REGULATORY CONSIDERATIONS FOR NANOPESTICIDES AND VETERINARY NANOMEDICINES

A Draft APVMA Report

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EXECUTIVE SUMMARY

Advances in nanoscale science, engineering and technology have paved the way for developing novel applications, devices and systems in agriculture and animal husbandry. Currently, the use of nanotechnology in these sectors is not widespread but is expected to change rapidly since more than 3000 patent applications have been lodged in the past decade for nanopesticides alone.

The interest in nanopesticides appears to focus predominantly on three formulation types: polymer-based nanoformulations, inorganic nanoparticles such as silica and titanium dioxide, and nanoemulsions. The benefits of these formulations compared to existing formulations include the release of active ingredients in a slow and targeted manner, protecting active ingredients against degradation and increasing the apparent solubility of active ingredients that are poorly water-soluble. Other benefits such as a network of wireless sensors able to detect and locate pest-infested portions of a crop and communicate the information via satellite to a laptop computer, and nanoclay devices installed in drip irrigation lines that release agrochemicals on demand, are also envisioned. Deploying such technologies will reduce the environmental footprint and off-site impacts of chemicals through the use of smaller quantities and more targeted application.

Nanotechnology in animal husbandry is an important area of Research & Development, though to date, only one nanoproduct is registered for use in Australia. In particular, the use of veterinary drugs and vaccines is anticipated to increase in the short-term. The benefits of nanotechnology in drug delivery predominantly stem from improved stability and/or apparent solubility; an increased concentration of a drug at the intended site of action (increased efficacy); a decreased concentration of a drug in healthy non-target tissues (reduced systemic toxicity) and modified pharmacokinetics, including controlled release. Increased bioavailability as well as improvements in the ability to target and control drug delivery should improve safety-efficacy profiles. Advances in vaccine technology due to nanotechnology will include safer antigens consisting of synthetic peptides and recombinant proteins as well as novel nanoparticle-based adjuvants that can be highly tuned and engineered so vaccines may be administered less frequently. Nanotechnology-enabled products will increasingly find applications in food-producing animals, such as modifying animal feeds, maintaining herd health, improving fertility, promoting growth and preserving animal identity.

The unique physical and chemical characteristics of nanomaterials that offer so much promise to agriculture and animal health and livestock production may also pose risks to human health and the environment. The opportunities and potential risks associated with the use of nanomaterials in crop production and animal husbandry are discussed in Chapter 1.

The novel properties of manufactured nanomaterials are attributed to a combination of their small size, chemical composition, physicochemical properties and surface structure. As well as offering great benefits, these same properties may give rise to toxicity – the so-called ‘nanomaterials paradox’. This begs the question: ‘Are nanotechnology products safe?’ The OECD Working Party on Manufactured Nanomaterials has published a Series on the Safety of Manufactured Nanomaterials. These guidance documents will be amended and refined as necessary to reflect a rapidly growing knowledge base and are directly relevant to regulators and industry. In Australia, it is the role of the APVMA to ensure that the use of AgVet nanoscale chemicals and chemical products do not harm human or animal health or the environment (see Chapter 2).

Information obtained from the characterisation of nanomaterials is a starting point for risk assessment. Characterising the relevant physical and chemical properties of nanomaterials may require access to
specialised instrumentation that is not available in many test facilities. Also, nanoparticles may need to be characterised at various stages of their life-cycle because their functionalities may change when exposed to different media. The characterisation of nanomaterials is addressed in ‘Report of the OECD Expert Meeting on the Physical and Chemical Properties of Manufactured Nanomaterials and Test Guidelines’, No. 41, and is covered in Chapter 3 of this report.

The manner in which nanomaterials are manufactured plays a significant role when considering risk. Because the relationship between manufacturing processes and risk is complex, it is not possible to make generalisations yet. What is of concern is the possibility that small changes to manufacturing processes may introduce unpredictable risks. Chapter 4 of the report discusses ‘top-down’ and ‘bottom-up’ nanofabrication as well as the numerous methods for manufacturing nanomaterials.

A key conclusion of the OECD Working Party on Manufactured Nanomaterials, as reported in ‘Important issues on risk assessment of manufactured nanomaterials’, No. 33, was that there is no significant evidence that the toxicological endpoints prescribed in the current Test Guidance document about ‘normal-sized’ materials are not adequate for nano-sized ones. However, some aspects of the risk assessment paradigm may need refining to reflect the increasing understanding of nanomaterial behaviour. A case in point is the self-assembly of certain nanomaterials into new structures in the body, which is not well captured within the current approaches to hazard assessment. The form of nanomaterials used in toxicology studies must be well-characterised. Issues such as physicochemical characterisation, preparation and characterisation of dosing suspensions and dose metrics are likely to need more detailed examination. Chapter 5 of the report reviews the potential risks to human health associated with AgVet nanomaterials.

Chapter 6 discusses the regulatory considerations for nanoscale AgVet nanomaterials in the environment. The adequacy of the current state of knowledge about the behaviour of nanomaterials in both terrestrial and aquatic environments is the basis for considering their potential environmental fate and effects. These can be very different compared with those of non-nanoscale chemicals. A whole life-cycle approach needs to be applied to the assessment of nanomaterials and should be considered during product development.

The report aims to inform and stimulate discussion about emerging nanotechnology and highlights the key regulatory considerations for AgVet chemical nanomaterials based on the current state of knowledge. It systematically explores the opportunities and risks of these substances in Australian agriculture and animal husbandry and reviews the published work relevant to the registration of nanoscale AgVet chemicals. It is not the report’s purpose to provide formal guidelines since the field is advancing so rapidly they would likely be obsolete soon after their publication. Nor is the purpose of this report to describe a regulatory framework for AgVet nanomaterials. The general consensus is that, for the foreseeable future, existing regulatory frameworks developed for macroscale chemicals will be used to regulate nanomaterials. Over time, however, the framework will evolve as new information highlighting limitations in the current risk assessment paradigm becomes available.
1 NANOTECHNOLOGY IN AGRICULTURE AND ANIMAL HUSBANDRY: AN INTRODUCTION

1.1 Background and historical context

A nanometre is one-billionth of a metre. To put nanoscale dimensions between approximately 1 and 100 nm into perspective, a sheet of paper is about 100,000 nm thick; a human hair is approximately 80,000 nm in diameter and most animal cells are 10,000 to 20,000 nm in diameter. The nanoscale dimension was described by Klaine et al (2012) in the following way: ‘Imagine shrinking the moon to the size of a tennis ball. This is the same as shrinking the tennis ball to the size of a Buckminsterfullerene molecule. This molecule made up of 60 carbon atoms is also known as a buckyball. It’s spherical and has a diameter of about 5 nm.

Nanotechnology promises benefits in a wide range of applications, from material sciences to healthcare, food, cosmetics, chemicals (including industrial chemicals, pesticides and veterinary medicines), information and communication technology, transport and space, and energy generation and storage. The potential benefits to society include lighter and stronger materials, ‘lab-on-a-chip’ technology, environmental remediation technology, remote sensing and tracking devices related to food quality and spoilage, enhanced renewable energy from solar cells using silicon nanocrystals, increased computer speeds and self-cleaning surfaces.

But what is nanotechnology and how did it come about?

Nanotechnology is the application of nanoscience to develop new materials and products, and involves manipulating matter at the nanometric scale (Health Canada Fact Sheet, 2011). Although it has only recently attracted public attention, the field of nanotechnology had its roots in 20th century advances in materials science and high-resolution imaging and analytical techniques (Maynard et al, 2011). Indeed, it was a speech titled "There’s plenty of room at the bottom", delivered by Richard Feynman to a meeting of the American Physical Society at the California Institute of Technology way back in 1959 that is now credited with heralding the coming of nanotechnology.

Feynman’s speech not only addressed manipulating and controlling matter on a smaller scale, he also anticipated many scientific and technical fields that are well established today. These include electron-beam and ion-beam fabrication, molecular-beam epitaxy, nanoimprint lithography, projection electron microscopy, atom-by-atom manipulation, quantum-effect electronics, spintronics, and microelectromechanical systems (Roukes, 2007).

Many significant events relating to the emergence of nanotechnology have occurred since Feynman’s speech but only key events are included in the following timeline.

1959 Richard Feynman’s speech titled ‘There’s plenty of room at the bottom’
1974 Norio Taniguchi coined the term ‘nanotechnology’

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1 Nanoscience is the study of materials at dimensions between approximately 1nm and 100nm and the process for their manipulation.
1981 Alexei Ekimov discovered nanocrystalline, semiconducting quantum dots in a glass matrix.

1985 Gerd Binnig and Heinrich Rohrer invented the scanning tunneling microscope, allowing scientists to ‘see’ individual atoms for the first time.

1985 Harold Kroto, Sean O’Brien, Robert Curl and Richard Smalley discovered Buckminsterfullerenes (C_{60}).

1985 Louis Brus discovered colloidal semiconductor nanocrystals (quantum dots).

1986 Gerd Binnig, Calvin Quate, Christoph Gerber invented the atomic force microscope.

Don Eigler and Erhard Schweizer manipulated 35 individual xenon atoms to spell out the IBM logo.


1991 Sumio Iijima is credited with discovering carbon nanotubes (CNT).

2003 Naomi Halas, Jennifer West, Rebekah Drezek and Renata Pasqualin developed gold nanoshells.

2007 Nanotoxicology journal was launched.

2014 Lai-Sheng Wang and colleagues discovered borospherenes (B_{40})².

The advent of nanotechnology has unleashed enormous prospects for the development of new products and applications for a wide range of industrial and consumer sectors. The scope of the potential impacts of nanotechnology is highlighted by Richard Smalley³, in a list of the Top Ten Problems Facing Humanity over the next 50 years:

1. Energy
2. Water
3. Food
4. Environment
5. Poverty
6. Terrorism and war
7. Disease
8. Education
9. Democracy
10. Population

The world’s population in 2003 was 6.3 billion people and is predicted to increase to 8–10 billion people by 2050. This is an exponential increase that will result in a greater need for food, water,

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² In early 2014, Wang and his colleagues reported clusters of 36 boron atoms forming one-atom-thick disks which they referred to as borophene. A short time later Wang and his research team reported clusters of 40 boron atoms forming a molecular cage. The new structure, referred to as borospherene, consists of 48 triangles, four seven-sided rings and two six-membered rings. Wang suggested the borospherene might have application in hydrogen storage.

³ The late Professor Richard Smalley was awarded the Nobel Prize in Chemistry in 1996 for his role in discovering the Buckminsterfullerene (C_{60}) in 1985.
energy, healthcare and shelter in a world that is already struggling to meet these demands. Nanotechnology offers solutions to many of these problems.

However, many of the same novel properties that give nanotechnologies the capacity to solve problems relating to the essential needs of humanity and the environment may also present novel and as yet unthought-of risks. Importantly, these potential risks may extend across the life-cycle of nanoproducts covering design and production, shipping, storage, use, and recycling or disposal. All must be carefully considered and managed if society is to accept the new products and developments arising from the technology.

Nanotechnology is cutting-edge science offering considerable opportunities to develop innovative products and applications for numerous industrial and consumer sectors. It draws from a wide range of fields including physics, material science, supramolecular and polymer chemistry, interface and colloidal science, and from chemical, mechanical, biological, and electrical engineering. Cross-disciplinary research will be needed to overcome the major technical problems faced by researchers if they are to realize the paradigm-shifting advances they seek.

From a global industry perspective, one of the reasons for the excitement around nanotechnology and its alluring investment opportunities is the 2008 Lux Research forecast of a US$3.1 trillion market for nanotechnology-related industry by 2015. Due to the global economic slowdown and some concerns about the safety of nanomaterials, this expected market growth never took place. The current assessment is that the nanotechnology industry will grow to $81 billion by 2015 (Technology Strategy Board, 2009).

1.2 Definitions and terminology

Many definitions of nanotechnology-related terms have been developed by expert bodies and regulatory agencies and are detailed in Chapter 3 of this report. Only a small sub-set of definitions need be presented here.

The prefix ‘nano’ comes from the Greek word for ‘dwarf’ and nanoscience is the study of materials at dimensions between approximately 1 nm and 100 nm and the processes for their manipulation (ISO/TS 80004-1).

Nanotechnology is the application of scientific knowledge to manipulate and control matter in the nanoscale in order to make use of size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk or ‘normal-sized’ materials (ISO/TS 80004-1).

Nanoscale is the size range from approximately 1 nm to 100 nm (ISO/TS 80004-1).

Nanomaterial is material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale (ISO/TS 80004-1).

Nano-object is material with one, two or three external dimensions in the nanoscale (ISO/TS 80004-1).

The APVMA’s working definition of a nanomaterial is ‘an intentionally produced, manufactured or engineered substance with unique properties that are directly caused by size features with 10% or more of the number size distribution of these features lying in the range approximately 1–100 nm (the nanoscale)’. However, the APVMA acknowledges that biological and health, safety and environmental
(HSE) issues may require a different size range above 100 nm.

The terminology for describing the manufacture of nanomaterials can be summed up in three definitions:

1. **Top-down** nanofabrication implies that structures are made increasingly small by progressively removing matter, usually by etching.
2. **Bottom-up** nanofabrication implies that atoms or molecules are distributed and rearranged to build new, functional nano-objects.
3. **Self-assembly** is the process in which a disordered system of molecules spontaneously forms an organized structure or pattern that is at equilibrium or in a quasi-equilibrium state. The product formed has reduced free energy compared to the initial state of the disorganized molecules.

### 1.3 Properties and behaviours of nanomaterials

The physical, chemical, and biological properties of nanomaterials may differ in important ways from the properties of bulk materials and single atoms or molecules. Differences in magnetic properties, electrical conductivity and optical sensitivity attributed to quantum mechanics phenomena become prevalent at the nanoscale (Nel et al, 2006). For example, gold is very stable to oxidation as the bulk material but burns spontaneously at sizes below a few nanometers (Donaldson and Tran, 2002).

Batley and co-workers (2012) described seven main classes of manufactured nanomaterials: carbonaceous nanomaterials (eg carbon nanotubes), semiconductors (eg quantum dots), metal oxides (eg zinc oxide), nanopolymers (eg dendrimers), nanoclays, emulsions (eg acrylic latex) and metals (eg silver). Further, the researchers noted that these nanomaterials may exist in single, aggregated, or agglomerated forms and have various shapes, coatings and surface functionality.

It is important from a regulatory perspective to understand and consider the unique properties of nanomaterials and formulations (Eifler et al, 2011). For example, the similarity in size between natural biomolecules and manufactured nanomaterials raises concerns about nanomaterials interfering with biological processes both on the cell membrane and within the cell. This point is illustrated by the diameters of a DNA double helix and a buckyball, which are approximately 2 nm and 5 nm respectively. The increased ability of nanosized particles to migrate into organisms and body tissues compared to non-nanoscale materials creates additional health concerns.

Table 1.1 illustrates the effect of particle size on particle number and the particle surface area/volume ratio for a given mass of a carbonaceous substance (Maynard et al, 2011). In this conceptual model, sample A is comprised of micron-sized spherical particles (10 µm particle diameter) and sample B is comprised of nanoscale spherical particles (10 nm particle diameter). Sample A and sample B each contain 1 mg of particles. In this model, when particle diameter is decreased by three orders of magnitude, the number of particles increases by nine orders of magnitude, and the surface area/volume ratio of particles increases by two orders of magnitude.
Table 1.1 Effect of particle size on particle number and surface area/volume ratio

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass (mg)</th>
<th>Particle diameter (nm)</th>
<th>Number of particles</th>
<th>Particle surface area/volume ratio (nm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>10⁴</td>
<td>~ 10¹²</td>
<td>0.006</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>10</td>
<td>~ 10²¹</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The relationships between particle diameter and particle number, and particle diameter and particle surface area/volume, have two important implications for nanomaterial behaviour. First, for a defined weight of nanoparticles, a larger number of smaller nanoparticles can increase the potential for disposition to more and different locations. Second, the surface area/volume ratio is higher for smaller particles and this is conducive to greater chemical reactivity since a greater proportion of atoms are located on the particle surface rather than in the inner bulk lattice.

The physicochemical properties of nanomaterials are addressed in detail in Chapter 3 of this report.

1.4 Examples and applications of nanomaterials

The applications of nanotechnology in healthcare and food and in the devices used in these sectors, as well as in the evolution of material science, are all relevant to advancements in the agricultural and animal health sectors due to the cross-fertilisation between these sectors. All need to be discussed. This analysis is limited to nanotechnology products and applications already on the market, or in the research and development pipeline.

Healthcare

In the healthcare sector, nanomedicines hold enormous promise to improve the prevention, detection, diagnosis and treatment of disease. Applying nanotechnology to drug reformulation has allowed some otherwise toxic drugs to be delivered more safely and effectively. These novel approaches offer great hope in overcoming problems resistant to conventional therapy. Nanotechnology also offers novel nanomedicine applications, of which there are many examples. Some of these demonstrate multiple functionalities such as diagnostics, targeted drug delivery, therapeutics and an ability to report back on the effectiveness of therapy. Another example involves magnetic nanoparticles, which are being investigated for a wide variety of biomedical applications, including improved contrast for magnetic resonance imaging (MRI), targeted drug delivery and hyperthermia treatment to destroy cancer cells. Cornell dots, which were first developed in 2005, received FDA approval in 2011 for human trials into improved cancer imaging and drug delivery. Cornell dots (also known as C-dots) comprise a silica shell less than 8 nm in diameter encapsulating near-infrared fluorescent dyes and a chemotherapeutic agent. The silica shell is coated with polyethylene glycol to increase residence time in the body and with cancer-targeting molecules. Researchers anticipate using C-dots to diagnose and treat cancer, in

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4 Data in columns 2, 3, and 4 are from Maynard et al., 2011.

5 Surface area and volume of spherical nanoparticles were estimated as 4πr² and 4πr³/3 (where r = radius), respectively.
the staging of cancer disease, and assessing tumour burden via lymph node mapping (Benezra et al, 2011).

‘Nanosponges’ and ‘nanojuice’ have been reported in recent scientific literature. ‘Nanosponges’ are approximately 3000 times smaller than a red blood cell and comprise a biocompatible polymer core coated with segments of red blood cell membranes derived from the host (Hu et al, 2013). When injected into the bloodstream, the nanosponges attract pore-forming toxins produced by snakes, insects, bacteria etc which would otherwise perforate the outer membranes of erythrocytes causing cell lysis. In 2014, a new imaging technique involving nanoparticles suspended in liquid that patients drink (referred to as ‘nanojuice’) was reported. A laser light is used to activate naphthalcyanine dyes contained in the nanoparticles when the formulation reaches the small intestine, which is then imaged using photoacoustic tomography. Human studies are now underway to determine whether this novel contrast agent is superior to those currently available for patients with celiac disease, Crohn’s disease or irritable bowel syndrome (Zhang Y et al, 2014). Further, doctors expect to use nanotechnology for regenerative medicine, such as repairing spinal cord injuries (Gelain et al, 2011).

Many more novel materials with applications in nanomedicine are forthcoming. Zhang S et al (2014) have reported working on a class of molecules called amphiphilic Janus dendrimers used to form evenly sized, stable vesicles. The unique onion-like structure of these vesicles may open the door to next generation nanomedicine through serial delivery of a drug from each of the 20 layers of the vesicle, or the release of a different drug from each layer of the vesicle.

Food

While there are no applications for manufactured nanomaterials in the food sector in Australia, packaging and nanoencapsulation are reported to be the main applications received by agencies overseas (FAO/WHO, 2010). Examples of food packaging applications include plastic polymers with nanoclay to reduce oxygen permeability, nanosilver and nanozinc oxide for antimicrobial action, nanotitanium dioxide for UV protection, nanotitanium nitride for mechanical strength and as a processing aid, and nanosilica for surface coating. While nanotechnology in food packaging has demonstrable benefits, there are also health concerns that nanomaterials might migrate from the packaging into food, and environmental concerns that when the packaging is disposed of, nanoparticles may enter landfills and cycle into other living organisms, and even the food chain. While such impacts are unproven and uncertain, consumers expect to know what is in their food. Insurance professionals also require information on product labelling to evaluate underwriting risks.

Nanoencapsulation in the food sector, in the form of micelles, liposomes or biopolymer-based carrier systems, enables the development of delivery systems for additives and supplements in food and beverage products. Nano-encapsulated food additives include minerals, antimicrobials, vitamins and antioxidants. The most common objective of nanoencapsulation is to enhance the uptake and bioavailability of food additives; other benefits include improving taste, consistency, stability and texture (Chaudhry et al, 2008). One report describes the development of a colourless and tasteless beverage containing nanoencapsulated ingredients or additives that can be activated by a consumer at a particular microwave frequency. This activates selected nanocapsules, thereby releasing only the preferred flavour, colour or nutrients (Cientifica, 2006).

