures should not be so high as to produce effects that are due to processes that are not relevant to man. For example, pathologic changes associated with exposures exceeding the maximal tolerated dose can be extremely difficult to interpret.

*Species differences* have not been extensively investigated in the existing literature on engineered NPs. However, many ultrafine particulates are NPs that are not products of nanotechnology and species differences in their effects have been investigated. In addition, studies of the effect of fine particulates may be relevant to studies of NPs. In inhalation studies at high exposure concentration, rodents have a tendency to sequester micron-sized particles in alveolar macrophages and develop overload of clearance pathways, inflammation, and an alveolar epithelial proliferative response to inhaled particles. This response is believed to cause some of the lung tumors that are specific to high-dose particle exposures in the rat. Careful interpretation of findings from inhalation studies of high doses of fine particulate matter is important, and tumors produced through chronic inflammation pathways may not be relevant to low-dose exposures that do not cause chronic inflammation. However, lung fibrosis is a response that is seen in both rodents and humans. As mentioned above, workers sometimes develop very high lung burdens of inhaled particulates and can respond with fatal

interstitial fibrosis – a process that is also seen in rodent models.

Existing studies of ultrafine particle inhalation have demonstrated increased interstitial deposition of inhaled NPs relative to fine particles. The interstitial dose of NPs appears to be a major determinant of pulmonary fibrosis. What recent studies of NPs have also provided is an understanding that endocytic pathways of intracellular trafficking and lymphatic transport pathways between tissues are not necessarily the same for NPs as for fine particulates. This suggests that conclusions based upon the effects of fine particulates will not necessarily be true for NPs, although the effects of fine particulates can certainly generate hypotheses regarding the effects of NPs.

The accumulating human data do indeed suggest that at least some inhaled NPs can deposit in the human lung and cause inflammation and fibrosis. A worker who inhaled nickel NPs with an estimated diameter of less than 25 nm died less than 2 weeks after exposure with severe pulmonary inflammation and damage consistent with adult respiratory distress syndrome. Seven young female workers in a print plant exposed to nanoparticulate polyacrylate (~30 nm in diameter) developed pulmonary inflammation and fibrosis with pleural effusions. A worker exposed to nanoscale $TiO_2$ in polyester paint powder developed bronchiolitis obliterans organizing pneumonia. Since some NPs can enter cells through endocytic pathways that exclude micrometer-sized particulates, NPs have increased interstitial deposition relative to micrometer-sized particulates, NPs appear to be transported extracellularly in the lymphatics, and at least some NPs are associated with fatal pulmonary inflammation and fibrosis in workers, these smallest of particulates may not have the same interspecies toxicity differences that have been described for micron-sized particulates in the lung.

## 7. FUTURE TRENDS IN NANOPATHOLOGY AND NANOTOXICOLOGY

For workers, as the body of knowledge in nanopathology and nanotoxicology grows, the prudent implementation of engineering controls (containment, local exhaust ventilation), personal protective equipment (respirators), and administrative controls (training in safe handling practices) is advisable. Current evidence indicates that filtration and ventilation are highly effective in limiting worker exposure.

A current emphasis area in nanotoxicology is the determination of which physiochemical properties influence bioactivity. Such information will drive "safety by design" – i.e., the modification of NPs to reduce toxicity while maintaining novel properties desired in commercial applications. Therefore, the goal is that information obtained by nanopathology and nanotoxicology will lead to the safe production and application of nanomaterials and allow the economic growth of the nanotechnology industry.

Toxicologic pathologists will play an essential role in identifying changes that can occur in the *in vivo* environment and may not necessarily be predictable through existing knowledge of particle toxicology. Such opportunities for discovery have already been demonstrated by the interactions between SWCNTs and the mitotic spindle, and between MWCNTs and the pulmonary lymphatics. Toxicologic pathologists, with their multi-system training, are also critical members of the research teams that will investigate the potential to target nanomedicine to tissues, such as the central nervous system, and to cells, such as tumor stem cells, that are difficult to target with current pharmaceuticals. An essential part of those discoveries and opportunities will be recognition that NPs or their breakdown products can potentially be delivered to cells and subcellular compartments that are not the traditional sites of exposure for xenobiotics. In-depth understanding of the toxicologic pathology of NPs may also reveal mechanisms of toxicity associated with well-known particulate hazards, such as asbestos. Most importantly, toxicologic pathology studies of NPs will be essential to safely harnessing the enormous promise of nanotechnology.

## 8. CONCLUSIONS

Nanotechnology is growing rapidly, and is currently a major economic force. Nanomedicine is also rapidly growing. The first nanomedical products include products with improved

imaging and chemotherapy capabilities compared with previous products. Toxicologic pathologists evaluating the safety of the new products of nanotechnology will need important skills.

One important skill is an ability to interact within a multidisciplinary environment. Interactions with engineers, chemists, physicists, cytogeneticists, toxicologists, pharmacologists, and molecular biologists, and some understanding of the literature in those fields, can be particularly helpful. The nanotechnology and nanomedicine scientific literature is expanding at such a rate that it is a huge challenge to find the studies in those fields which are relevant to toxicologic pathology. In many cases, the papers are written by scientists who have not considered the potential hazard of the NP, and keywords may not help in the literature search. Many publications are outside of the standard medical literature and may not be recovered using common search engines such as PubMed. For that reason, the extensive suggested reading list for this chapter includes the scientific publications supporting statements made in the chapter.

A second important skill is understanding the similarities and differences between the NPs creating adverse effects and those that do not. When size, shape, and composition are considered, a virtually infinite variety of NPs is possible. Increasingly, the products of nanotechnology will include designer NPs, able to inform our understanding of medicine, disease pathogenesis, and particle toxicology. Toxicologic pathologists will play a critical role in identifying NP features that predict toxicity or safety.

Another important skill is the ability to follow the pathway of the NP, even if it has gone where it cannot be seen in a standard H&E slide at a standard magnification. In this chapter we have provided examples of some of the tools, such as FESEM and enhanced darkfield imaging, which have helped us to see NPs in tissue sections. There are also examples of tools which have allowed us to see damage in targets that are not traditionally evaluated by pathologists, including lymphatics and the mitotic spindle. Following the pathway of the NP also means understanding the limits of light and electron microscopy so that the dose will be sufficient to demonstrate the NP in tissue sections.

A final critical skill of toxicologic pathologists evaluating NP studies will be their understanding of diverse cells, tissues, and organs. The medical background, pathology training, and toxicology training of the toxicologic pathologist can facilitate an understanding of how these smallest of particulates may be a key to new medical breakthroughs or to adverse consequences.

Nanotechnology and nanomedicine are changing our world. Through understanding the toxicologic pathology of NPs, toxicologic pathologists will play a major role in allowing the safe use of these revolutionary new products.

## DISCLAIMER

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.


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## THIRD EDITION

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