The food industry overseas is also using nanocarrier technology. Examples include rendering water-soluble compounds like vitamin C to become fat dispersible, and rendering fat-dispersible compounds like vitamin A to make them water dispersible (FAO/WHO, 2010). New developments in nanotexturing achieve new taste sensations and improved textures in foods, and improved consistency and stability in food emulsions.
Nanosensors have been developed for food packaging to add an ‘intelligent’ function. These sensors are designed to ensure the integrity of foods packed under vacuum or an inert atmosphere by detecting leaks, indicating time-temperature variations such as occurs with freeze-thaw-refreezing, or revealing when food has spoiled. An example of the latter is a label for poultry meat based on a reaction between hydrogen sulphide and a nanolayer of silver (Smolander et al, 2004). The nanosilver layer is opaque light brown, but when meat starts to deteriorate silver sulphide is formed and the layer becomes transparent.

‘Smart’ labels that have radio frequency identification displays (RFIDs) are being developed for foods with a limited shelf-life. The objective is rapid and accurate distribution of products. Self-healing nanomaterials that will repair small holes/tears in food packaging and respond to environmental conditions are also in the pipeline (Garland, 2004).

Further, an electronic ‘tongue’ for beer classification has been developed that uses an array of sensors comprised of 21 ion-selective electrodes. These allow it to distinguish between different varieties of beer with 82% accuracy (Cetó et al, 2013).

Material sciences

Several industry sectors are investigating using carbon-based nanomaterials as ultralight, high-strength composites and fibres. Carbon nanotubes have very high tensile strength. They are considered to be 100 times stronger than steel while being only one-sixth of its weight, making them potentially the strongest, smallest fibres known. Because of their strength, researchers are investigating the potential use of single-walled carbon nanotubes (SWCNTs) as reinforcing agents for intercalation matrices in polymer composites. Researchers are also developing smart nanomaterials with increasing functionalities, including responsiveness to external stress, electric and magnetic fields, temperature, moisture and pH.

Smart surface technology is another area that is advancing rapidly. It has potential applications within drug delivery systems, lab-on-a-chip analytic systems, self-cleaning systems, liquid and chemical sensor systems, and filtration systems. For example, nanostructured coatings for dirt-repellent surfaces have been reported with a cleaning action due to a ‘lotus effect’—the phenomenon that water beads and runs off the surface of lotus leaves due to nanoscale wax pyramids on the leaves’ surface.

1.5 Nanodevices and related technologies

Sensors

Nanotechnology is exerting remarkable influence on the development of new sensing devices. Sensors are analytical instruments that generate quantifiable output signals, usually as a result of an analyte binding to a recognition element. In the case of biosensors, recognition elements include biological receptors such as antibodies, enzymes, aptamers and peptides. The aim is to create nanodevices with new functions made possible due to the unique properties of nanomaterials, some of which can be precisely tuned. When coupled with materials capable of responding to external stress, electric and magnetic fields, temperature, moisture and pH, nanodevices can provide real-time, highly sensitive, analytical outputs. A recent development in the field of sensors is molecular imprinting, which is a powerful tool for generating tailor-made receptors for recognition elements in nanodevices. Molecularly imprinted materials are easier to prepare than biogenic antibodies and equilibrate with analytes faster.
Lab-on-a-chip devices

A laboratory-on-a-chip integrates laboratory functions on a chip that is only millimeters or centimeters in size. The technology promises faster reaction times, reduced use of materials and high product yields. It has the potential to improve and reduce the cost of healthcare. Lab-on-a-chip devices are already used in a wide range of applications, including fast and accurate hand-held sensors for environmental monitoring, medical diagnosis and process control in manufacturing. Lab-on-a-chip devices equipped with integrated electronic sensors will allow scientists and healthcare professionals to make better informed analyses (Brisk et al, 2014).

1.6 What nanotechnology could mean for agriculture and animal husbandry

Advances in nanoscale science, engineering and technology have paved the way for novel applications in agriculture and animal husbandry. While the main advantages that nanotechnologies offer over existing technologies arise from the improved or novel functionalities of nanomaterials, not all nanomaterials have relevance to the agricultural and animal husbandry sectors. To date, relatively few applications have been commercialized in these sectors globally and only one product has been registered in Australia. However, this situation is expected to rapidly change as more nanoproducts move through the R&D phase.

A diverse array of potential nanotechnology-derived applications for the agricultural and animal husbandry sectors has been reported (Scott and Chen, 2002; Chen and Yada, 2011; Underwood and van Eps, 2012). Existing applications, and those expected in the immediate future, include nanoformulations that promise enhanced efficacy, better product stability and smaller environmental footprints; ‘smart field systems’ able to detect pests as well as adverse conditions in field crops and apply pesticides, water and fertilizers to crops only as needed; ‘smart herd systems’ that detect and treat subclinical illness in a single infected animal in a herd; nanoscale identity preservation for the continuous tracking and recording of the history of agricultural commodities, and ‘smart fabrics’ able to monitor the vital signs of the wearer.

In the agrochemicals sector, nanotechnology will offer significant advances, such as pesticides delivered to plants by novel routes. An increasingly important consideration when formulating nanopesticides is reducing potential harm to the environment. As well as reducing pesticide use and off-site impacts through more targeted pesticide application, ‘greener’ nanopesticides are being developed to achieve environmental sustainability benefits. Potential candidates include naturally occurring active ingredients, such as pheromones and essential oils, and safer adjuvants such as biodegradable polymers.

The commercial application of nanotechnology-enabled products in the animal health sector is in its infancy, but anticipated applications for companion animals will include diagnostics, targeted drug delivery and effective therapy associated with minimal adverse side effects. Such applications are not dissimilar to those used in human nanomedicine. Consequently, the research underpinning certain human nanomedicines will likely be used to develop veterinary nanomedicines for companion animals. By comparison, nanotechnology-derived products for food-producing animals are expected to focus on modifying animal feeds, maintaining herd health, improving fertility, promoting growth and preserving animal identity.
1.6.1 Nanotechnology in agriculture

Australia has a modern agricultural system and its farmers are among the most efficient in the world. Even so, innovation through R&D will be required to increase current food production levels while improving the sustainability of production. Nanotechnology has the potential to increase the amount of food and sustain the systems that produce it.

More than 3,000 patent applications for nanopesticides have been lodged in the past decade (Kah et al, 2012). Therefore, despite few nanopesticides being marketed so far, considerable activity is occurring. A more recent literature review into the different types of nanopesticides identified polymer-based nanoformulations, inorganic nanoparticles (eg silica and titanium dioxide) and nanoemulsions as the formulation types most reported (Kah and Hofmann, 2014). The authors noted that polymer-based nanoformulations have greater efficacy compared to commercial formulations and have multiple applications such as the release of active ingredients in a slow and targeted manner, protecting active ingredients against degradation and increasing the apparent solubility of active ingredients that are poorly water soluble.

Nanotechnology may modify the behaviour of agrochemicals by one or more of the following mechanisms:

- increasing the apparent solubility of poorly soluble active ingredients
- releasing active ingredients in a slow/targeted manner
- protecting the active ingredient against premature degradation.

The mechanisms by which nanoformulations increase efficacy have not been characterised, though the behavioural effects noted above may contribute to the observed increase. For example, increasing the apparent solubility of poorly water-soluble active ingredients results in improved tank mixing, cuticle penetration and uptake. The drivers for developing slow release formulations include improved operator safety and reduced application rates due to less pesticide losses from degradation, leaching and/or volatilisation. Slow/targeted release formulations are particularly important with active ingredients that degrade rapidly.

**Polymer-based nanoformulations**

A range of polymers is anticipated to be used in agrochemical formulations in the short term. The main types of polymer-based nanoformulations are polymeric nanospheres and polymeric capsules. These allow the rate of release of active ingredients to be adjusted by changing the proportions and molecular weights of the polymers used. For example, the release half-life of carbofuran in water ranged from 7.5 to 55 days depending on the polymer matrix used. Other possible indications of polymer-based formulations include drift control agents, foam control agents, and improved safety in case of accidental ingestion.

**Inorganic nanoparticles**

The majority of studies investigating inorganic nanoparticles have considered silica, titanium dioxide, silver and copper. Data generated by Yuvakkumar et al (2011) in laboratory and field studies showed that silica nanoparticles increased seed germination and water use efficiency. However, other workers reported that comparable application rates of silica nanoparticles and diatomaceous formulations were required to achieve similar effectiveness (Debnath et al, 2011). Meanwhile, the evidence base showing the potential beneficial effects of titanium dioxide in agriculture is growing. Owolade et al (2008) and Moaveni et al (2011) reported increased yields from cowpeas and barley respectively, while Zheng et al (2005) reported improved spinach seedling growth. Titanium dioxide nanoparticles have also been shown to reduce the incidence of some diseases in the field (Owolade and Ogunleti, 2008). The presumed active mechanisms of titanium dioxide nanoparticles include protection against
disease and increased photosynthesis. Nanoparticles of silver and copper were trialled in laboratory, glasshouse and field studies and shown to curtail the growth of fungal and bacterial plant pathogens (Rai and Ingle, 2012; Lamsal et al, 2011).

**Porous hollow silica nanoparticles**

Porous hollow silica nanoparticles are promising agents in applications requiring sustained pesticide release, especially for photosensitive active ingredients. They have a shell thickness of approximately 15 nm, a pore diameter of four to five nanometers, and facilitate a high pesticide loading. The UV-shielding properties of porous hollow silica nanoparticles have been demonstrated to significantly improve the photostability of avermectin entrapped in the hollow core of the nanoparticle carrier. Moreover, the entrapped avermectin demonstrated sustained-release behaviour (Li et al, 2007). Controlled delivery from porous hollow silica nanoparticles has also been reported for the watersoluble pesticide validamycin (Liu et al, 2006).

**Nanoemulsions**

Nanoemulsions are mixtures of two immiscible liquids. Their major use in the agrochemical sector is to increase the apparent solubility of poorly soluble active ingredients while limiting the concentration of surfactants present in the formulation. They have achieved efficacy similar to or slightly greater than that of current formulations. The greater efficacy is thought to result from a slower release of labile active ingredients from the protective environment of the nanoemulsion (Kah and Hofmann, 2014).

**Solid lipid nanoparticles**

Solid lipid nanoparticles have been investigated for controlling the release of pesticides (Frederiksen et al, 2003) and protecting pesticides from photodegradation (Nguyen, 2012a, b).

**Nanodispersions**

Nanodispersions (also called nanosuspensions) result from the dispersion of nano-crystals (crystalline or amorphous particles consisting of 100% active ingredient) in liquid media. The aim is to maximize the surface area (relative to volume) of the active ingredient to increase the dissolution of poorly water-soluble compounds. Nanodispersions have relatively low production costs and reduced impact on the environment.

**Nanogels**

Nanogels are composed of a crosslinked polymer network or hydrogel. Those proposed for use in agriculture tend to be insoluble in water and therefore less prone to swelling or shrinking with changes in humidity. They also demonstrate good pesticide loading and release profiles.

**Electrospun nanofibres**

Electrospun nanofibres are being investigated for plant protection purposes. These fibres are obtained by electrospinning (using an electrical charge to draw the fibres from a liquid) and their release profiles are superior to those of spheres and capsules (Xiang et al, 2013).
Nanoclays

Nanoclays are thin sheets of silicate materials in the order of 1 nm thick and 70-150 nm wide. They are derived from montmorillonite clays commonly found in volcanic ash and their size is reduced and surface modified to form nanoclays that are biocompatible and have low toxicity. A promising group of these inorganic materials are the layered double hydroxides, or so-called anionic clays. They are layered solids consisting of cationic layers and exchangeable interlayer anions. They have already been used as hosts for the controlled release of plant growth regulator α-naphthaleneacetate and for the controlled release of the herbicide 2,4-dichlorophenoxyacetate (Bin Hussein et al, 2005). Other potential uses include the slow/targeted delivery of pesticides, plant nutrients, and fertilisers.

Carbon nanotubes

Carbon nanotubes are reported to have demonstrably positive effects on plant growth. Khodakovskaya and coworkers (2009) reported carbon nanotubes penetrating tomato seeds and increasing their germination and growth rates. In laboratory studies, carbon nanotubes were found to improve shoot and root growth in chickpeas (Tripathi et al, 2011).

Biosensors

Nanotechnology also has potential applications in agricultural biosensors. These nanodevices are likely to be used increasingly to detect environmental contaminants, including pesticides. Other potential uses for biosensors are the detection of diseases and/or pests including in imported agricultural produce, and testing food safety at the farm gate.

Wireless sensor networks

Wireless sensor networks are collections of very tiny, ultra-low-power sensor nodes, capable of sensing and communicating within a few tens of metres to fulfil complex, large-scale monitoring tasks. They have a wide variety of potential applications, including in precision agriculture. Researchers believe wireless sensors will be able to detect and locate portions of a crop that are pest-infested and communicate the information via satellite to a laptop computer. Another benefit of this technology will be targeted application of smaller quantities of pesticides, thereby reducing their environmental footprint.

Environmental Remediation

Nanotechnology can also be used to remediate agricultural land. For example, methods involving nanotechnology are being used to reverse the effects of pesticides in soil and groundwater (Baruah and Dutta, 2009). Similar technologies are available to treat waste water streams to remove pesticide contaminants (Mueller and Nowack, 2010).

1.6.2 Nanotechnology in animal husbandry

Nanotechnology has the potential to revolutionise animal health. Many applications for companion animals and food-producing animals have been reported, some of which exist currently while others are in the R&D phase. Nanosensor devices such as a ‘lab-on-a-chip’ for in vitro applications are also envisioned. The discussion that follows focuses on the benefits of nanotechnology-derived products in the animal health sector.

An area expected to increase in the short term is the delivery of veterinary drugs and vaccines. The benefits of nanotechnology in drug delivery are predominantly the result of improved stability and/or
apparent solubility; an increased concentration of drug at the site of action (increased efficacy); a decreased concentration of drug in healthy non-target tissues (reduced systemic toxicity) and modified pharmacokinetics, including controlled release. Increased bioavailability as well as improvements in the targeted and controlled delivery of existing drugs and their application through nanotechnology should make administering them easier while also improving their safety-efficacy profiles. The number of drugs available to a veterinarian may also be extended. Drugs that previously were not available due to their pharmaceutical behaviour (eg poor solubility), pharmacokinetics (eg too rapid elimination), pharmacodynamics (eg adverse side effects), or therapeutic response (eg lack of efficacy for a specific condition) may soon be safely used. Nanotechnology offers opportunities to address many of these shortcomings and overcome problems resistant to conventional therapy. Advancements in vaccine technology include safer antigens consisting of synthetic peptides and recombinant proteins (Nordly et al, 2009); novel, nanoparticle-based adjuvants that are highly tunable and can be engineered so that vaccines may be administered less frequently and via a convenient administration route (Scheerlinck et al, 2006) and, in humans, administration methods that allow patients to safely treat themselves (eg the NanoPatch vaccine).

An important advance is in ‘smart’ drug delivery which allows specific sites to be targeted and drug release to be controlled. The strategy generally involves attaching targeting ligands such as monoclonal antibodies to the surface of nanoparticles, which are then transported in the systemic circulation to the target tissue. With tumours, infections and inflammation, the situation is different due to the vasculature being permeable to nanoparticles, allowing for their extravasation and accumulation in the target tissue. This phenomenon is known as the ‘enhanced permeability and retention’ (EPR) effect, and it facilitates both active and passive targeting of nanoparticles to specific sites. Passive targeting involves the movement of small particles through leaky vasculature to the target tissue. Active targeting relies either on targeting ligands attached to the surface of the nanoparticles, or on an alternating magnetic field to direct magnetic nanoparticles to the desired site of action. Schiffelers et al (2001) report targeting intracellular parasitic, fungal and viral infections in cells of the mononuclear phagocyte system with uncoated nanoparticles, which showed rapid cellular uptake. This novel approach is counter to the one generally practised whereby nanoparticles are coated with hydrophilic substances such as polyethylene glycol to reduce opsonisation and prolong circulation time.

A variety of nanoformulations for animal drug delivery are in use, or are proposed for the foreseeable future. The following is a brief account of these different nanoformulation types.

**Drug nanocrystals**

When the bioavailability of poorly water-soluble drugs is limited by their rate of dissolution, nanosizing can markedly improve bioavailability. The observed improvement is attributed to the surface area (relative to volume) of a drug nanocrystal being orders of magnitude greater than that of its conventional counterpart. Another advantage is less variability in bioavailability for the fed and fasted state. Drug nanocrystals may be produced using either top-down technology (ie subjecting micronized particles to milling or grinding) or bottom-up technology (ie the nanoprecipitation of molecules).

**Liposomes**

Liposomes are self-assembled vesicles comprised of a central aqueous cavity surrounded by a lipid membrane(s) or lamella(e). Hydrophilic and hydrophobic drugs in the core or lamella of liposomes, respectively, are protected from degradation during the absorptive and distributive phases following oral administration. This protection is lost if a drug is prematurely released into the gastrointestinal tract. The circulation time of liposomes is prolonged by coating them with polyethylene glycol. On contact with biological cells, liposomes tend to unravel and merge with the membrane of the cell, releasing their payload of drugs or other agents. Liposomes encapsulating imaging contrast agents
have also been used in in vivo diagnostics. However, such use has been restricted following concerns over toxicity and safety relating to complement-mediated hypersensitivity reactions. The latter occur in 5-45% of human patients during liposome administration (Szébeni et al, 2007).

**Polymer-drug conjugates**

Drugs conjugated with polymers demonstrate slower degradation than drugs alone and the degradation time varies with different polymers. Polymers synthesized for this purpose are generally biodegradable. Slower degradation of polymer-drug conjugates results in a prolonged circulation time which offers two benefits. First, such conjugates may be administered less frequently to maintain effective blood levels. Second, a prolonged circulation time allows for greater extravasation of a drug by the EPR effect, resulting in higher drug concentrations at the site of action. Polymer-DNA conjugates or polyplexes, which are similar in concept to polymer-drug conjugates, are used in gene therapy.

**Dendrimers**

Dendrimers are highly branched polymers consisting of an initiator core, interior layers composed of repeating units and terminal moieties that can be functionalised to modify the solubility, miscibility, and reactivity of the resulting macromolecule. The synthetic process controls the size and structure of dendrimers as well as their biocompatibility and biodegradability. High loadings of a drug can be incorporated in the dendrimer core, or attached to the terminal moieties on the dendrimer surface.

**Polymeric micelles**

Polymeric micelles comprise a core protected by a hydrophilic outer shell formed by amphiphilic block copolymers. The advantages of polymeric micelles for drug delivery include solubilisation of poorly soluble molecules and sustained drug release attributed to the drug’s encapsulation protecting it from degradation and metabolism. Polymeric micelles can also enhance the delivery of drugs to desired biological sites, thereby improving therapeutic efficacy and reducing unwanted side effects. An example is micelles containing attached sugar-group ligands that specifically target glycol-receptors in cellular plasma membranes.

**Solid lipid nanoparticles**

Solid lipid nanoparticles demonstrate excellent physical stability and protect an incorporated drug from chemical degradation. A disadvantage is that they have low drug loading capacity. It is likely that solid lipid nanoparticles will be developed that are suitable for delivery by most routes of administration.

**Polymeric nanoparticles**

The two main forms of polymeric nanoparticles are polymeric nanocapsules and polymeric nanospheres. From a drug delivery perspective, polymeric nanocapsules demonstrate a high drug loading capacity. They also allow for increased drug bioavailability and controlled drug release compared with the conventional drug counterpart. Other applications of polymeric nanocapsules include the detection (including imaging), diagnosis and treatment of disease. Unlike with polymeric nanocapsules, the drug in polymeric nanospheres is physically and uniformly dispersed in a dense polymeric matrix.
Magnetic nanoparticles

Drug-coated magnetic nanoparticles, generally larger than 50 nm in size, are used in drug delivery. After drug delivery, a magnetic field is used to direct the magnetic nanoparticles to the desired site of action and to keep them there. Smaller magnetic nanoparticles, often approximately 5 nm in diameter, are used for therapeutic hyperthermia. This is created by applying an external alternating magnetic field to the tissue in which the magnetic nanoparticles have accumulated to cause localised cellular necrosis in, for example, a targeted cancer.

Nanoclays

Minerals that exist in nature, and volcanic ash in particular, are processed to form nanoclays. The layered double hydroxides (LDHs), which comprise two layers of positive charge balanced by intercalated hydrated anions, are one category of nanoclays. An example of an LDH is nanobiohybrids, which are nanoclay hosts with various negatively charged biomolecules intercalated between the layers. Replacing the hydrated anions with DNA using ion exchange results in a nanobiohybrid with applications for gene therapy. Following delivery to a biological system, nanobiohybrids are phagocytosed and the biological material released from the inorganic host either by dissolution of LDHs in the acidic environment of lysosomes, or through reverse ion-exchange within the cellular fluids.

Gold nanoshells

An advantage of gold nanomaterial is its biocompatibility. Gold nanoparticles are used to diagnose and treat diseases such as cancer. In this application, gold nanoparticles are coated with surface moieties specific for the tissue being targeted (e.g., monoclonal antibodies) and a hydrophilic substance such as polyethylene glycol to prolong circulation time. The gold nanoparticles are irradiated with near infrared to visualise and destroy gold-targeted cancer cells.

Carbon nanotubes

These nanomaterials have a high optical absorbance at infrared frequencies and may in future have similar applications to gold nanoshells. Carbon nanotubes are also being investigated as nanovector systems. However, there are concerns under investigation into possible long-term toxicity due to bioaccumulation.

Quantum dots

Quantum dots are colloidal semiconductor nanocrystals with unique optical properties. Their in vitro toxicity is related to the composition of the core (typically cadmium selenium) and can generally be overcome by coating the core with other metals, such as zinc sulfide, or adding a protective hydrophilic coating like polyethylene glycol. Quantum dots demonstrate high level fluorescence, long-term stability, simultaneous detection of multiple signals, and tunable emission spectra. They hold promise as a multifunctional therapeutic for lymph node mapping, identifying molecular targets, photodynamic therapy, drug delivery, and surgical oncology. Further research is necessary to evaluate the long-term stability of quantum dots in vivo.
Vaccines and vaccine adjuvants

Nanoparticle vaccine delivery systems for more than 40 animal diseases have been reported as either successfully developed or under development (Scheerlinck and Greenwood 2008). A shift from antigens comprising inactivated microorganisms to safer synthetic peptides and recombinant proteins has also been reported (Nordly et al, 2009), while nanoparticle adjuvants including emulsions, liposomes, nanobeads, immune stimulating complexes (ISCOMs) and inorganic particles are being investigated to improve immunogenicity (Scheerlinck et al, 2006). They may be engineered to be highly tunable to elicit prolonged immunogenic responses and to allow for convenient administration.

Nanotechnology-enabled vaccines have been noted to decrease unwanted inflammatory responses at injection sites in food-producing animals. This is thought to be caused by nanosized adjuvants that mimic the size of viruses being well tolerated by cells. Advancements are also being reported in vaccine delivery. The NanoPatch® is an example of a nanotechnology-enabled device that delivers vaccines dermally to humans, though the concept applies equally to animals. Studies in mice have found that a comparable immunogenic response is elicited by one one-hundredth of the dose delivered conventionally by a needle and syringe, a finding consistent with skin having more immune cells than muscle. The NanoPatch® promises significant benefits, particularly in developing countries where access to healthcare professionals and facilities is limited.

Nanotechnology applications in food-producing animals

In food-producing animals, nanotechnology-enabled products will increasingly find other applications. For example, animal feeds may incorporate nutritional supplements in nanoparticulate form to increase the bioavailability of a mineral or vitamin. In this respect, fat-soluble vitamin E is more stable in the aqueous environment of the gastrointestinal tract of animals when encapsulated in liposomes as a nanodispersion. The bioavailability of vitamin E in this formulation is increased compared to the conventional counterpart. Nanotechnology-enabled feed additives to protect animals against mycotoxins or to remove food-borne pathogens in the gastrointestinal tracts of livestock have also been reported. An example of the latter are nanoparticles that adhere to E. coli; the nanoparticles used consist of a polystyrene base, a polyethylene glycol (PEG) linker, and a mannose-targeting biomolecule. Other opportunities that nanotechnology offers for food-producing animals relate to growth promotion, fertility and breeding, and animal health. All of these applications may involve implantable self-regulating drug delivery systems. One report envisages a system for the detection, diagnosis and therapy of sub-clinical bacterial infections in food-producing animals (Scott and Chen, 2002). Major benefits of using smaller quantities of antibiotics are reduced pressure for the emergence of antibiotic resistance and markedly reduced residues of antibiotics in food commodities.

In vitro nanosensor devices

In vitro nanosensor devices will play an increasingly important role in the animal health sector. A lab-on-a-chip is an example of an in vitro nanosensor device. A lab-on-a-chip integrates laboratory functions and promises faster reaction times (allowing for point-of-care diagnostics), reduced material use and a high product yield. The very latest advances in plasmonics, nanofabrication, microfluidics and surface chemistry underpin the capabilities of nanosensor devices. As a result, novel diagnostic assays are available that link functionalized nanoparticles to biological molecules such as antibodies, peptides, proteins and nucleic acids (Driskell et al, 2005; Luchini et al, 2010). A lab-on-a-chip able to detect protein cancer markers in blood is a case in point. A drop of blood injected into the chip circulates through the micro-channels and any cancer markers present will stick to gold nanoparticles located on the microchannels, setting off changes in ‘plasmonic resonance’ that are monitored by the device. In the veterinary field, a multitude of nanoparticle-based detection systems have been successfully validated to detect viral, parasitic and bacterial pathogens (Kumanan et al, 2009; Yuan et al, 2009).
1.7 Potential risks posed by nanomaterials

While nanotechnologies offer a multitude of opportunities for innovation in agriculture and animal husbandry, a balanced view is critically important because the unique physical and chemical characteristics of nanomaterials that offer so much promise may also pose risks to human health and the environment (SCENIHR, 2006). Eifler et al (2011) noted that effective regulation of nanotechnology-enabled products requires an understanding and consideration of their unique properties. In reality, nanoparticle application in the various industries has outpaced the research that is needed to determine which of their characteristics might pose unique hazards. A better understanding of the link between nanomaterial properties and biological behaviour in humans and the environment is also needed to guide risk assessment paradigms. Detailed accounts of the regulatory considerations for assessing the risks of nanomaterials are contained in subsequent chapters. The discussion that follows briefly addresses only a few elements of the potential risks posed by nanomaterials.

Manufactured nanomaterials are found in many consumer products on the market today, including sunscreens, cosmetics and clothing (RIVM, 2011). The public want to know if these products are safe; some people contend that the risks posed by nanomaterials are unknown and there have been calls nationally for mandatory labelling and a register of nanomaterials as a fundamental right to know. Both of these issues have been considered by a previous Australian Government and dismissed.

The safety of nanomaterials is an emotive and controversial matter that often features in the popular press. The three cases that follow are typical of those reported.

This first case involves safety concerns relating to nanoparticle-based sunscreens. Media reports assert that nanoparticles incorporated in some UV filters penetrate the skin, enter the bloodstream, and cause possibly adverse effects. Scientists contend there is no definitive evidence for nanoparticles in sunscreens penetrating the skin (TGA, 2013). Rather the nanoparticle ‘marker ions’ detected in the blood and urine of human trial subjects who have applied sunscreens containing nanoparticles result from the dissolution and ionization of nanoparticles on the skin — so it’s the ions and not nanoparticles per se that have been absorbed percutaneously (Gulson et al, 2010). Dermal absorption studies including the findings of a review of nanoparticle-based sunscreens are discussed in Chapter 5 of this report.

Nanoscale silver (nanosilver) has been the subject of numerous media reports. In addition the National Institute for Public Health and the Environment (RIVM) located at Bilthoven, the Netherlands, conducted a hypothetical registration of nanoscale silver according to the EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation in 2009. Most products containing nanosilver are used for their antibacterial properties; in the home to keep surfaces clean or to reduce odours, as a coating in medicinal applications, such as artificial joints and pacemakers, and in ceramic filters for water purification. The principal concern about nanosilver is environmental risk, which is addressed in Chapter 6. There are also concerns that overusing nanosilver could lead to antimicrobial resistance. Nanosilver highlights the importance of life-cycle considerations, including production, transport, storage, use and disposal or recycling, when regulating nanomaterials.

A third case focuses on media reports about research published by Poland et al (2008), who reported that injecting carbon nanotubes (CNTs) into the abdominal cavities of mice resulted in asbestos-like lesions. The study demonstrates that CNTs conform to a structure-activity relationship based on
aspect ratio⁶, to which asbestos and other macro- and micron-sized pathogenic fibres conform. However, while the findings confirm that fibres with high aspect ratios can result in long-term harm, this behaviour is not restricted to nanofibres but applies to all fibres, regardless of size.

**Size matters!**

Size affects the potential risks posed by nanomaterials in at least three ways. First, materials at the nanoscale dimension are of similar size to natural biomolecules. For example, the diameter of a DNA double helix is about 2 nm while the diameter of buckyballs is about 5 nm (Klaine et al, 2012). The similarity in size translates into an increased potential for manufactured materials to interfere with biological processes. This could include the behaviour of cell membranes, biochemical pathways in cells, or even the genetic code itself. Second, the surface areas (relative to volume) of nanomaterials are orders of magnitude larger than those of non-nanomaterials. A consequence of a large surface-to-volume ratio is that the properties of the surface molecules dominate (Klaine et al, 2012). It therefore follows that surface chemistry is an important determinant of nanomaterial hazard. Third, nanoparticles may translocate to locations in the human body or the environment not accessible to their conventional counterparts. All three of these size-related nanomaterial behaviours pose potential risks to human health and the environment, and are addressed in registration applications and assessed by Government regulators.

A complete and accurate characterization of manufactured nanomaterials is required in order to fully understand both the benefits and the potential toxicity of nanoparticles in biological systems (Royal Society, 2004). When a full characterization is not possible and it becomes necessary to prioritise the parameters for characterisation, Oberdorster et al (2005) propose that the following criteria be considered:

- the context within which a material is being evaluated
- the importance of measuring a specific parameter within that context
- the feasibility of measuring the parameter within a specific context.


Testing the toxicity of nanomaterials also requires special considerations and are covered in the OECD Working Party Manufactured Nanomaterials Report, No. 33, titled ‘Important issues on risk assessment of manufactured nanomaterials’ (OECD, 2012). Traditional test methods need to be applicable to the nanomaterial under consideration. For example, whether a material is soluble, insoluble, or partially soluble may affect the suitability of a traditional toxicity test. If a traditional toxicity testing method cannot be satisfactorily modified to be fit for purpose, new methods may be needed. In addition, caution is needed if extrapolating the results of in vitro studies to an in vivo assessment since a direct translation seldom applies. It is also important to keep in mind that in vitro tests are most useful in providing information on mechanistic processes and in clarifying mechanisms and modes of action suggested by studies in whole animals.

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⁶ Aspect ratio describes the primary dimension over the secondary dimension(s).
Biokinetics, which deals with the absorption, distribution, metabolism and excretion (ADME) of substances in the body, determines the internal exposure of organs to potentially toxic substances. In addition to a sound knowledge of the biokinetics of a nanomaterial being necessary, the possibility of nanoparticles having a ‘Trojan horse’ effect by acting as carriers of contaminants (Shipley et al, 2008) must also be considered.

The impact of nanomaterials on the environment has been the subject of intense and ongoing research. The impact is more pronounced with non-biodegradable nanoparticles that persist and accumulate in the environment. The current knowledge base highlights the need to track the full lifecycle of manufactured nanomaterials and adopt ‘safe by design’ concepts for reducing, or even preventing altogether, the detrimental impact of nanomaterials on the environment. Two examples of ‘safe by design’ concepts applied to nanoparticles are those designed to dissolve very slowly, and those coated with inert compounds. The OECD Working Party on Manufactured Nanomaterials, Report No. 33, discusses in details the environmental risk assessment framework as well as elements that need to be considered when assessing ecological risks (OECD, 2012).

Nanotechnology-enabled products used in food-producing animals and crops need to be evaluated for their potential to leave residues in food. A recent expert meeting noted that the current risk assessment approaches used by FAO/WHO and Codex for residues in food are suitable for manufactured nanomaterials, but any additional safety concerns arising from the characteristic properties of nanomaterials would need to be addressed. (FAO/WHO, 2010). It is also noted in reference to Codex standards that: ‘neither the specifications nor the ADI for food additives that have been evaluated in other forms are intended to apply to nanoparticulate materials’ (WHO, 2007).

### 1.8 Regulation of nanomaterials in Australia

A question asked increasingly by consumers and scientists alike is ‘Are nanotechnology products safe?’ A related question is ‘What is known about the fate of nanotechnology products in the body and the environment?’ While it is generally accepted that many areas of nanotechnology do not present new hazards, there remain information gaps in our understanding of nanotechnology products that only research can fill.

In Australia, the APVMA is responsible for regulating AgVet chemicals and chemical products, including those based on nanotechnology, up to the point of retail sale. Protecting human health and the environment from these substances is a seminal legislative responsibility of the APVMA. Regulating an emerging technology such as nanotechnology is not a unique problem and the challenge confronting the APVMA has been reduced because, to date, it has received only one nanotechnology-enabled product application for registration. Currently, the APVMA practices a case-by-case approach to the risk assessment of nanomaterials. The general consensus is that, for the foreseeable future, the existing regulatory framework developed for non-nanoscale chemicals, in conjunction with a case-by-case approach, will be used to regulate nanomaterials. Over time, however, the framework will evolve as new information highlighting limitations in the current risk assessment paradigm becomes available. The development of a rational regulatory framework for nanopesticides and veterinary nanomedicines will be guided by a better understanding of the biological behaviour of nanomaterials in humans and the environment.
1.9 Conclusion

It is critical that strategies are available for minimizing the potential risks such that the social, environmental, and economic benefits of nanotechnology are fully realized. The APVMA has a key role in ensuring that nanotechnology-enabled AgVet chemicals and chemical products are introduced to Australian agriculture and animal husbandry in a safe and responsible manner.

The objective of this report is to highlight the regulatory issues that need to be considered when bringing AgVet products of nanotechnology into the Australian market. Chapters of the report address relevant aspects of nanotechnology including definitions, metrology, physicochemical properties, manufacture and the potential impacts on human health and the environment. Every attempt has been made to ensure the information on this rapidly evolving field was current at the time of writing. The report represents a first attempt to offer a blueprint on the regulatory considerations applicable to nanotechnology in Australian agriculture and animal husbandry.
1.10 References


2 LEGISLATIVE AND POLICY CONSIDERATIONS IN THE AUSTRALIAN REGULATORY FRAMEWORK

2.1 Legislative and Policy Considerations

2.1.1 Global context

Worldwide regulatory approaches to nanotechnology vary but are becoming increasingly consistent. In 2007 when Ludlow et al reviewed nanotechnology and applicable Australian regulatory frameworks for industrial, human therapeutic and AgVet chemicals, international regulators were also only just commencing their own considerations.

Since then there have been a number of significant steps taken overseas. Charriere and Dunning (2014) provide a detailed timeline and overview of nanotechnology policy and regulation in Canada, Australia, the European Union, the United Kingdom and the United States.

Collectively, the data identifies challenges common across international regulators that include terminology, definitions, testing methods and standards, standardised measurement, calibration and reference materials (Purushotham 2014).

2.1.2 Australian context

The APVMA has specific regulatory oversight of nanomaterials where they constitute, or are intended for use in, agricultural or veterinary (AgVet) chemical products. APVMA control occurs at several stages of the ‘nanofamily lifecycle’ identified by Ludlow et al (2007:29). These stages include importation, manufacture and supply.

Importing substances that are active constituents (that are neither approved nor exempt) for a proposed or existing chemical product requires APVMA import consent. Likewise, importing a chemical product that is not registered or exempt requires consent.

The Agricultural and Veterinary Chemicals Code Act 1994 prohibits supply of AgVet chemical products or active constituents unless the substances have been authorised by the APVMA via registration, approval, permit or some other form of exemption. There are some exceptions to this requirement provided in the Agricultural and Veterinary Chemicals Code Regulations 1995. Regulation 40 allows quantities of active constituents and products to be imported without consent where the quantities imported meet certain quantity requirements, and are for the purpose of research.

The base regulatory triggers relate primarily to the function and intended purpose of a chemical substance and are informed by understanding its chemical composition and any risks arising from its proposed use.

The schedule to the Agricultural and Veterinary Chemicals Code Act 1994, defines agricultural chemical products as follows:

‘… a substance or mixture of substances that is represented, imported, manufactured, supplied or used as a means of directly or indirectly:

a) destroying, stupefying, repelling, inhibiting the feeding of, or preventing infestation by or attacks of, any pest in relation to a plant, a place or a thing; or
b) destroying a plant; or

c) modifying the physiology of a plant or pest so as to alter its natural development, productivity, quality or reproductive capacity; or

d) modifying an effect of another agricultural chemical product; or

e) attracting a pest for the purpose of destroying it'.

(Agvet Code s.4)

The schedule to the Agricultural and Veterinary Chemicals Code Act, 1994, defines veterinary chemical products as:

‘... a substance or mixture of substances that is represented as being suitable for, or is manufactured, supplied or used for, administration or application to an animal by any means, or consumption by an animal, as a way of directly or indirectly:

a) preventing, diagnosing, curing or alleviating a disease or condition in the animal or an infestation of the animal by a pest; or

b) curing or alleviating an injury suffered by the animal; or

c) modifying the physiology of the animal:

d) so as to alter its natural development, productivity, quality or reproductive capacity; or

e) so as to make it more manageable; or

f) modifying the effect of another veterinary chemical product’.

(Agvet Code s.5)

Additional provisions and regulations further refine these definitions by specifying the inclusion and exclusion of certain substances in certain circumstances (Schedules 3 and 3AA to the Agricultural and Veterinary Chemicals Code Regulations 1995).

Amendments to AgVet legislation took effect on 1 July 2014. The AgVet Code restates provisions clarifying that the health and safety of human beings, animals and the environment are the priority of the regulatory system. Among other things, the amendments emphasise the need to align regulatory effort with risk. New provisions are drafted to emphasise that the AgVet Code must be implemented using science-based risk analysis processes (Explanatory Memorandum: 2010-13: p19).

The legislation now includes reference to safety criteria, trade criteria and efficacy criteria and outlines the circumstances in which these criteria are relevant to the APVMA’s consideration of product registration or active approval, or issue of an APVMA permit.

The safety criteria reflect the pre-2014 consideration of safety. However, there is now scope for the APVMA to determine when the criteria become relevant, based on risk. The APVMA must have regard to safety criteria before deciding whether to approve a new active constituent. Similarly, before registering a chemical product, the APVMA must have regard to the safety criteria and trade criteria or an established standard for the product, and in certain circumstances the APVMA must also have regard to efficacy criteria. The APVMA’s satisfaction with regard to safety must endure, and the APVMA may reconsider products or active constituents to determine whether they continue to meet the safety criteria.
Additional guidance is provided in legislative instruments and guidelines prepared under Section 6A of the AgVet Code (http://apvma.gov.au/node/981).

2.1.3 Safety criteria

For the purposes of being satisfied whether an active constituent meets the safety criteria the APVMA:

a) must have regard to the following:

i. the toxicity of the constituent and its residues, including metabolites and degradation products, in relation to relevant organisms and ecosystems, including human beings;

ii. the method by which the constituent is, or is proposed to be, manufactured;

iii. the extent to which the constituent will contain impurities;

iv. whether an analysis of the chemical composition of the constituent has been carried out and, if so, the results of the analysis;

v. any conditions to which its approval is, or would be, subject;

vi. any relevant particulars that are, or would be, entered in the Record for the constituent; (vi-a) whether the constituent conforms, or would conform, to any standard made for the constituent under section 6E to the extent that the standard relates to matters covered by subsection (1);

vii. any matters prescribed by the regulations; and

b) may have regard to such other matters as it thinks relevant.

For the purposes of being satisfied as to whether a chemical product meets the safety criteria, the APVMA:

a) must have regard to the following:

i. the toxicity of the product and its residues, including metabolites and degradation products, in relation to relevant organisms and ecosystems, including human beings;

ii. the relevant poison classification of the product under the law in force in this jurisdiction;

iii. how the product is formulated;

iv. the composition and form of the constituents of the product;

v. any conditions to which its registration is, or would be, subject;

vi. any relevant particulars that are, or would be, entered in the Register for the product; (vi-a) whether the product conforms, or would conform, to any standard made for the product under section 6E to the extent that the standard relates to matters covered by subsection (1);

vii. any matters prescribed by the regulations; and

b) may have regard to one or more of the following:
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i. the acceptable daily intake of each constituent contained in the product;

ii. any dietary exposure assessment prepared under subsection 82(4) of the Food Standards Australia New Zealand Act 1991 as a result of any proposed variation notified under subsection 82(3) of that Act in relation to the product, and any comments on the assessment given to the APVMA under subsection 82(4) of that Act;

iii. whether any trials or laboratory experiments have been carried out to determine the residues of the product and, if so, the results of those trials or experiments and whether those results show that the residues of the product will not be greater than limits that the APVMA has approved or approves;

iv. the stability of the product;

v. the specifications for containers for the product;

vi. such other matters as it thinks relevant.

2.1.4 Trade criteria

5C Definition of ‘meets the trade criteria’:

1. A chemical product meets the trade criteria if use of the product, in accordance with instructions approved, or to be approved, by the APVMA or contained in an established standard, does not, or would not, unduly prejudice trade or commerce between Australia and places outside Australia.

2. For the purposes of being satisfied as to whether a chemical product meets the trade criteria, the APVMA must have regard to the following:

(a) any conditions to which its registration is, or would be, subject;

(b) any relevant particulars that are, or would be, entered in the Register for the product;

(ba) whether the product conforms, or would conform, to any standard made for the product under section 6E to the extent that the standard relates to matters covered by subsection (1);

(c) any matters prescribed by the regulations.

3. For the purposes of the operation of this Code in relation to a particular chemical product, the APVMA is required to have regard to the matters set out in subsections (1) and (2) only:

(a) to the extent prescribed by the regulations; or

(b) if there are no such regulations—to the extent that the APVMA thinks the matters are relevant.

2.1.5 Efficacy criteria

5B Definition of ‘meets the efficacy criteria’:

1. A chemical product meets the efficacy criteria if use of the product, in accordance with instructions approved, or to be approved, by the APVMA for the product or contained in an established standard, is, or would be, effective according to criteria determined by the APVMA by legislative instrument.
2. For the purposes of being satisfied as to whether a chemical product meets the efficacy criteria, the APVMA must have regard to the following:

(a) whether any trials or laboratory experiments have been carried out to determine the efficacy of the product and, if so, the results of those trials or experiments;

(b) any conditions to which its registration is, or would be, subject;

(c) any relevant particulars that are, or would be, entered in the Register for the product;

(ca) whether the product conforms, or would conform, to any standard made for the product under section 6E to the extent that the standard relates to matters covered by subsection (1);

(d) any matters prescribed by the regulations.

3. For the purposes of the operation of this Code in relation to a particular chemical product, the APVMA is required to have regard to the matters set out in subsections (1) and (2) only:

(a) to the extent prescribed by the regulations; or

(b) if there are no such regulations—to the extent that the APVMA thinks the matters are relevant.

### 2.1.6 Standards for chemical products and actives

Section 87 of the Agricultural and Veterinary Chemicals Code Act 1994 requires chemical products to comply with the standard prescribed for the product (if any). (For the purposes of s.87, Regulation 43 of the Agricultural and Veterinary Chemicals Code Regulations 1995 prescribes all chemical products.)

Where a standard applies to a constituent of a chemical product, the constituent must comply with that standard. Standards that may be applied are in the form of a cascade. Standards developed by the APVMA take precedence, followed by (for veterinary substances) standards specified in the British Pharmacopoeia, the British Pharmacopoeia (Veterinary), the European Pharmacopoeia or the United States Pharmacopoeia, followed by standards specified in the FAO and WHO Specifications for Pesticides. If no standard is applicable then the standard is as set out in the table at Regulation 42(4) of the Agricultural and Veterinary Chemicals Code Act Regulations 1995.

The APVMA has not yet needed to develop standards for nanomaterials as no applications have been made for it to approve nanomaterial-active constituents. Additional guidance is provided in APVMA guidelines prepared under Section 6A of the Agvet Code ([http://apvma.gov.au/node/981](http://apvma.gov.au/node/981)).

In 2007 the Monash Review (CIECS: 2011: 30-31) identified various areas of potential concern regarding the Australian regulatory oversight of nanomaterials. These remain relevant to the future APVMA approach to regulating chemical products based on nanotechnology and are issues also identified by international regulators.

Key issues can be grouped into three main topics: whether nanoform materials should be considered new substances; metrology and definitions, including threshold measures for triggering regulatory action; and risk assessment methodologies.
2.1.7 Nanomaterials as new substances

The first issue is whether materials in nanoform should be considered ‘new’ substances. International regulators are still grappling with this issue, for instance the US is considering policy in 2014 to decide whether nanomaterials are ‘new’.

The APVMA is not limited in this regard, however it has proved an issue for overseas regulators. Irrespective of ‘nanoform’, the APVMA legislative framework allows it to consider products (mixtures of chemical substances) if they are represented or intended for use as agricultural or veterinary chemical products that would require approval or registration. Regulatory guidelines prepared by the APVMA include the requirement to indicate whether any constituent used in a product has nanoscale properties. Similarly the Regulatory Guidelines for chemistry and manufacture (http://new.apvma.gov.au/node/473) include detailed advice about the information required where nanoscale materials are included.

2.1.8 Metrology, definitions and thresholds

Threshold weights or quantities of nanomaterials which trigger regulatory oversight vary internationally. The EU trigger quantity is one tonne of materials. While risks are mitigated via mandatory reporting schemes, this figure is currently under review.

APVMA regulatory triggers do not rely on threshold weights or volumes of materials, although there are exemptions in APVMA regulations for quantities of materials involved in research activities.

Relevant to these quantum issues, specific exemptions for research and development uses that currently apply to conventional materials may assume greater significance for potentially hazardous nanomaterials and their products.

Authorisations via an APVMA permit require similar consideration of safety criteria as for products and actives. Applications for permits require a declaration regarding nanomaterial content.

A related factor is consideration of the threshold definitional values for nanomaterials. For instance, what are the threshold values for considering an active or product to require consideration as a nanomaterial? While the APVMA currently requires a declaration from an applicant for active approval or product registration if it contains nanomaterials, this declaration relies on the definition, and also on an applicant reasonably understanding the presence or absence of nanomaterials. The APVMA has prepared regulatory content including the definition of nanomaterials (http://new.apvma.gov.au/definition-of-terms/n).

APVMA Regulatory guidelines state that, where size distribution shows that, by number of particles, 10% or more of a substance is at the nanoscale, the substance will be considered a nanomaterial for risk assessment purposes. This is consistent with the European Food Safety Agency guidelines regarding safety and uncertainty (http://www.euractiv.com/health/meps-reject-commissions-definiti-news-533499). The APVMA’s partner regulator for Industrial Chemicals (NICNAS) adopts a similar approach (see Chapter 3).
2.1.9 Other issues relating to the regulation of nanomaterials

A less-defined series of issues identified in the 2007 study relate to the understanding of risks associated with nanoform materials. Without a clear understanding of the particular risks (or absence thereof), regulatory processes triggered by threshold risks may not be invoked where effects of engineered nanomaterials on human health are currently unknown. There is scope also for risk to accrue based on a variety of factors including the inherent toxicology of the parent material when rendered in nanoform and risks inherently introduced via the manufacturing process.

Irrespective of the presence of nanomaterials, the APVMA needs to be satisfied with the safety criteria (see above) before approving an active constituent or registering a chemical product. Later incorporation of nanoscale-registered constituents into already registered products would render the product non-compliant with particulars recorded at the time of registration.

The APVMA currently requires applications for permits, product registration or active constituent approval to include a declaration about whether the proposed activity, product or active includes nanoparticles. This is in effect a notification scheme for new products or actives, but will not capture research activities covered by general APVMA permits (eg PER7250) or where the weights of materials concerned are excluded by Regulation.
2.2 References


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3 DEFINITIONS, NANOMETROLOGY, PHYSICOCHEMICAL PROPERTIES

3.1 Introduction

The regulation of nanomaterials requires a definition and validated methods and instrumentation to detect, characterise, and analyse nanomaterials.

This report begins by examining the current situation regarding the definition of a nanomaterial. This is followed by a review of the physico-chemical parameters needed for risk assessment and a brief account of the instruments and techniques used to measure these parameters, focusing on nanoparticles. A detailed account of the instruments and techniques used in nanometrology is presented in Appendix 1 of this report.

3.2 Defining Nanomaterials

The term ‘nanomaterial’ informally refers to materials with external dimensions or an internal structure in the nanometre length range, a nanometre being one billionth of a metre. These materials typically exhibit different or additional properties and behaviour compared to the same material without nanoscale features.

Stakeholders such as government, industry, the scientific community, standards organisations, non-government organisations and others have expended much effort in recent years in attempting to define a nanomaterial. The result has been a wide range of definitions which are largely consistent and have common elements.

Various stakeholders have different ‘framesets within which challenges and options are discussed, varying interpretations of what a nanomaterial is, and confusion over the underlying science and its implications to risk’ (Maynard et al. 2011). The result is a ‘wicked’ public policy problem — in which stakeholders are unable to agree on the nature of the problem (to the degree that it exists at all) or on the most desirable solution (Klijn 2008).

Regulatory definitions are used to identify those substances that are captured within regulatory frameworks. Risk assessments for regulatory purposes determine the hazard and exposure of humans and the environment to these substances and, where it is possible identify measures, where possible, to manage any potential risks identified. The protection of human health and the environment is the primary objective of the risk assessment. Regulatory definitions are also important in enforcement activities. Recommended general reading on regulating nanotechnology includes Hodge et al. 2007, Hodge et al. 2010, Hodge et al. 2013.

Research has established that the point at which nanomaterials change their behaviour from conventional to unconventional behaviour depends on the particular material and the context. Thus, the boundary between nanoscale and non-nanoscale material behaviour is often indistinct and may depend on many parameters, including size, particle shape, porosity, surface area and chemistry. This has led some to suggest that a ‘one size fits all’ general definition of an engineered nanomaterial will inevitably fail to capture what is important for addressing risk (Maynard 2011). Regulators consider all of these factors when assessing the potential risks posed by nanomaterials.

A detailed analysis of 27 existing definitions of nanomaterials from four different sources, namely academic institutions and scientific advisors, regulators, non-government organizations and four
international organizations (Saner and Stoklosa 2013) shows that the most common elements of these definitions are size, structure and properties/novel phenomena.

Note that the word ‘size’ is used commonly in the literature, with units of nanometres, when what is meant is ‘length’. ‘Size’ strictly refers to the dimensions, proportions, amount, or extent of something and can include mass, volume, area and number, whereas ‘length’ should be used when referring to a distance. To avoid confusion, this report will continue to use the word ‘size’.

The following are reviews of some of the more significant published definitions of the term ‘nanomaterial’ and related terms. A more detailed review may be found in Lövestam et al, 2010.

### 3.2.1 International Organisation for Standardisation (ISO) definition

ISO is the world’s largest developer of standards. It is a non-governmental network of the national standards bodies of 157 countries, supported by the Central Secretariat based in Geneva, Switzerland. The principal deliverables of ISO are international documentary standards embodying the essential principles of global openness and transparency, consensus and technical coherence. ISO standards are developed by experts nominated by the national member bodies contributing to the work of the particular committee responsible for the subject matter under consideration.

ISO Technical Committee TC229 (TC229) was formed in 2005 and is the main ISO technical committee responsible for international standardisation work related to nanotechnologies. It was created to complement and coordinate the nano-relevant standardisation work already undertaken by other ISO technical committees. TC229 has formal liaisons with several other major players in the nanotechnology standardisation arena, including (1) the Organisation for Economic Cooperation and Development (OECD), which has devoted two working parties to the topic (Working Party on Manufactured Nanomaterials, WPMN, and Working Party on Nanotechnology, WPN), (2) the International Bureau of Weights and Measures (BIPM), (3) the European Commission’s Joint Research Centre (JRC), a research based policy support organisation and (4) the Versailles project on Advanced Materials and Standards (VAMAS), which is active in pre-normative research.

Initially, three working groups were established by TC229, namely WG1: Terminology and Nomenclature, convened by Canada, WG2: Measurement and Characterisation, convened by Japan and WG3: Health, Safety, and Environmental Aspects of Nanotechnologies, convened by the USA.

The work of WG1 continues to be a critical foundation and priority for ISO TC229, as the development of standards for measurement, characterization and health and safety cannot be completed until consensus on terminology, a controlled vocabulary and nomenclature is reached. It follows that regulations, legal contracts and health and safety guidelines cannot be written until agreement on terminology is reached.

TC229 has published six Technical Specifications on nanotechnology terminology so far, namely

ISO/TS 27687: 2008 Nano-objects—nanoparticle, nanofibre, nanoplate
ISO/TS 80004-1: 2010 Core Terms
ISO/TS 80004-3: 2010 Carbon nano-objects
ISO/TS 80004-4: 2011 Nanostructured materials
ISO/TS 80004-7: 2011 Diagnostics and Therapeutics for healthcare
a) TS 80004-1 (Core Terms)

Important definitions in TS 80004-1 are:

- **Nanoscale**
  Size range from approximately 1 nanometre (nm) to 100 nm.
  
  *Note 1 –* Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties the size limits are considered approximate.
  
  *Note 2 –* The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as nano-objects or elements of nanostructures, which might be implied by the absence of a lower limit.

- **Nanotechnology**
  Application of scientific knowledge to manipulate and control matter in the nanoscale in order to make use of size and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules, or with bulk materials.

- **Nanomaterial**
  Material with any external dimension in the nanoscale, or having internal structure or surface structure in the nanoscale.
  
  *Note 1 –* This generic term is inclusive of nano-object and nanostructured material.
  
  *Note 2 –* See also engineered nanomaterial, manufactured nanomaterial and incidental nanomaterial.

- **Nano-object**
  Material with one, two or three external dimensions in the nanoscale.
  
  *Note –* Generic term for all discrete nanoscale objects.

- **Nanostructure**
  Composition of interrelated constituent parts, in which one or more of those parts is a nanoscale region.
  
  *Note –* A region is defined by a boundary representing a discontinuity in properties.

- **Nanostructured material**
  Material having internal nanostructure or surface nanostructure.
  
  *Note –* This definition does not exclude the possibility for a nano-object to have internal structure or surface structure. If external dimension(s) are in the nanoscale, the term nano-object is recommended.

- **Engineered nanomaterial**
  Nanomaterial designed for a specific purpose or function.

- **Manufactured nanomaterial**
  Nanomaterial intentionally produced for commercial purposes to have specific properties or specific composition.

TS 80004-1 is currently being reviewed, and this is due for completion in 2015. Significantly, the term ‘nanomaterial’ is excluded from this review. The review is considering removing the term ‘approximately’ from the definition of the nanoscale and extending the range above 100 nm for biological and environmental, health and safety (EHS) reasons. A definition of ‘bulk material’ is likely to be added.
b) TS 27867 Nano-objects — nanoparticle, nanofibre, nanoplate

TS 27867 is also currently being reviewed and is scheduled for completion in December 2014. It will then be renamed ISO/TS 80004-2: Vocabulary for nano-objects, nanoparticle, nanoplate, and nanofibre.

Important definitions are:

- **Nanoparticle**
  Nano-object with all three external dimensions at the nanoscale.
  \*Note – If the lengths of the longest and the shortest axes of the nano-object differ significantly, the terms nanorod or nanoplate should be considered. ‘Significantly’ is considered to mean more than three.
  Section 4 of TS 27687 is concerned with assemblies of particles and defines agglomerates and aggregates as:

- **Agglomerate**
  Collection of loosely bound particles or aggregates, or mixtures of the two, where the resulting external surface area is similar to the sum of the surface areas of the individual components.
  \*Note 1 – The forces holding an agglomerate together are weak forces, for example van der Waals forces, as well as simple physical entanglement.
  \*Note 2 – Agglomerates are also termed secondary particles.

- **Aggregate**
  Particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components.
  \*Note 1 – The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.
  \*Note 2 – Aggregates are also termed secondary particles and the original source particles are termed primary particles.

The review of TS 27867 is likely to introduce the definition of a ‘primary particle’.

The ISO definition of a nanomaterial is very broad. It includes all nano-objects (nanoparticles, nanofibres and nanoplates) and nano-structured materials (including aggregates and agglomerates). The central use of the term ‘nanoscale’ means that a nanomaterial is essentially categorised according to the size of its constituent parts. It does not require a nanomaterial to display unique or specific properties or have a specific risk.

The 1–100 nm range specified in the ISO definition of nanoscale is commonly used as a threshold in the field of nanotechnology. However, there is no scientific evidence to support this. Indeed, it is now apparent that, in many cases, the unique properties and phenomena associated with size and shapes in engineered materials extend above and below the nanoscale, with applications in medicine, cosmetics and food. Also, single thresholds do not take into account the fact that the constituents of most nanomaterials have a size distribution. When only a part of the nanomaterial has a size within the size range of the definition, it should be clear whether, and when, such a material would be considered a nanomaterial (European Commission Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) 2010).
3.2.2 Organisation for Economic Co-operation and Development (OECD) definition

The OECD established a Working Party on Manufactured Nanomaterials (WPMN) in 2006 and adopted the draft TC229 definition of nanomaterial as a working definition for the term 'manufactured nanomaterial'. This was later modified to:

Manufactured nanomaterials: Nanomaterials intentionally produced to have specific properties or specific composition, a size range typically between 1 nm and 100 nm and material which is either a nano-object (ie that is confined in one, two, or three dimensions at the nanoscale) or is nanostructured (ie having an internal or surface structure at the nanoscale) (OECD Working Party on Manufactured Nanomaterials 2008).

This definition adds intention to produce unique physico-chemical properties to the size-based ISO definition. The ISO and OECD definitions specify an approximate size range for nanomaterials; however they do not address the issue of size distributions and are difficult to apply in a regulatory context.

3.2.3 European Union (EU) Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) definition

In a 2007 report the European Union (EU) Scientific Committee on Emerging and Newly Identified Health Risks (European Commission Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) 2007), concluded that the term nanomaterial is a categorization of a material due to its size, and defined a nanomaterial as:

Any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less.

SCENIHR also stressed that size distribution should be taken into account when defining a nanomaterial. This accounts for the materials in which only a part has a size within the size range of the definition. It is suggested that this could be achieved by considering the percentage of the number size distribution that is above or below the threshold. For example, a material could be considered a nanomaterial when more than 0.15% of the material, as indicated by the number concentration, has a size below the designated upper size limit.

In 2010, SCENIHR published a report on the scientific basis for the definition of nanomaterial. It was concluded that:

‘Whereas physical and chemical properties of materials may change with size, there is no scientific justification for a single upper and lower size limit associated with these changes that can be applied to adequately define all nanomaterials.

There is no scientific evidence for a single methodology (or group of tests) that can be applied to all nanomaterials.'
Size is universally applicable to define all nanomaterials and it is the most suitable measure. Moreover, an understanding of the size distribution of a nanomaterial is essential and the number size distribution is the most relevant consideration.

In order to define an enforceable definition of “nanomaterial” for regulatory use it is proposed to set an upper limit for nanomaterial size and to add to the proposed limit additional guidance (requirements) specific for the intended regulation. Crucial in the guidance that needs to be provided is the extended description of relevant criteria to characterise the nanoscale. Merely defining single upper and lower cut-off limits is not sufficient in view of the size distributions occurring in manufactured nanomaterials.

Alternatively, a tiered approach may be required depending on the amount of information known for any specifically manufactured nanomaterial and its proposed use.

The scientific opinion recognises however that specific circumstances regarding risk assessment for regulatory purposes for certain areas and applications may require the adaptation of any overarching definition.

It should be stressed that “nanomaterial” is a categorization of a material by the size of its constituent parts. It neither implies a specific risk, nor does it necessarily mean that this material actually has new hazard properties compared to its constituent parts or larger sized counterparts.’

The SCENIHR report was the first to highlight the need to consider a size distribution as well as a size range in defining a nanomaterial. The report also emphasised the lack of a scientific basis for a simple size range such as 1-100 nm, implying that the use of such a size range in a nanomaterial definition would be a policy decision rather than a scientific one.

### 3.2.4 Joint Research Center (JRC) of the EU definition

The JRC published a reference report (Lövestam et al. 2010) that did not include a specific definition for a nanomaterial. But it did recommend that a definition for regulatory purposes should use size as the defining property, should include size distribution considerations and, significantly, only concern particulate materials.

The justification for restricting a definition for regulatory purposes to materials which are in a particulate form at the nanoscale, and which are mobile in their immediate environments, is that it is only these materials that raise environmental, health and safety (EHS) concerns.

The JRC 2010 report specifically targeted a nanomaterial definition for regulatory purposes. It therefore focused on identifying materials that may pose risks to health, safety or the environment. This leads to the restriction of a definition to particulate materials, a significant reduction in generality.
3.2.5 EU Definition

The EU has definitions of nanomaterial in several regulations, including one on cosmetics (European Commission 2009):

Nanomaterial means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm.

and one on food (European Commission Scientific Committee on Emerging and Newly Identified Health Risks SCENIHR 2010):

Engineered nanomaterial means any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale.

The most significant definition of a nanomaterial published by the EU was a recommendation in October 2011 (European Commission 2011) based on the JRC report (Lövestam et al. 2010), the SCENIHR opinion (European Commission Scientific Committee on Emerging and Newly Identified Health Risks SCENIHR 2010) and the definition of nanomaterial developed by the ISO. The recommended definition was:

Nanomaterial means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm to 100 nm.

In specific cases, and where warranted by concerns for the environment, health, safety or competitiveness, the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.

The definitions of particle, agglomerate and aggregate are essentially the same as the ISO definitions for these terms.

The recommendation includes a statement that by derogation, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials. Also, compliance with this definition may be determined on the basis of the specific surface area by volume, namely a material should be considered as falling under the definition where the specific surface area by volume of the material is greater than 60 m²/cm³. This recommendation has recently been incorporated into a regulation for biocidal products (European Union (EU) 2012).

The EU recommendation:

- includes incidental and natural materials as well as engineered materials
- is restricted to nanoparticles
- includes aggregates and agglomerates of nanoparticles
- focuses on the size of the nanoparticles
- includes a specific size distribution requirement, and
• allows the number distribution threshold to be varied depending on environmental, health and safety concerns.

It is puzzling that the EU definition did not use the ISO defined term ‘nano-object’, choosing instead ‘nanoparticles’ which excludes nanoplates and nanofibres, both of which could have EHS concerns.

The EU recommendation has been controversial and has prompted global debate (Foss Hansen et al. 2013; Bleeker et al. 2013). Member states, Union agencies, and economic operators within the EU are invited to use the definition, but very few regulations have been introduced that use the recommendation. It is not harmonised with other jurisdictions, including the USA.

Indeed, some have argued that the EU definition uses criteria that are not supported by current data on nanomaterial risk and that perhaps nanomaterials should not be explicitly defined at all (Maynard 2011). The alternative view is that a definition is required for labelling purposes, and would assist industry and regulators in identifying where specific safety assessments might be necessary (Stamm 2011).


### 3.2.6 North American definitions

The United States National Nanotechnology Initiative (NNI) describes nanotechnology as ‘the understanding and control of matter at dimensions between approximately 1 and 100 nanometres, where unique phenomena enable novel applications’ (National Nanotechnology Initiative 2009).

Adding intention to this leads to the NNI definition of an engineered nanomaterial as:

A material that has been purposely synthesized or manufactured to have at least one external dimension of approximately 1 to 100 nanometres – at the nanoscale – and that exhibits unique properties determined by this size (National Nanotechnology Initiative 2011).

The United States Food and Drug Administration (FDA) states that while there is no formal agency definition, it does offer ‘guidance’ on its ‘current thinking’. When considering whether an FDA-regulated product contains nanomaterials, or otherwise involves the application of nanotechnology, the FDA will ask:
In June 2014 the FDA updated its ‘guidance for industry’, saying it would only consider products to be ‘engineered’ to have certain dimensions or exhibit certain properties if they have been subject to ‘deliberate and purposeful manipulation’ by nanotechnology. It said the incidental presence of particles in the nanoscale range in ‘conventionally-manufactured’ products did not fall under the guidance if they had not been deliberately manipulated to be their size. ‘Familiar’ biological and chemical nanoscale substances such as microorganisms and proteins are also not covered by the guidance.

The FDA added that while it has no opinion on whether nanotechnology is ‘inherently safe or harmful,’ its use ‘may result in product attributes that differ from those of conventionally-manufactured products and thus may merit particular examination’.

The United States Environmental Protection Agency (EPA) also has no formal agency definition. However, it has outlined key criteria across several documents including:

- particle size between 1 and 100 nm in at least one dimension
- the material exhibits unique properties compared to larger sized particles
- the material is engineered at the nanoscale
- inclusion of aggregates and agglomerates, and
- a distribution of particles with greater than 10% by weight less than 100 nm.

The Office of Pesticide Programs (OPP), part of the EPA, has a working definition of a nanoscale material (U.S. Federal Register 2011), namely:

An ingredient that contains particles that have been intentionally produced to have at least one dimension that measures between approximately 1 and 100 nanometres.

Health Canada

Health Canada published a definition of nanomaterials in a policy statement that applies to all substances that it regulates including consumer products, industrial substances, food, therapeutic and AgVet products (Health Canada 2011) namely:

Health Canada considers any manufactured substance or product and any component material, ingredient, device, or structure to be nanomaterial if it is at or within the nanoscale in at least one external dimension, or has internal or surface structure at the nanoscale, or if it is smaller or larger than the nanoscale in all dimensions and exhibits one or more nanoscale properties/phenomena.

For the purposes of this definition:

- ‘Nanoscale’ means 1 to 100 nm, inclusive.
- ‘Nanoscale properties/phenomena’ means properties which are attributable to size and their effects; these properties are distinguishable from the chemical or physical properties of individual atoms, individual molecules and bulk material.
- ‘Manufactured’ includes engineering processes and the control of matter.
Health Canada is the only North American organisation to formally define a nanomaterial. US organisations and agencies typically do not formally define nanomaterials, instead giving a list of key criteria that a nanomaterial must satisfy. However it is noted that the Canada-US Cooperative Council Nanotechnology Initiative is developing common criteria for identifying characteristics of nanomaterials of concern, or no concern (http://actionplan.gc.ca/en/page/rcc-ccr/nanotechnology-work-plan).

3.2.7 Australian Definitions

National Industrial Chemicals Notification and Assessment Scheme (NICNAS) Australia

NICNAS is the only Australian Government department that has developed a working definition for regulatory purposes (NICNAS 2010, 2013). This working definition is broadly consistent with other available international definitions.

The NICNAS working definition is:

…industrial materials intentionally produced, manufactured or engineered to have unique properties or specific composition at the nanoscale, that is a size range typically between 1 nm and 100 nm, and is either a nano-object (i.e. that is confined in one, two, or three dimensions at the nanoscale) or is nanostructured (i.e. having an internal or surface structure at the nanoscale).

Notes to the working definition:

- Intentionally produced, manufactured or engineered materials are distinct from accidentally produced materials.
- ‘Unique properties’ refers to chemical and/or physical properties that are different because of a material's nanoscale features when compared with the same material without nanoscale features, and result in unique phenomena (e.g. increased strength, chemical reactivity or conductivity) that enable novel applications.
- Aggregates and agglomerates are considered to be nanostructured substances.
- Where a material includes 10% or more number of particles that meet the above definition (size, unique properties, intentionally produced) NICNAS will consider this to be a nanomaterial.

The NICNAS definition emphasizes intention and engineering. It is not restricted to primary nanoparticles but specifically includes aggregates and agglomerates.

NICNAS has also published detailed guidance in relation to nanomaterials, which is accessible at http://www.nicnas.gov.au/regulation-and-compliance/nicnas-handbook/handbook-appendixes/guidance-and-requirements-for-notification-of-new-chemicals-that-are-industrial-nanomaterials/specification-conditions-for-requesting-additional-data-requirements

Australian Pesticides and Veterinary Medicines Authority (APVMA).

The APVMA is currently developing a working definition for a nanomaterial based closely on the NICNAS definition. The review of current definitions of a nanomaterial given above would indicate that such a working definition should consider the following;

A nanomaterial should be an intentionally produced, manufactured or engineered substance with unique properties that are directly caused by size features with X per cent (to be determined) of the number size distribution of these features lying in the range approximately 1-100 nm (the nanoscale). There should be recognition that biological and EHS issues may require a different size range above 100 nm.

3.3 The Metrology of Nanomaterial

3.3.1 The parameters used to characterise nanomaterials

The appropriate characterisation of manufactured nanomaterials is critical for many fields, including manufacturing, regulation, environmental, health and safety risk assessments, food, toxicology, cosmetics, medicine, pharmaceuticals and pesticides. There have been numerous studies on which physico-chemical parameters should be used to characterise nanomaterials generally (for example (Stone et al. 2010, Stintz et al. 2010), but it is now well established that a single list of parameters, covering all fields, is not possible.

There is, however, some consensus on the parameters needed for risk assessment and hence regulation (OECD Chemical Committee 2009). Lists of such parameters, developed in recent years, include the following.

In May 2008, WG3 of TC229 (private communication) produced a focused list of Physico-Chemical Characteristics of Engineered Nano-Objects for Toxicological Assessment namely:

- agglomeration state/aggregation
- composition (eg chemical composition and structure)
- concentration
- hydrophobicity
- manufacturing process
- oxidizing properties
- particle size/size distribution
- purity
- shape
- solubility
- stability
- surface area
- surface chemistry
- zeta potential.

A SCENIHR report concentrates on the risk assessment of products of nanotechnologies (Scientific Committee on Emerging and Newly Identified Health Risks (SCENHIR) 2009) concluding that the main physico-chemical parameters of interest with respect to nanoparticle safety are:
Physical properties
- size, shape, specific surface area, aspect ratio
- agglomeration/aggregation state
- size distribution
- surface morphology/topography
- structure, including crystallinity and defect structure
- solubility.

Chemical properties
- structural formula/molecular structure
- composition of nanomaterial (including degree of purity, known impurities or additives)
- phase identity
- surface chemistry (composition, charge, tension, reactive sites, physical structure, photocatalytic properties, zeta potential)
- hydrophilicity/lipophilicity.

The OECD published a list focused on the safety aspects of nanomaterials. It concluded that the majority of the end-points and the test guidelines regarding physicochemical, environmental fate, ecotoxicological and toxicological properties found in existing OECD test guidelines were relevant and applicable to nanomaterials (OECD Chemical Committee 2009), (OECD 2010), (OECD Environment Directorate 2010).

In 2010, the OECD WPMN listed 17 physico-chemical properties for characterising nanomaterials (OECD 2010) namely:
- shape
- agglomeration/aggregation
- water solubility/dispersability
- crystalline phase
- dustiness
- crystallite size
- representative electron microscopy (TEM) picture(s)
- particle size distribution – dry and in relevant media
- specific surface area
- zeta potential (surface charge)
- surface chemistry (where appropriate)
- photocatalytic activity
- pour density
- porosity
- octanol-water partition coefficient, where relevant
- redox potential
- radical formation potential.

A report from a workshop on nanoparticle (NP) metrology (Stintz et al. 2010) summarised the properties of interest as follows:

Morphology
- characteristic length and areas in 2D-projection
- parameters describing aggregates/agglomerates
- shape parameters from morphology data, eg sphericity, aspect ratio, fractal dimension.

Size-related properties based on hydrodynamics and/or interaction with external fields
- diffusion coefficient, hydrodynamic diameter (of translation)
- settling velocity, Stoke’s diameter
- aerodynamic diameter
- acoustophoretic mobility
- (partial) scattering or extinction cross section.

**Surface area of the dispersed phase**
- via adsorption of gases
- via small angle X-ray scattering
- via titration experiments with surfactants, polyelectrolytes etc.

**Chemical composition and phase**
- crystallinity (amorphous fraction vs crystalline fraction)
- phase fractions of different crystallographic phases.

**Concentration of particles**
- mass, surface, number concentration
- total or fractional concentration.

**Interfacial properties**
- surface charge
- zeta potential
- surface conductivity
- pristine point of zero charge and iso-electric point (for different charge determining ions).

**Interaction with continuum/suspendants**
- solubility and dissolution kinetics
- ROS (radical oxidising species) potential
- wettability.

The ‘Report of the OECD Expert Meeting on the Physical Chemical Properties of Manufactured Nanomaterials and Test Guidelines’ in the Series on the Safety of Manufactured Nanomaterials, No. 41 was published recently (OECD, 2014). Guidance is provided for assessing the aggregation and agglomeration of nanomaterials and determining the size, surface area, porosity and surface reactivity of nanopesticides.

It is important to note that for all of these lists typically only a few of the parameters will need to be measured for a given application. It is also apparent that size and number size distribution are the two parameters universally applicable in characterising nanomaterials (Linsinger et al. 2012). The other parameters most commonly used are state of agglomeration and aggregation, shape, surface area, surface and bulk chemistry and zeta potential.

Before addressing the specific measurement issues involving nanomaterials, general matters concerning metrology and nanometrology need to be addressed.
3.3.2 Metrology - the science of measurement

Metrology, the science of measurement, is a well-developed scientific discipline with a long history. Metrology in the nanoscale is known as nanometrology. For further reading see Miles (2007), Miles (2010) and Jamting and Miles (2013).

The current international measurement system began in 1875 when 17 nations signed the Metre Convention, recognising the need for measurements to be uniform internationally. A further 37 member states are now signatories, with Australia signing in 1947. The Metre Convention established the structure and processes for worldwide uniformity in measurement, firstly through a harmonised set of units of measurement, the International System of Units (SI), and secondly through a recognised method of establishing measurement standards that realise these units. The international structures established under the Metre Convention cover scientific and industrial measurements and are described by the International Bureau of Weights and Measures (BIPM), located in Sèvres, France. They are overseen by the peak international expert metrology body, the International Committee for Weights and Measures (CIPM). Australia’s National Measurement Institute (NMIA) is Australia’s official representative to the Metre Convention’s activities.

The SI system is a set of agreed definitions duplicated in many countries. BIPM’s mission is worldwide consistency of measurements traceable to the SI. Traceability (see below) relates a measurement result, or the value of a standard, to references at higher levels ending at a national primary standard. In doing so it uses a chain of comparisons, all having stated uncertainties. International traceability allows nationally realized standards to be linked and known in terms of the SI units.

The BIPM cooperates with appropriate national authorities, normally the relevant National Metrology Institute (NMI). All Member States of the Metre Convention support a NMI that has, in general, the role of maintaining national measurement standards, ensuring their suitability for national needs, and transferring measurement traceability, metrological expertise and knowledge to national users through high level calibration services, advice, and other assistance.

Some of the key terms and concepts used in the field of metrology (Joint Committee for Guides in Metrology 2008) include:

Measurand – the quantity intended to be measured. This needs to be clearly defined and understood. For example, the measurand for the size of a complex-shaped nanoparticle may involve lengths in three dimensions, the aspect ratio, the temperature and the measuring technique used. The correct and full description of the measurand is a prerequisite for a successful measurement.

Reference – a measurement unit, a measurement procedure, a reference material, or a combination of them all. For example, the length of a given rod may be 5.34 m, a product of a number and a measurement unit, namely the metre.

Calibration – an operation that, under specified conditions:

- in a first step establishes a relation between the values of the quantity to be measured with measurement uncertainties provided by measurement standards, as well as corresponding indications with associated measurement uncertainties, and
- in a second step uses this information to establish a relation for obtaining a measurement result from an indication. This complex definition may be summarized as the operation that relates a measurement standard to the reading of an instrument.
Measurement Uncertainty – non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used. The measurement uncertainty is an estimate of the range of values within which the true value lies. It is a fundamental parameter, as important as the measurement result itself. For example, the diameter of a nanoparticle may be written as 10 nm ± 2 nm, where ± 2 nm is the measurement uncertainty.

Estimating the measurement uncertainty involves considering all known sources of uncertainty in the measurement process, and has to be done in accordance with the ISO Guide to the Uncertainty of Measurement (International Organization for Standardization and International Electrotechnical Commission). Typically, the measurement uncertainty is reported as the standard uncertainty multiplied by a coverage factor \( k = 2 \), which for a normal distribution corresponds to a coverage probability of approximately 95 %, i.e. the correct value of the measurand is within the range (measured value ± expanded uncertainty) at a confidence level of about 95 %.

Metrological Traceability – property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty. The traceability of measurements typically relates measurements to the SI, maintained by NMIs, which regularly perform international key and supplemental comparisons to ensure that their national references agree with those of other countries. Establishing metrological traceability is crucial if measurements are to be compared and accepted internationally. It allows meaningful comparison of measurement results, made at different times and different locations.

High quality measurements that may be relied upon for legal and regulatory purposes require clearly specified measurands and measurements that are traceable and made with calibrated measuring instruments by skilled observers in a suitable measuring environment. The measurement uncertainty must be properly determined and appropriate for the needs of the measurement.

### 3.3.3 Nanometrology

Significant efforts are ongoing nationally and internationally to achieve a harmonised and valid nanometrological measurement system. Programs are now in place in many countries, including Australia, to develop capability for performing high quality nanometrological measurements (NMIA).

Traceability for length measurements in the nanoscale is typically achieved at the NMI level by transferring the realisation of the primary standard for the metre down to measurements at the nanometre level. This is normally done using primary length standards to calibrate high magnification microscopes, such as an electron microscope (EM) or an atomic force microscope (AFM), fitted with optical interferometers on the translation axes. These microscopes are then used to calibrate the grids, gratings and line scales that are used to calibrate secondary AFMs or EMs. These secondary instruments are in turn used to calibrate reference standards or materials for the calibration of instruments in testing and industrial laboratories.

More generally, properly certified reference materials are being developed internationally and nationally (Roebben et al. 2011 and Roebben et al. 2013) as they are crucial for instrument calibration. Instrument manufacturers often provide reference materials to monitor the performance of their instruments, but these can lack traceability.

High quality nanometrology requires laboratories with independently proven competence, normally achieved by third party accreditation (NATA in Australia: [http://www.nata.asn.au/](http://www.nata.asn.au/)) using laboratory audits and interlaboratory comparisons to support method validation.
A significant complexity of physico-chemical characterisation of nanomaterials is that the properties may depend both on the employed methodology as well as the properties of the medium supporting the sample. The requirement that measurements be performed in various media adds significant complexity to the design of instruments, the handling of reference materials and the test methods.

It cannot be stressed too strongly that the results for the measurement of a given physico-chemical parameter of a nanoparticle will differ depending on the instrument and technique used and the supporting medium. It is equally important to realise that characterising a given nanomaterial demands the use of more than one measurement method.

a) Instruments and techniques used in nanometrology

Nanometrology uses a very large range of instruments, techniques and physical principles. Because the trend in regulating nanomaterials is to focus on nanoparticles, the rest of this report will likewise focus on the characterisation and nanometrology of nanoparticles.

Particle characterisation techniques may be classified into three different classes. Firstly, there are ensemble techniques that average over a large number of particles and measure an average for the system as a whole. These techniques provide good statistical representation of the particle system but are often unable to resolve contributions from individual particles or from small parts of a broad particle size distribution.

Secondly, there are single particle analysis techniques that measure the properties of individual particles and can resolve particle size distributions in great detail but are limited by small sample sizes. Although it is possible to increase the number of particles that are measured, the time and expense involved is often prohibitive.

Thirdly there are separation techniques. These are based on a separation step before applying detection and measuring techniques. Fractionation allows the sample to be separated into smaller volume fractions which can be detected with either an ensemble technique, now capable of detecting contributions to the measurement from each fraction, or further analysed using single particle analysis techniques.

Particle characterisation techniques may also be classified according to the parameter of interest, and then technique, as follows:

- **Size and shape**

  **Microscopy**, including scanning electron microscopy (SEM), transmission electron microscopy (TEM), scanning probe microscopy (SPM), atomic force microscopy (AFM), scanning tunneling microscopy (STM), near-field scanning optical microscopy (NSOM), fluorescence microscopy (FM) and confocal optical microscopy (COM).

  **Scattering techniques**, including dynamic light scattering (DLS), small angle x-ray scattering (SAXS), small angle neutron scattering (SANS) and particle tracking analysis (PTA).

  **Aerosol characterisation**, including condensation particle counting (CPC), differential electrical mobility classification (DEMC) and a differential mobility analysing system (DMAS).

  **Separation techniques**, including field flow fractionation (FFF), differential centrifugal sedimentation (DCS) and size exclusion chromatography (SEC).
Surface area measurement, including the Brunauer-Emmett-Teller (BET) method.

- **Chemistry**

Surface and bulk chemical analysis, including fluorescence spectroscopy, UV–Vis spectroscopy, fluorescence correlation spectroscopy (FCS), Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, electron energy loss spectroscopy (EELS), auger electron spectroscopy (AES), secondary ion mass spectrometry (SIMS), x-ray photoelectron spectroscopy (XPS), inductively coupled plasma mass spectrometry (ICP-MS), nuclear magnetic resonance spectroscopy (NMR) and energy-dispersive x-ray spectroscopy (EDS).

- **Charge in suspensions**

Zeta potential.

- **Mass**

Quartz crystal microbalance (QCM), differential scanning calorimetry (DSC).

- **Crystallinity**

X-ray diffraction, electron backscatter diffraction (EBSD)

b) **Sampling and dispersion**

Prior to measuring any parameter related to a nanoparticle, it is critical to first prepare a representative sample (Jamting and Miles 2013). Some of the issues with sampling are:

- ensuring that any sub-sample is representative of the whole sample
- minimizing sampling errors
- ensuring that the main sample is well mixed before sampling, and
- choosing appropriate sampling techniques.

If the nanomaterial is already a dilute liquid suspension, the sampling process is straightforward but care needs to be taken to ensure that the main sample is well dispersed before measurement. If the nanomaterial is in the form of a dry powder, dispersion of the sample must be concerned with:

- choice of a suitable suspendant
- choice of surfactant (if any)
- method of dispersing the powder into the suspendant
- wettability of the dry particles by the suspendant, and
- suspension stability.

Sample dispersion can also be a challenge, particularly for dry powders. Nanoparticles have a strong tendency to aggregate when dried and some form of energy, such as ultrasonication or vortexing, is often required to break up the aggregates in the suspension. Care has to be taken when using ultrasonication, as high power levels can damage the particles themselves.

There are two ISO standards that deal with the issues related to sampling and dispersion: (International Organization for Standardization 2007b), (International Organization for Standardization 2001b). These standards, along with other publications (Allen 1997), (Jillavenkatesa et al. 2001), (Merkus 2009) provide guidelines for sampling and dispersion of particles.
The remainder of this report now focuses not only on the characterisation and nanometrology of nanoparticles, but more specifically on measurements of size and shape. The nanometrology of the other parameters listed above (chemistry, charge, mass and crystallinity) will not be considered for reasons of economy.

c) Reference materials

The dependence on the instrument for a measurement result, as well as technique and supporting media in nanometrology, means that reference materials (RMs) are very important. They allow the instrument performance to be checked and verified under conditions very similar to the actual measurement conditions. Most of the particle characterization documentary standards referenced in this report recommend regular verification procedures be established.

Reference materials (Joint Committee for Guides in Metrology 2008) must be sufficiently homogeneous and stable regarding their specified properties, which have been established as fit for intended use in measuring or examining nominal properties. The German Federal Institute for Materials Research and Testing (BAM) has developed a database in collaboration with TC229, which lists RMs with properties at the nanoscale.

A Certified Reference Material (CRM) is a reference material accompanied by documentation issued by an authoritative body. It provides one or more specified property values with associated uncertainties and traceabilities using valid procedures (Joint Committee for Guides in Metrology 2008). Metrological traceability of a technique or measurement may be established using CRMs.

Certified reference materials are one of the most important tools for ensuring appropriate quality and reliability of measurements. Private companies do not typically provide the more complex reference materials for calibration and quality control, such as nanomaterial CRMs, in sufficient variety, quantity and quality. Government intervention is therefore necessary to remove this obstacle to the free movement of goods and innovation. The JRC’s institute for Reference Materials and Measurements (JRC-IRMM) develops and markets CRMs for standardization and metrology in nanotechnology (http://irmm.jrc.ec.europa.eu/).

d) Size and shape techniques and instruments

Measurements of size and the number size distribution are considered the most universally applicable and suitable measurands for nanoparticles (Scientific Committee on Emerging and Newly Identified Health Risks (SCENHIR) 2009).

The adequate description of three-dimensional objects, such as nanoparticles, poses a challenge. If the particles are spherical, their size could be described by a single diameter but for non-spherical particles, other descriptors have to be used. Standard terminology and methodology has been developed specifically for this purpose (International Organization for Standardization 2008a).

A common method is to use the equivalent diameter, namely the diameter of a sphere that produces a response by a given particle-sizing method, which is equivalent to the response produced by the particle being measured. For example:

- Volume diameter, $x_v$, the diameter of a sphere having the same volume as the particle (measurand in, for example, laser diffraction measurements).
- Surface volume diameter, $x_{sv}$, the diameter of a sphere having the same surface to volume ratio as the particle (measurand in, for example, gas adsorption measurements).
- Stokes’ diameter, $x_{Stk}$, the free-falling diameter of a particle in the laminar flow region (measurand in, for example, disk centrifugation measurements).
- Projected area diameter, $x_a$, the diameter of a circle having the same area as the projected area of the particle resting in a stable position (measurand in, for example, microscopy based image analysis measurements).
- Projected area diameter, $x_p$, the diameter of a circle having the same area as the projected area of the particle resting in random orientation (measurand in, for example, dynamic image analysis measurements).
- Perimeter diameter, $x_c$, the diameter of a circle having the same perimeter as the projected outline of the particle.
- Feret’s diameter, $x_F$, the mean value of the distance of parallel tangents the projected outline of the particle position (measurand in, for example, microscopy based image analysis measurements).
- Martin’s diameter, $x_M$, the mean chord length of the projected outline of the particle (measurand in, for example, microscopy-based image analysis measurements).
- Hydrodynamic diameter, $x_h$, the diameter of a sphere which has the same drag coefficient as the particle position (measurand in, for example, DLS and PTA measurements).
- Radius-of-gyration, $x_{Gyr}$, a measure of the distribution of mass about a chosen axis, given as the square root of the moment of inertia about that axis divided by the mass (measurand in, for example, static light scattering, small angle neutron scattering and small angle x-ray scattering).

As discussed above, particle characterisation techniques comprise three classes: ensemble techniques, single particle analysis techniques and separation techniques. Detailed information on specific techniques and instruments used to measure the size and shape of nanoparticles is presented in Appendix 1.

3.4 Conclusion

The proposed APVMA working definition of a nanomaterial must be suitable for both nanopesticides and veterinary nanomedicines. Currently, there is no universal consensus regarding the definition of either a nanopesticide or a veterinary nanomedicine. Several workers (Kah et al, 2012; Kay and Hofmann, 2014; and Kookana et al, 2014) have highlighted a number of issues that need to be considered when developing a definition of nanopesticides. For example, the nanomaterial found in a nanopesticide may be either the active ingredient or a non-active ‘carrier’; the size may exceed the traditionally accepted upper limit (100 nm) of the nanoscale dimension; and the durability of a nanopesticide varies markedly such that the retention of the nano characteristic may be either transient or persistent. The SCENIHR (2010) report cited earlier notes that ‘additional guidance (requirements) specific for the intended regulation’ may be necessary. Given that the concepts discussed apply equally to nanopesticides and veterinary nanomedicines, it is proposed that the APVMA working definition of a nanomaterial should have an upper limit to the nanoscale dimension of 1000 nm and should govern the regulation of both nanopesticides and veterinary nanomedicines. The SCENIHR (2010) report also notes the need for an enforceable definition that covers risk assessments conducted by regulatory agencies.

There are challenges associated with nanometrology, particularly in characterising nanoparticle-based nanomaterials. The measurement of parameters such as size and size distributions lead to
unique problems, for example, different measurement methods may not provide comparable results. The analysis of nanomaterials in complex media is especially challenging due to a lack of validated and cost-effective measuring methods.

Using several different techniques that complement each other as well as providing some redundancy is a more suitable approach to better understand nanoparticle systems. A sample of nanoparticles may have a very uniform distribution of shapes and sizes, but often they are more complex. The challenge is then to characterise an ensemble of particles by a small number of descriptors such as, for example, size. Also, it is often necessary to use separation techniques. These present the particle ensemble to the measurement technique in such a way that sub-populations can be measured separately. Details of measurement techniques and instrumentation are presented in the Appendix of this report.

For most of the techniques presented in this report there are established protocols, such as ISO standards, that can be used to calibrate instruments and verify measurement techniques. These documentary standards also provide a greater insight into the limitations of the applied method. Using RMs and CRMs in combination with test protocols ensures that the instruments are functioning correctly and giving accurate, reliable results.
3.5 References


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Accessed 2013

4 MANUFACTURE OF NANOMATERIALS

4.1 Introduction

In much the same way as manufacturing processes influence the quality of non-nano active constituents, manufacturing processes for nanomaterials play a significant role when considering risk. Manufacturing techniques can dictate particle qualities, including impurity, and are significant for assessing occupational exposure during subsequent stages of product manufacture. However, the relationships between manufacturing processes and risk are not straightforward and it is not possible to make generalisations yet. What is of concern is the possibility that small changes to manufacturing processes may introduce unpredictable risks.

Regulations authorising the marketing of nano-pesticides and veterinary nanomedicines are product-based rather than manufacture or process-based. However, regulators must also consider the handling of these materials as they move through their ‘life-cycle’ from being raw materials to become products that are retailied, used and discarded.

Nanoform materials may offer advantages over conventional manufacturing and accord with ‘Green Chemistry’. Explanatory information about green chemistry is presented on the US Environmental Protection Agency (EPA) website at Green Chemistry and Nanotechnology and in a presentation by Naidu (2009) presentation on nanochemicals. Twelve principles define what is a ‘greener’ chemical, process or product. These are: waste prevention, synthetic methods that consume all ingredients, less hazardous syntheses, chemicals that are safe by design, use of safer solvents and excipients, renewable raw materials, reduction of derivatives, catalytic reagents in favour of stoichiometry, designed to innocuously decay, manufacturing interventions to prevent pollution and choice of the safest chemical forms to prevent accidents. The American Chemical Society defines the 12 principles.

Aside from the need to consider manufacture of the nanoparticles, introducing nanomaterials into conventional manufacturing also needs consideration. Nanomaterials can offer a range of ‘green’ advantages over conventional manufacturing, including waste and by-product reduction, elimination of impurities, and more efficient use of chemical resources. They can also introduce new risks, particularly around occupational exposure during manufacturing processes.

Manufacturing aspects of nanomaterials are relevant when considering the various statutory criteria which must satisfy the APVMA before it will approve or register active constituents or products. For example, the APVMA’s Manufacturing Licencing Scheme (MLS) applies to conventional and nanoform veterinary medicines alike. As a result, the nanotech manufacturing process will be assessed and may become a relevant particular requirement for enduring approval of the active constituents.

4.2 Manufacturing risks

Approaches to nanomaterial regulation internationally have not yet resulted in any standardised risk-based approaches based on manufacture. Nanomaterial manufacture is often described as either ‘top-down’ or ‘bottom-up’, though within these broad generalisations there are many specific manufacturing pathways for engineered nanomaterials. The top-down or bottom-up descriptors distinguish between nanomaterials made by breaking ‘down’ larger matter via a range of processes, and nanomaterials made by agglomerating molecules ‘up’ into nanostructured materials, also via a
range of processes. There are also hybrid processes incorporating both top-down and bottom-up elements.

Some issues arise via manufacture but are a result of materials needed to facilitate the inclusion or operation of the nanomaterials. As the US FDA (USFDA 2014) notes: ‘Changes in the manufacturing process, including use of different solvents, time/temperature conditions and changes to the starting chemicals (eg alternative starting materials, different purity levels or different concentrations of the chemicals used in the process) may change the types and/or quantities of impurities in the final product. Additional agents, such as dispersing agents and surface modifiers, are often used in the manufacture of nanomaterials. These additional agents and impurities should be considered in the safety substantiation for nanomaterials.’

### 4.3 Methods for manufacturing nanomaterials

Whitesides and Love (2007) eloquently described nanofabrication methods, which can be divided into two categories: top-down methods, which carve out or add aggregates of molecules to a surface, and bottom-up methods, which assemble atoms or molecules into nanostructures. Advances in top-down nanofabrication techniques yield almost atomic-scale precision and will most likely remain the method of choice for building complex devices for some time to come. Top-down fabrication is used to fabricate electronic devices such as microchips, whose functions depend more on their patterns than on their dimensions.

The bottom-up method starts with atoms or molecules and builds up to nanostructures. This method is used to fabricate the smallest nanostructures with dimensions between two and 10 nanometers. These include quantum dots and carbon nanotubes.

#### 4.3.1 Top-down nanofabrication

Top-down manufacturing involves precision engineering and the cutting, etching and grinding of a starting material. The four most common approaches are:

1. **Mechanical**: cutting, rolling, beating, machining, compaction, milling, atomisation, pearl/ball milling and high pressure homogenisation.

2. **Thermal fabrication**: annealing, chill-block melt spinning, electrohydrodynamic atomisation, electrospinning, liquid dynamic compaction, gas atomisation, evaporation, extrusion, template synthesis and evaporation, sublimation, thermolysis, combustion and carbonisation of copolymers.

3. **High-energy and particle fabrication**: arc discharge, laser ablation, solar energy vaporisation, ion milling, electron beam evaporation, reactive ion etching, pyrolysis, combustion and high-energy sonication.

4. **Chemical fabrication**: chemical etching, chemical mechanical polishing, electropolishing, anodising and combustion.
Lithography in its simplest form is a planographic printing process that makes use of the immiscibility ('unmixability') of grease and water. It has since been considerably refined for use with new technologies. A diverse array of methodologies is available:

LIGA techniques, photolithography, immersion lithography, deep violet lithography, extreme ultraviolet lithography, x-ray lithography, electron beam lithography, electron beam writing, electron beam projection lithography, focused ion beam lithography, microcontact printing methods, nanoimprint lithography, nanosphere lithography, scanning AFM nanostencil, scanning probe nano lithography and 2-photon polymerisation.

4.3.2 Bottom-up nanofabrication

Bottom-up manufacturing involves atomically precise engineering – chemical synthesis. The five most common approaches are:

1. **Gas-phase fabrication**: chemical vapour deposition, atomic layer deposition, combustion, thermolysis, metal oxide organometallic vapour phase epitaxy, molecular beam epitaxy, ion implantation, gas phase condensation and solid template synthesis.

2. **Emulsification**: diffusion and supercritical fluid precipitation.

3. **Liquid-phase fabrication**: molecular self-assembly, supramolecular chemistry, nucleation and sol-gel processes, reduction of metal salts, single crystal growth, electrodeposition, electroplating, electroless deposition, anodising, electrolysis in molten salt solutions, solid template synthesis, liquid template synthesis and supercritical fluid expansion.

4. **Solid-lithographic fabrication**: nanolithography, dip-pen methods, nanosphere template methods, nanopore template methods, block copolymer lithography, local oxidation nanolithography and scanning tunnelling microscope writing.

5. **Biological and inorganic fabrication**: protein synthesis, nucleic acid synthesis, membrane synthesis, inorganic biological structures and crystal formation methods.

4.3.3 Hybrid and other processes

**Self-assembly** involves atoms or molecules arranging themselves into ordered nanoscale structures via physical or chemical interactions. This method of manufacture is used for producing smart objects, crystals, films and tubes. Hybrid processes of manufacture incorporate both top-down and bottom-up nanofabrication

**Positional assembly**, which involves the deliberate manipulation of molecules, is reportedly used in the manufacture of modular composite nanosystems (Wong H, 2013) and printed electronics (Fachot M, 2013)
4.4 Common methods of production for AgVet nanomaterials

The methods described below are used to produce most types of nanomaterials popular in the agricultural and animal health sectors. The figures are not intended as exact representations of the production methods used, rather they are generic illustrations of the processes.

These methods are also commonly used in the early stages of the process of synthesizing more complex nanomaterials such as liposomes, nanoemulsions, nanosuspensions, chitosan and poly lactic-co-glycolic acid (PLGA)-loaded nanoparticles.

a) Polymerization methods (Patel et al, 2006)

Factors that are critical when producing a given polymer-drug combination include:
- the type of homogeniser used
- the time and intensity of homogenization
- the amount and type of emulsifier used (examples of emulsifiers that may be used are polyvinyl acetate (PVA), polysorbate, sodium dodecyl sulphate (SDS), gelatin and poloxamer)
- a particle hardening step that involves the removal of solvent
- avoidance of aromatic organic solvents (toluene or benzene) and non-aromatic organic solvents (cyclohexane, DMSO, diethylether) where possible to reduce the toxicity of the nanomaterial being synthesized.

b) Production of nanospheres

The production of nanospheres involves dispersing monomers in water with an emulsifier at a concentration between 0.05% and 7%. The steps involved are:

1) The dispersal of liquid monomers in emulsifier micelles
2) The diffusion of monomers in water
3) Nucleation of small oligomers
4) The growth of oligomers and phase separation with polymer precipitation, leading to the formation of a micelle
5) The growth of micelles, whose size is controlled by the amount of surfactant present in the synthesis, leading to the formation of nanoparticles.

The method of incorporating a drug in the nanosphere depends on the properties of the drug. Lipophilic drugs are dissolved in the liquid monomer directly or via an organic solvent whereas ionic hydrophilic drugs are encapsulated using counter-ion coupling.
c) Production of nanocapsules:

The production of nanocapsules involves several steps:

1) A liquid monomer and drug are dissolved in a solvent that is miscible with water.
2) The mixture at 1) is mixed dropwise to the aqueous phase containing hydroxyl ions.
3) Anionic polymerization results in the precipitation of polymers at the droplets interface.
4) The organic solvent is evaporated.
5) The nanospheres are separated by ultracentrifugation.

The emulsification/solvent evaporation method involves dissolving insoluble drug polymers in a water-insoluble drug polymer, then homogenising the mixture in the presence of surfactants and evaporating off the solvent. A suspension of the nanoparticular drug results.

![Diagram of emulsification/solvent evaporation method]

- Drug polymer + organic solvent
- Homogenization
- O/W Emulsion
- Vacuum heating
- Nanoparticle suspension


When insoluble polymers are dissolved in water-miscible organic solvents such as acetone, the latter is prevented from diffusing in water by adding suitable salts. A large dilution in water allows the solvents to mix and the nanoparticles to harden.

![Diagram of diffusion/emulsification/solvent evaporation method]

- Drug polymer + organic solvent
- Homogenization
- O/W Emulsion
- Distilled water
- Nanoparticle suspension
f) **Emulsification/solvent diffusion method** (Hartmann et al, 2011, Gosselin et al, 2003; Freitas and Müllerä et al, 1998; Tripathi, 2011)

The emulsification/solvent diffusion method is used with water-insoluble polymers dissolved in a mixture of water-miscible (eg. Acetone) and immiscible (eg. Dichloromethane) organic solvents.

Mixing of the miscible solvent (eg water-acetone) with water causes the surface tension to decrease, leading to the spontaneous emulsification of the immiscible solvent (eg dichloromethane). A suspension of nanoparticles is obtained by evaporating the solvent.

![Diagram of emulsification/solvent diffusion method](image)

- Drug polymer in acetone/dichloromethane
- Water + PVA
- Stirring at reduced pressure
- Solvent evaporation
- O/W Nanoemulsion
- Nanoparticle suspension


g) **Coacervate method** (Sales and Palmer, 2008)

The coacervate method is used to obtain nanoparticles from water-soluble polymers (eg chitosan) and PLGA-loaded nanoparticles.

The separation of these small droplets (ie the coacervate) from a polymer-rich viscous phase can be obtained by one of the following processes:

- adjusting the pH to the isoelectric point of the polymer
- adding salts (de-solvation)
- mixing with polar organic solvents (anti-solvent action).

The graphic below depicts one of the three processes de-solvation. The active ingredient is initially suspended in an anionic polyelectrolyte. A cationic polyelectrolyte is then added, resulting in the formation of a polyelectrolyte complex which entraps the active ingredient.

![Diagram of coacervate method](image)

- Cationic polyelectrolyte
- Anionic polyelectrolyte
- Active ingredient

- Active ingredient + anionic/polyelectrolyte
- Addition of a cationic polyelectrolyte
- Formation of a polyelectrolyte complex
- Entrapment of the active ingredient
4.4.1 Methods of production that are specific to nanomaterials

The most common and safest methods of production for more specific types of nanomaterials are described below.

As a result of using organic solvents, a few of these methods of synthesis produce nanomaterials that are toxic.

4.4.1.1 Liposomes

There are two main methods for producing liposomes, the thin layer evaporation method and the reverse phase evaporation method. These are described below.

a) Thin layer evaporation method (Brailoiu et al., 1994; Maestrelli et al., 2006)

The production of liposomes by this method involves the following steps:
1) The dissolution of lipid in organic solvent
2) Solvent evaporation and thin film formation
3) Hydration with aqueous drug solution and the formation of multilamellar vesicles (MLVs, encapsulated)
4) Re-sizing (this step is optional)
b) Reverse phase evaporation method (Otake et al, 2003)

The reverse phase evaporation method for producing liposomes uses three steps. MLVs or unilamellar vesicles (ULVs) are produced and the encapsulation efficiency is good.

**Step 1** Solubilisation of lipids in diethyl ether, re-sizing of the lipid aggregates formed, and removal of the non-encapsulated drug.

**Step 2** Formation of a viscous gel consisting of lipids in a water phase.

**Step 3** The aggregates formed at Step 2 are diluted with excess water to form ULVs or MLVs.
4.4.1.2 Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are composed of a solid lipid matrix stabilized by surfactants. SLNs contain a dissolved or dispersed active ingredient. Highly purified lipids are used in the production process.

The incorporation of drug will depend on:
- the solubility of the drug in the lipid melt
- the structure of the lipid matrix (expulsion of the drug from the lipid melt can result in toxicity and must be avoided)
- the polymorphic state of the lipid matrix.

Six methods for producing stable SLNs are depicted below.

a) Hot high pressure homogenization method (Note: this method is also suitable for the production of nanoemulsions) (Mantovani et al, 2011; Eldem et al, 1991; Speiser, 1990)

In this method, lipid at a temperature 5°C above its melting point is pre-emulsified with an aqueous surfactant solution and then homogenized at high pressure (500 bar) to form a nanoemulsion.

The particle size of the SLNs formed is determined by the operational conditions including:
- the type of homogenizer
- the number of homogenization cycles
- the pressure and temperature used during production.
b) Cold high pressure homogenization method (Raj et al, 2012; Speiser, 1990; Sjöström and Bergenstähl, 1992)

The cold, high pressure homogenization method is suitable for temperature-sensitive drugs and hydrophilic molecules. The method is also suitable for producing nanoemulsions. Immediately after drug dispersion, the lipid melt is ground under liquid nitrogen to form lipid microparticles. The lipid microparticles are pre-dispersed in a surfactant-aqueous solution while stirring at high speed. SLNs are subsequently obtained by high pressure homogenization (5 cycles at 500 bar) at room temperature. The method is depicted in the following graphic:


The supercritical fluid (SCF) method for the production of SLNs is applicable in producing nanosuspensions and liposomes.

The properties of SCFs such as polarity, viscosity and diffusivity can be modified during the process by varying the operating temperature and/or pressure.

At its supercritical state, the physicochemical properties of CO₂ are intermediate between a liquid and a gas (see phase diagram below).
CO₂ can act as:

- a solvent in the rapid expansion of supercritical solution (RESS) process
- an anti-solvent in the gas/supercritical anti-solvent (SAS) process
- a solute/plasticizer in the particle from gas saturated solutions (PGSS) process.

Note: In addition to modifying particle solubility, an anti-solvent can have an impact on crystallization mechanisms including primary and secondary nucleations, crystalline growth, and agglomeration. These modifications can have an effect on the polymorphic nature of the particles obtained, particle morphology, and crystal size.

d) Rapid expansion of supercritical solution method

The rapid expansion of supercritical solution (RESS) method for the production of SLNs is depicted below. It entails the release of a drug and/or polymer dissolved in SCF from a high pressure vessel, which decreases pressure and CO₂ density. This results in drug precipitation in SLNs.
e) Supercritical anti-solvent method

The supercritical anti-solvent (SAS) method for producing SLNs involves spraying a solution of drug in organic solvent into supercritical CO₂. The supercritical CO₂ extracts the solvent and precipitates the drug. Different powders are produced by changing parameters such as pressure, temperature, mass flow and concentration of the drug in the initial solution.

f) Particle from gas saturated solutions method

The first step in the particle from gas saturated solutions (PGSS) method is solubilising the supercritical fluid in the melted solute. Once saturated in gas, the solution is sprayed through a nozzle in a low pressure expansion chamber. This causes the gas to vaporize and cool rapidly due to the Joule–Thomson effect, resulting in the precipitation of particles which exit the vessel via a nozzle. The SLNs thus formed are deposited in a collection chamber.

The PGSS method is similar to the RESS method, the difference being that in the PGSS method, the solute is in a melt whereas in the RESS method, the solute is in a solution.

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7 The Joule–Thomson effect describes the temperature change of a gas or liquid when it is forced through a valve or porous plug while kept insulated to prevent heat exchange with the environment (124).
g) Spray drying method (Mukherjee et al, 2009; Yang et al, 1999)

The spray drying method for producing solid lipid nanoparticles transforms aqueous SLN dispersions into drug products. It is an alternative procedure to lyophilisation (also known as freeze drying).

A disadvantage of the spray drying method is particle aggregation caused by the high temperature of the air in the drying chamber.

The spray drying method is also suitable for producing PLGA-loaded nanoparticles.

4.4.1.3 Polymer-based nanoparticles

Numerous production processes for synthesizing polymer-based nanoparticles are available, some of which are described here.

a) Polymer interfacial deposition method (Yu et al, 2013; Fessi et al, 1989)

The polymer interfacial deposition method is usually preferred for obtaining nanocapsules from insoluble polymers dissolved in non-polar solvents.

A water-miscible solvent (e.g. acetone) is mixed with water. When this mixture is added to a non-polar solvent (oils e.g. benzyl benzoate and Miglyol are commonly used) containing the water-immiscible drug polymer, the surface tension decreases resulting in the formation of an oil-in-water emulsion. The water-immiscible polymer precipitates at the surface of the dispersed droplets forming a nanocapsule membrane.
The polymer interfacial deposition method facilitates a high loading of the dispersed droplets with lipophilic drugs.

b) Gelification method (Shah and Londhe 2011; Nagavarma, 2012)

The gelification method is used to produce nanoparticles from water-soluble polysaccharides. Certain polyelectrolytes undergo gelification under particular conditions. For example, anionic and cationic polymers such as sodium alginate and cationic polymers, respectively, are commonly used in the gelification method, and require surface modification in order for the reaction to occur.

Also shown (bottom two figures) are the chemical structures of alginate and chitosan. The chemical groups likely to be linked with other components to facilitate surface modifications are shown in colour.

Alginate

Chitosan
c) Emulsion droplets coalescence method (Guo et al, 2013; Fan and Striolo, 2012)

The emulsion droplets coalescence method is depicted below (Key: chitosan [yellow]; drug [blue] and sodium hydroxide [grey]).

Chitosan is a cationic polymer of animal origin formed from the deacetylation of chitin. It exhibits a number of properties that make it suitable for transmucousal drug delivery including biocompatibility, mucoadhesion and an ability to reversibly open tight junctions.

The emulsion droplets coalescence method entails the production of polymer-based nanoparticles, which involves precipitating chitosan at neutral-basic pH

![Emulsion droplets coalescence method diagram]

d) Ionotropic gelation method (Calvo et al, 1997)

The ionotropic gelation method of nanoparticle production relies on electrostatic interaction to achieve cross-linking. The complexation of oppositely charged polyelectrolytes is achieved through mixing; additional coating procedures may be required.

In the graphic (upper panel), anionic groups of alginate particles interact with cationic groups of the cross-linking agent. The alginate nanoparticles are subsequently trapped in a cationic network to form a hydrogel.
The graphic (lower panel) depicts an example of a hydrogel, comprised of alginate and a Ca$^{2+}$ system.
4.4.1.4 Metallic nanoparticles

Four methods for producing metallic nanoparticles are described below.

a) Sol-gel method (Liu et al, 2013; Mammeri et al, 2005)

The sol-gel method is used to produce metallic nanoparticles and is also suitable for producing nanoclays. Inorganic nanostructures are formed through formation of a colloidal suspension (commonly referred to as sol) followed by gelation and integration into a network in a continuous inert liquid phase (commonly referred to as gel).

The production process selected will ultimately depend on the product being synthesized. For example, metallic thin film coatings and metallic powders are produced using the sol method whereas metallic particles are produced using the gel method.

![Diagram of sol-gel method]

b) Laser ablation method (Compagnini et al, 2003)

The laser ablation method for processing metallic nanoparticles depicted below is a novel approach that uses an ultrafast pulsed laser beam. Colloidal nanoparticle solutions are produced directly in liquid inorganic solvents (eg ethylene glycol) without the need to use stabilizing agents. The laser ablation method both increases the metallic nanoparticle surface area available for binding biomolecules and reduces the activation energy required for the binding reaction. The result is that both the binding efficiency and total loading are increased.

The laser ablation method is also suitable for producing carbon nanotubes.
c) Hydrothermal and solvothermal synthesis (INTECH, 2011; Wahi et al, 2006)

The hydrothermal method for producing metallic nanoparticles allows the synthesis of single crystals (depicted as a nanocube in the graphic below). The method uses an aqueous precursor solution and depends on the minerals being soluble above the boiling point of water and under high pressure.

Hydrothermal synthesis uses two approaches: 1) crystal growth and dissolution; and 2) direct nucleation. Only the first of these approaches is described and depicted below.

In the crystal growth and dissolution approach, crystal growth is performed in an autoclave. A temperature gradient is maintained across the growth chamber containing a nutrient supply and water. The higher temperature facilitates nutrient dissolution whereas the lower temperature facilitates crystal seeding and growth.
In the solvothermal method, the reaction is carried out in organic solvents at temperatures some 200-300°C higher than their boiling points. The solvothermal method provides the benefits associated with both the sol-gel and hydrothermal methods.

**d) Inert Gas Condensation method (Gracia-Pinilla et al, 2010)**

The Inert Gas Condensation (IGC) method is used in the production of metal oxide nanocrystals. Metals are evaporated in an ultra-high vacuum chamber filled with helium or argon gas. The small particles formed grow by Brownian coagulation and coalescence and finally form nanocrystals. The method is depicted below.

4.4.1.5 **Nanoclays**

There are several methods for producing nanoclays and three of these are described below.

**a) Solution induced intercalation method (Gao, 2004)**

This involves solubilizing the polymer in a solvent, then dispersing the clay in the resultant solution, followed by either evaporation of the solvent (as shown in the graphic below) or precipitation of the polymer.

Disadvantages of this method include:
- poor clay dispersion
- the high costs of solvents
- the need to use large volumes of solvent to achieve appreciable filler dispersion
- technical difficulties encountered with phase separation
- health and safety issues.

The potential toxicity concerns associated with the use of certain organic solvents are allayed when the method is used to produce water-soluble polymers.
b) In situ polymerization method (Patel et al, 2006)

With this method, the dispersed layers of clay are polymerized in the matrix before the silicate layers, in conjunction with the polymerization initiator and/or the catalyst, are mixed with the monomer.

c) Melt-processing method (Patel et al, 2006; Gao, 2004)

This method of producing nanoclays is depicted below (polymers are shown in red; silicates are in blue).

The silicate layers are directly dispersed into the polymers during the melt. The method requires the silicates to have been previously surface-treated by organo-modification.
4.4.1.6 Nanoemulsions

Two methods for producing nanoemulsions are emulsification by the temperature-dependent phase inversion method and the microfluidization method.

a) Emulsification by the temperature-dependent phase inversion method (Mantovani et al, 2011; Raj et al, 2012)

The starting material for emulsification by the temperature-dependent phase inversion method is an oil-in-water emulsion stabilized using non-ionic emulsifiers. The phase inversion from oil-in-water to water-in-oil results from modifying the composition (eg by adding emulsifiers) or a temperature increase. In order for phase inversion to occur, the hydrophilic and lipophilic properties of the mixed emulsifier need to be balanced.

b) Microfluidization method (Mantovani et al, 2011; Raj et al, 2012)

The microfluidization method uses a device called a microfluidizer with a high-pressure positive displacement pump. The pump forces the starting material, a macroemulsion, through an interaction chamber where it is further processed (eg the macroemulsion may undergo chemical reactions) and through a heat exchange unit to form a stable nanoemulsion.
4.4.1.7 Dendrimers

Dendrimers are produced by several methods, four of which are described below.

a) Divergent-growth method (Svenson, 2004)

In this method, the initiator core is reacted with a reagent comprised of a reactive branch unit (grey) with protective groups (blue) to form a first generation dendrimer (also known as a 1G dendrimer). The production of higher generation dendrimers (ie 2G dendrimers and 3G dendrimers) requires the protective groups to be removed and the ‘growth’ reaction repeated until a dendrimer of the required size is formed.

A disadvantage of the divergent-growth method is the number of steps involved.
b) Convergent-growth method (Gohel et al, 2009)

The convergent-growth method for producing dendrimers was developed in response to divergent synthesis being considered too slow. Convergent growth begins at what will be the surface of the dendrimer, and works inwards by gradually linking surface units together. The graphic depicts a surface unit comprised of a reactive branch unit and surface groups, and a growth unit comprised of a focal point and a reactive branch unit.
c) Hypercores and branched monomers growth method (Pushkar et al, 2006)

Oligomeric species are pre-assembled as depicted in the graphic below. A ‘wedge’ is pre-assembled by the convergent-growth method and a ‘hypercore’ is pre-assembled by the divergent-growth method. These pre-assembled oligomers are linked to produce dendrimers at higher yields. The production of a 4G dendrimer is depicted below.
d) Double exponential growth method (Pushkar et al, 2006)

The double exponential growth method requires the preparation of monomers for both divergent and convergent growth from a single starting point. The two monomers are reacted to give an orthogonally protected dendrimer, which is used to repeat the growth process.
4.4.1.8 Quantum dots

Quantum dots may be synthesized by a number of methods, including the colloidal process method, the molecular beam epitaxy method, a combination of the colloidal process method and the molecular beam epitaxy method, the chemical vapour deposition method and the contact printing process. Only the contact printing process is described below.

Contact printing process (Kim et al, 2011; Panzer et al, 2012)

The contact printing process for synthesizing quantum dots is a very simple and cost-effective method of forming thin films of quantum dots. The device does not come into contact with the solvents during the printing process. A silicon master is used for moulding polydimethylsiloxane. Its top side is coated with a thin film of Parylene-c, which is a chemical vapor deposited on an aromatic organic polymer. The ink-coated parylene stamp is produced by spin-casting colloidal quantum dots suspended in organic solvent. When the solvent evaporates, the quantum dot monolayer gets transformed on the substrate by contact printing.

The discussion points below refer to the numbered arrows shown in the graphic:

1) Modifying the donor surface and spin-casting quantum dots.

2) Applying an elastomer stamp on the quantum dot film with appropriate pressure.

3) Quickly peeling the stamp from the donor substrate.

4) Contacting the inked stamp to the device stack for transfer printing of red quantum dots, and slowly peeling back the stamp.

5) Sequential transfer printing of green quantum dots.

6) Sequential transfer printing of blue quantum dots.

7) Micrograph-like quantum dot strips transfer-printed onto a glass substrate that fluoresces when excited by 365 nm UV radiation.

Key: The Parylene-C and polydimethylsiloxane mixture is shown in yellow; quantum dots displaying different emission wavelengths are shown in blue, red and green.
4.5 **Major classes of excipients used in AgVet formulations**

Excipients are included in AgVet formulations for many reasons, including:
- to stabilize solutions, suspensions, dispersions or emulsions
- to act as antimicrobials
- to aid in the manufacture of dosage forms
- to control and/or target drug delivery
- to minimize pain upon injection.

Examples of excipients (147) and their function include:
- buffering agents to control pH
- surfactants to inhibit protein adsorption to interfaces
- preservatives to prevent microbial growth
- carbohydrates as bulking agents to facilitate lyophilisation
- polymers to increase solution viscosity
- salts or sugars to stabilize proteins and obtain physiological tonicity and osmolality.

Described below are some excipient categories used in AgVet nanoformulations.

### 4.5.1 Stabilizers

Stabilizers play two important roles in nanoformulations: first, they stabilize the native conformation of proteins and second, they prevent the aggregation of metallic and magnetic nanoparticles. Examples of stabilizers are:
- polyols
- sugars
- amino acids
- amines
- salting out salts.

Sucrose and trehalose are the most frequently-used stabilizers in nanoformulations and in general, sugars and large polyols are better stabilizers than smaller polyols.

Stabilizers act by a number of mechanisms and behave differently with different protein formulations. However, in nearly all cases, thermodynamically unfavourable excipient-active principle interactions (147) exclude excipients from the protein surface.

### 4.5.2 Surfactants

Toxicity is an important consideration when selecting surfactants to control the size and shape of nanomaterials. Consequently, alternatives to surfactants may need to be considered for stabilizing and controlling nanoparticle size and shape during synthesis and when new functionalities are being incorporated on the particle surface (148, 149).
Non-ionic surfactants are generally non-toxic and are widely used to:
- stabilize nanosuspensions/nanodispersions
- suppress aggregation
- assist in protein refolding.

In this respect, polysorbate 80 (Tween 80) and Polysorbate 20 (Tween 20) have been widely incorporated in marketed protein pharmaceuticals at concentrations in the 0.0003-0.3% range (147).

A separate consideration is the extent to which a polymer or surfactant can cover the surface of a nanomaterial without adversely affecting the nanomaterial’s desired effect. A case in point is the use of surfactants, such as Bry 35, Triton X-10, Pluronic F12 and sodium dodecyl sulphate (SDs), or polymers, such as chitosan and carbopol, during nanomaterial synthesis when the aim is to create a particular surface property. To achieve the desired effect, the surface must not be completely covered by the surface modifier and hence, it may be necessary to monitor the progress of reactions using techniques such as the measurement of zeta potential and hydrophobic interaction chromatography (HIC) analysis.

### 4.5.3 Polymers and proteins

Hydrophilic polymers are used to stabilize solutions and enhance protein assembly. Examples of the hydrophilic polymers used include dextran, hydroxyl ethyl starch (HETA), gelatin, and PEG-4000 (PEGs with higher molecular weights have been found to be more effective than those with smaller molecular weights). The non-polar moieties on certain polymers such as PEGs and pluronics can decrease water surface tension, which suppresses surface adsorption-induced aggregation.

The aggregation of metallic nanoparticles attributed to van der Waals or magnetic dipole-dipole interactions can be prevented by steric or electrostatic repulsion. Applying a coating of inert lipids and polymers to stabilize nanoparticles also avoids the aggregation of nanoparticles and reduces or eliminates the formation of an oxide layer on nanoparticles.

### 4.5.4 Coupling agents

Porous hollow silica nanoparticles are prone to agglomeration due to the presence of a terminal NH$_2$ group on the silane structure. Agglomeration in turn leads to an increased rate of hydrolysis which induces particle growth. This can be overcome using the coupling agent, 3-aminopropyltrimethoxysilane (APTMS), in a specific cocondensation process (151).

### 4.5.5 Amino acids

Amino acids including histidine, arginine, glycine, methionine, proline, lysine, glutamic acid and arginine mixtures are used to stabilize solutions of nanoparticles. A variety of mechanisms account for the improved stability, one of which allows for better molecular conformation through linking the amino acids to the nanoparticles (147).
4.5.6 Preservatives

Preservatives are included in formulations to prevent microbial growth, particularly for multi-dose formulations. Examples of preservatives include benzyl alcohol, phenol, m-cresol and antioxidants such as sulphites and ascorbic acid. In some situations, preservatives will cause the aggregation of nanoparticles, and this is especially prevalent in those nanoformulations containing proteins.

4.5.7 Chelators

Chelators or sequestrating agents are organic complexing agents that inactivate metallic ions (152). They reduce the aggregation of gold and other metallic/magnetic nanoparticles. Examples of chelating agents are phosphonates, ethylenediamine-N,N'-disuccinic acid (EDDS) and citric acid.

4.5.8 Thickeners and emulsifiers

These classes of excipients are used to:
- avoid phase separation of emulsions and suspensions
- improve the stability of solutions of nanoparticles
- dissolve or disperse lipophilic drugs
- increase the bioavailability of drugs administered orally.

Examples of thickeners and emulsifiers include alginates, saccharose (which is unstable in acidic solutions), cellulose and polysorbate 80.
4.6 References


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5 POTENTIAL HUMAN HEALTH RISKS ASSOCIATED WITH THE USE OF NANOTECHNOLOGIES IN AGRICULTURAL AND VETERINARY CHEMICALS

5.1 Introduction

Nanotechnology offers the opportunity to manufacture a diverse group of materials with properties that offer potential benefits for use in agricultural and veterinary chemicals. However, there has been some concern about the potential risks to human health and the environment that these materials may pose. One of the key concerns is that nanoparticles may cause harm in a manner that is not assessable or predictable based on current approaches to risk assessment.

This overview of the available literature on the toxicokinetics and toxicology on nanoparticles will consider whether the current risk assessment paradigm and toxicity testing methodologies apply. The risk assessment model depicted in Figure 5.1 is an adaptation of the classical risk assessment framework. By identifying the main stages of the risk assessment paradigm, it highlights the regulatory considerations applicable to the potential human health risks associated with the use of nanotechnology-enabled products in agriculture and animal husbandry. Where possible this Chapter will provide general guidance about assessing human health risks associated with using these materials in agricultural and veterinary chemicals. The intended audience is risk assessors, industry and other interested parties.

It is generally agreed that increased surface area, altered surface chemistry and latent qualities for dissolution, create a potential toxicity profile for nanoparticles that deviates from conventional materials of the same composition. This suggests regulators may need to give greater consideration to nanoparticle ingredients that remain in particulate form in the final product. This may include, for example, more or different testing to characterise the physicochemical properties of the test material, as well as the material’s properties in dosing suspensions.

Conversely, there is less likelihood of novel toxicities due to the particulate nature of nanoparticles if they are in materials that are soluble in the final product. Similarly, there is a reduced likelihood if they rapidly undergo dissolution or biodegradation in, for example, water, lipid, food or feed, or biological fluids to form soluble non-nanoform degradation products. The toxicity of these materials will primarily be caused by the constituent ions or monomers or metabolites, so novel toxicities would not be anticipated due to the particulate nature of the material. These materials should be assessed using conventional risk assessment processes and methodologies.

In conducting this review, the APVMA has considered available documents related to nanoparticle risk assessments produced by other national and international agencies and bodies such as the TGA, NICNAS, Safe Work Australia, US FDA, EMEA, FAO/WHO and EFSA. It also considers the recent work of the OECD Working Party on Manufactured Nanomaterials (WPMN), which has conducted one of the most comprehensive nanomaterial research programs into the health and safety of nanoparticles.
5.2 Applicability of the risk assessment framework

The classical risk assessment framework for human health includes four main steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation. A number of international agencies and bodies have evaluated the applicability of this framework and found it to be generally appropriate to address risks posed by nanomaterials, although some modifications in methods are anticipated (COT 2005; 2007, EFSA, 2009; 2011, FAO/WHO, 2009; FDA, 2007; OECD, 2012a; SCENIHR 2005; 2007).

The classical risk assessment framework has been applied successfully to a number of nanoscale materials in the food sector including cyclodextrins, silicon dioxide and large structured molecules and polymers (FAO/WHO, 2009). The United States (US) and European Union (EU) have similarly approved medical products composed of a range of nanoscale materials using the framework. Relevant examples include liposomes (Ambisome®, Doxil®, Visudyne®), nanoemulsions (Diazemuls®, Diprivan®, Intralipid®), micelles(Taxol®, Konakion MM®, valium MM®), polymer protein conjugates (PegIntron, Somavert) and polymeric substances (Copaxon) (EMEA, 2006; FAO/WHO, 2009).

Manufacturers of these materials have generally used standard processes which are well understood and which have a significant body of literature dealing with their preparation and safety. These materials are therefore not considered necessarily to represent an innovative or novel use of nanotechnologies.
5.3 Adequacy of existing test guidelines

The OECD WPMN reviewed health-related OECD test guidelines to determine their applicability to manufactured nanomaterials (OECD, 2009). It found the guidelines are generally applicable for investigating the health effects of manufactured nanomaterials. However, more consideration should be given to characterise the physicochemical properties of the test material. In some cases, test guidelines should also be modified, particularly in relation to toxicokinetics and inhalational toxicity testing. Those recommendations are addressed in subsequent sections (refer to Sections 5.6 and 6.9, respectively).

The WPMN found no significant evidence that the toxicological endpoints prescribed in the current test guidelines are not applicable to the testing of nanoparticles (OECD, 2009). Toxicological endpoints generally apply to the whole of an organism or tissue investigated (e.g., histopathology) and therefore should also apply to soluble chemicals and nanoparticles. However, the WPMN recognized that future research may identify modes of action unique to nanoparticles and, as such, it is possible that some modifications to toxicity testing endpoints may be necessary in the future.

The applicant is responsible for developing suitable methods and protocols to address particular toxicological concerns related to the use of potentially novel nanoparticles. The testing regimen should consider the physicochemical properties of the material. The APVMA would generally recommend that the applicant consult early with the Authority in relation to the safety of the end use of a product containing novel nanoparticles.

5.4 Physicochemical characteristics and sample preparations

Adequate particle characterisation is a necessary element in assessing the potential toxicity of nanoparticles in biological systems. This information ensures that the material used in toxicology studies has properties within the range of the material for which approval is sought. It should address the physicochemical properties of the naked particle and the nanoparticle in the final product, as well as any changes that may occur through the product’s life-cycle.

Particle characteristics that may require toxicological testing include: particle size, size distribution, aggregation, agglomeration state, shape, chemical composition, surface area, surface chemistry, dissociation constant, crystal structure, surface charge, zeta potential, Hamaker constant, interfacial tension, and porosity (OECD, 2010; OECD 2012b). Required particle characteristics will need to be determined on a case-by-case basis, depending on the nature, functionalities, and intended uses of the material.

The behaviour of the nanoparticles in dosing suspensions is also critical to interpreting toxicity studies. Nanoparticles can agglomerate or aggregate to form larger structures when dispersed in air, food/feed, liquid vehicles and biological media. Batch variations and ageing effects may also be more significant for nanoparticles than small molecules. Other factors, including nanoparticles adhering to the walls of vessels containing dosing suspension or drinking vessels, may result in significant overestimation of exposures.

Further information on the physicochemical characterisation and sample preparation for engineered nanomaterials in toxicity studies can be found in recent OECD guidance on sample preparation and dosimetry (OECD, 2012b).
5.5 Dose metrics

The relationship between toxicity and various dose metrics is a subject of continuing discussion in the scientific community (Oberdorster, 2005a; Warheit et al, 2005; 2006; Donaldson and Poland, 2013; Pauluhn, 2009). Despite structured reviews of this issue there is insufficient evidence as yet to recommend one dose metric in preference to another in all cases (Seaton et al, 2010; Maynard et al, 2006).

Doses are generally expressed as mass per kg body weight or mg/m$^3$, however other interrelated dose metrics, including particle number or surface area, may also need to be considered when describing nanoparticle dose-response relationships. On that basis, it is desirable to characterise the properties of the nanoparticle sufficiently (as above) to provide information so the mass dose can be converted to other metrics, if relevant. This may be useful when comparing the toxicity of a nanoscale particulate material with a conventional (non-nano) material of the same composition.

Scientific justification for the selection of the most appropriate dose metric should be provided.

5.6 Toxicokinetics

Understanding the toxicokinetics$^8$ of nanoparticles is critical to risk assessment to determine potential novel toxicities. It is an area generally recognized as under-researched for both nanoparticles and conventional particles, and presents challenges not typically encountered for soluble chemicals.

A summary of the available literature on the toxicokinetics of nanoscale and microscale particles following inhalational, oral and dermal exposure is presented below. In general, the available data indicate that the respiratory tract, gastrointestinal tract and skin represent substantial barriers to the absorption of nano- and microscale particulates.

5.6.1 Inhalation


The relative disposition of inhaled particles in the nasopharyngeal, tracheobronchial and alveolar regions of the respiratory tract depends on particle size, species and the structure of the respiratory tract. Moreover, the relationship between size and disposition is complex and may be non-monotonic, depending on the region of disposition. See Oberdorster et al, (2005a) for a description of predicted fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial, and alveolar region in the human respiratory tract following nose breathing.

The ICRP model (1994) predicts that approximately 90% of inhaled 1 nm particles are deposited in the nasopharyngeal region, 10% are deposited in the tracheobronchial region, and deposition in the alveolar region is negligible. Conversely, 5 nm particles are distributed relatively evenly in the three

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$^8$ Toxicokinetics can be described as the study of the absorption, distribution, metabolism and excretion of potentially toxic substances from the body. It essentially has the same meaning as pharmacokinetics but does not relate to pharmaceutical substances.
sections, while approximately 50% of 20 nm particles may be deposited in the alveolar region (Oberdorster et al., 2005a).

Following deposition in the respiratory tract, deposited particles are cleared either by chemical dissolution, or a physical translocation of particles (Oberdorster et al., 2005a). Particles which readily undergo dissolution can dissolve in lung fluid and act locally, or be absorbed systemically (Rogueda et al., 2007). Relatively insoluble nanoparticles are removed mainly by luminal macrophages and neutrophils, which internalize particles and degrade them, or carry them to the mucociliary escalator (Landsiedel et al., 2012; Rogueda et al., 2007).

The mucociliary escalator is an efficient transport system which pushes the mucus and trapped solid materials towards the mouth. The process of phagocytosis takes place within 6–12 hours, but the subsequent clearance is much slower. A retention half-time of solid particles in the alveolar region based on this clearance mechanism has been estimated to be approximately 50–70 days in rats in non-overloading conditions, and up to 700 days in humans (Rogueda et al., 2007; Oberdorster et al., 2005a; Pauluhn, 2009).

Several studies in rodents have demonstrated that microscale and nanoscale particles can translocate from the lung to the pulmonary interstitium and local lymph nodes. This is not a nanospecific effect and has been observed for larger particles and fibres, such as asbestos. (Reviewed in Donaldson and Poland, 2013). While the translocation of particles to local lymph nodes and the pleural cavity is well accepted, it has been a subject of debate as to whether insoluble nanoscale nanoparticles deposited in the lung are able to translocate to any significant extent to other secondary organs.

Some recent studies have attempted to measure particulate translocation from the lungs to secondary organs:

Kreyling et al., (2009) conducted inhalation studies in rats to estimate the amount of iridium (Ir) and carbon (C) nanoparticles translocated from lungs to the blood and secondary organs. Nanoparticles composed of chain aggregates (and agglomerates) with primary particle sizes of 2–4 nm (Ir) and 5–10 nm (C) were labelled with $^{192}$Ir. Rats were exposed via inhalation for 1 h and radioactivity was measured in the lungs, blood, liver, spleens, kidney, heart, brain and carcass. Virtually all radioactivity was recovered in the lungs at 24 h. Recovery of radioactivity in the liver, spleen, kidneys, heart, brain and bone was low (<0.05 expressed as a fraction of radioactivity recovered in tissues and excreta). In another study, Kreyling et al., (2010) found that approximately 2% of 20 nm TiO$_2$ nanoparticles deposited in the lungs of rats were translocated to secondary organs.

Klein et al., (2012) reported the results of organ disposition of a number of different nanoparticles administered by inhalation in a series of short-term inhalation toxicity studies. Concentrations of TiO$_2$, cerium oxide (CeO$_2$), Al-doped CeO$_2$, polymer-coated synthetic amorphous silica and barium sulfate (BaSO$_4$) were measured in the lung, mediastinal lymph node, liver, kidney, spleen, brain (olfactory bulb) and blood. The authors observed that, in general, test materials were not measured in tissues other than the lymph nodes at concentrations above the detection limit (0.5 µg per tissue). Polymer-coated synthetic amorphous silica was the exception and was detected in the spleen but the measured concentration was not reported.

Semmler-Behnke et al., (2008) reported that 24 h after intra-tracheal instillation of 18 nm gold nanoparticles, approximately 99.8% of the administered dose was retained within the lungs. Conversely, for 1.4 nm gold nanoparticles approximately 91.5% of the dose was retained in the lungs and 8.5% of the dose was recovered in secondary organs (lung, liver, blood, spleen, skin and carcass).

Nemmar et al., (2002), demonstrated an apparent rapid absorption of inhaled $^{99m}$Technetium-labelled carbon nanoparticles from the lungs of healthy humans. However, these findings could not be
replicated in similar studies using the same label (Brown et al, 2002; Hagens et al, 2007; Wiebert et al, 2006; Mills et al, 2006). Similarly, in a more recent study, Moller et al, (2008) found that most inhaled ultrafine carbon particles are retained in the lung periphery and in the conducting airways, without substantial systemic translocation or accumulation in the liver after 48 hours. As such, it can be reasoned that the studies of Nemmar et al (2002) reported the absorption of $^{99m}$Technetium species rather than the $^{99m}$-Technetium-radiolabelled nanoparticles (reviewed in Hagens et al, 2007).

Together, the available data suggest that systemic translocation of nanoparticles following deposition in the lung is generally very low at non-inflammatory exposure levels.

### 5.6.2 Oral exposure studies

Several comprehensive reviews are available on particulate absorption from the gastrointestinal tract (GIT), mainly focused on the potential use of nano- and microparticles for oral delivery of drugs and vaccines (des Rieux et al, 2006; Florence and Hussain, 2001; Hussain et al, 2001; O’Hagan, 1996; Shakweh et al, 2004). Other investigators have focused more generally on the potential human health hazards that may be associated with absorption of insoluble nanoparticles from the gastrointestinal tract (Hagens et al, 2007; Hoet et al, 2004; Landsiedel et al, 2012; Oberdorster et al, 2005a).

Conceptually, in order to be absorbed intact from the GIT, a particulate administered in the diet (nanoscale or microscale) must first resist dissolution and degradation in the stomach and intestine and be able to pass to the apical surface of the epithelial cells lining the mucosa. Dissolution can be defined as a dynamic process by which a particle, which has some solubility in the local environment, goes into a solution phase to form a homogenous mixture. The rate of dissolution is influenced by size, solute concentration, surface area, surface morphology, surface energy, dissolution layer properties and aggregation (reviewed in Borm et al, 2006a).

The gastrointestinal epithelium is permeable to substances of low molecular weight, including monomers of nutrients such as amino acids, fatty acids and saccharides, but relatively impermeable to macromolecules and particles. This low permeability means that most insoluble material has poor oral bioavailability and passes through the gastrointestinal tract to be eliminated from the body unchanged. Nevertheless, evidence accumulated over the past 40 years appears to indicate that some, albeit generally low, absorption from the gastrointestinal tract does occur (des Rieux et al, 2006; Florence and Hussain, 2001; Hussain et al, 2001; O’Hagan, 1996; Shakweh et al, 2004).

The mechanism of absorption is generally considered to involve M-cells, which are associated with submucosal lymphoid follicles of Peyer’s patches (PP) and other sites of gut-associated lymphoid tissue (GALT) (reviewed in Gebert et al, 1996). M-cells, named for their micro-fold and membranous appearance, are specialised epithelial cells which sample gut antigens. There is also some evidence that small amounts of nanoscale materials may be absorbed through normal columnar epithelial cells. Some relevant examples using different nanoscale particulates and experimental protocols are described below.

In a recent study, radiolabelled gold nanoparticles of different sizes (1.4–200 nm) with negative surface charge, and 2.8 nm nanoparticles with opposite surface charges, were administered by intra-oesophageal instillation to rats (Schleh et al, 2010). Radioactivity was measured in selected organs and excreta for 24 h by gamma-spectroscopy. Approximately 99.6% or greater of administered radioactivity was associated with the gastrointestinal tract or faeces. The maximum absorption (0.37% of administered radioactivity) was observed for negatively charged 2.8 nm gold nanoparticles. This is the only identified study in which the administered dose was completely recovered. However, the results of earlier more qualitative studies described below, were generally consistent in observing a low level of gastrointestinal absorption.
Mice received 20 ppm of 4, 10, 28 or 58 nm gold particles in drinking water for seven days ad libitum. The mice were killed and tissues including blood, brain, lung, heart, kidney, spleen, liver, small intestine and stomach samples were collected. Elemental gold was found above background levels in all nine tissues sampled for 4, 10 and 28 nm particles. Gold was not found in most tissues for 58 nm particles. Concentrations of gold in tissues ranged from approximately 5 ng/g in the brain to about 75 ng/g in the kidney for 4 nm particles. The authors reported that gold particle uptake occurred through single enterocytes that had died, and were in the process of being excluded from the villi. Uptake of gold particles was not seen through or between normal enterocytes, nor was it seen to occur through M-cells in the PP region of the ileum (Hillyer and Albrecht, 2001).

Titanium dioxide (TiO$_2$) particles (rutile; 500 nm) were administered by gavage as a 12.5 mg/kg bw suspension to rats, daily for 10 days. Urine and faeces were collected daily. Animals were killed 15 h after the final dose and the stomach, intestine, colon, peritoneal tissue, liver, spleen, kidney and heart were removed. Analysis was by light microscopy, scanning electron microscopy (SEM) and inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Microscopic examination found TiO$_2$ particles in the PP, connective tissues of the mesentery network and the mesenteric lymph node. Particles were also found in the Kupffer cells of the liver, but not in the kidney, heart or in lung macrophages. Using ICP-AES, the authors estimated that total systemic uptake of particles was approximately 6.5% of administered dose taking into account values in blood (0.02%), PPs and mesentery network and nodes (2.9%), liver (2.4%), lungs (1.2%), spleen (0.02%) and heart (0.04%) (Jani et al, 1994).

Polystyrene spheres (50, 100, 300, 500, 1000 and 3000 nm) with covalently-linked fluorescein were administered by oral gavage to rats at a dose of 1.25 mg/kg bw for 10 days. Stomach, small intestine, colon, liver, spleen, heart, blood, kidney and lungs were analysed for the presence of polystyrene by gel permeation chromatography. Uptake of polystyrene into the PPs was observed for all particle sizes. Distribution to liver, spleen, blood and bone marrow showed size dependence, and was estimated to be up to approximately 6% of administered dose for 50 nm particles; 1% for 1000 nm and 0% for 3000 nm particles (Jani et al, 1990).

In a longer term study, Joel et al, (1978) investigated the uptake of carbon particles (estimated to be of diameter 20-50 nm) from the intestinal tract of mice following gavage for 2-8 days, or administration in the drinking water (1.5% carbon suspension) for up to 12 months. Following gavage, carbon particles were identified in the PP but not in other tissues, including the mesenteric lymph nodes. After exposure in drinking water for two months, carbon particles were observed in the PP and the mesenteric lymph nodes, but not in the liver, spleen or lungs. Particles remained in PP and macrophages in the mesenteric lymph node for four months after exposure had ceased. Similar findings were observed after six months but there was some equivocal evidence of carbon particles in the liver, and macrophages containing carbon particles were frequently seen in the lung. There were no remarkable differences at 12 months.

Limited observations supporting intestinal absorption of non-biodegradable particulate material are also available for humans. Powell et al, (1996) reported the presence of granular pigment in GALT from surgically resected intestinal tissue. Pigmented macrophages containing numerous phagolysosomes, rich in submicron-sized particles, were characterized by morphological assessment, X-ray microanalysis and image electron energy loss spectroscopy to be: (1) spheres of TiO$_2$ (anatase 100-200 nm); (2) aluminosilicates characteristic of the natural clay mineral kaolinite; and (3) mixed environmental silicates without aluminium (100-700 nm in length). Bockmann et al, (2000) also reported that TiO$_2$ nanoparticles (160 and 380 nm) administered to human volunteers resulted in generally increased blood titanium levels for up to 24 h, which appears to provide some qualitative evidence that non-biodegradable particles can be absorbed across the intestinal mucosa, and reach the systemic circulation in humans.
5.6.3 Dermal absorption studies

The potential for nanoscale materials to be absorbed across the skin has received considerable attention due to their potential for use in sunscreens, cosmetics and therapeutics (Desai et al, 2010; Hagens et al, 2007; Landsiedel et al, 2012; Nohynek et al, 2007; Prow et al, 2011; Stern and McNeil 2008; TGA, 2013). In general, the consensus is that healthy skin represents a substantial barrier so the penetration of most environmental nanoparticles, including viruses, dusts, allergens and other materials is negligible. On that basis, it has been argued that, if nanoparticles are considered to resemble macromolecules of high molecular weight, significant absorption from healthy skin is considered unlikely (Maynard et al, 2011).

The penetration of zinc oxide (ZnO) and TiO$_2$ particles into animal and human skin appears to be the most studied of all nanoparticles in experimental studies due to their common use in sunscreens. The weight of evidence suggests that nanoparticles applied to the skin only penetrate into hair follicle openings and skin furrows, and that little material penetrates below the surface of the stratum corneum (Nohynek et al, 2008). This conclusion was supported more recently by the European Commission’s Scientific Committee on Consumer Safety (SCCS, 2012) which concluded there was no evidence that ZnO nanoparticles are absorbed through the skin.

Similarly, a recent review of the scientific literature by the Australian TGA found that the current weight of evidence suggests TiO$_2$ and ZnO nanoparticles do not reach viable skin cells or the systemic circulation, and that a potential for harm has not been demonstrated in short-term studies (TGA, 2013). This review considered the results of an Australian study which investigated the potential for systemic exposure of humans to nano-ZnO in a sunscreen formulation over a period of five days (Gulson et al, 2010). It concluded that while the results indicated increased levels of Zn (based on changes in $^{68}$Zn/$^{64}$Zn ratio) from $^{68}$ZnO particles, the analytical method could not differentiate between nano-particulate or ionic zinc.

There is some evidence suggesting that certain nanoparticles may penetrate into or through skin under certain conditions. For example, Ryman-Rasmussen et al, (2006) found that quantum dots (QD; particle size 4.6 or 12 nm) with neutral or cationic coatings may penetrate into the epidermis or dermis of intact porcine skin at alkaline pH, whereas QDs with anionic coating penetrated to a small extent into the epidermis after 24 h of exposure. This finding was further investigated in recent study in which QDs with three surface modifications, polyethylene glycol (PEG), PEG-amine (PEG-NH$_2$) and PEG-carboxyl (PEG-COOH), were evaluated for human skin penetration from aqueous solutions at pH 7.0, 8.3 (PEG, PEG-NH$_2$) and 9.0 (PEG-COOH). There was some penetration into intact viable epidermis of skin for the PEG-QD at pH 8.3, but not at pH 7.0 and no penetration into living skin was observed for any other QDs at the pHs used. However, all QDs penetrated through the viable epidermis and into the upper dermis within 24 h following tape-stripping of the stratum corneum (Prow et al, 2011).

Overall, while there is no evidence of significant penetration by nanoparticles in sunscreen through healthy human skin, there is some evidence that under certain conditions, including alkaline pH and compromised skin conditions, some limited penetration of certain nanoparticles through the stratum corneum to the viable dermis is possible. The toxicological significance of this finding is however unclear, given the limited amounts of dermal penetration likely to be encountered under normal conditions.
5.6.4 Parenteral administration

Typically, naked (uncoated) nanoscale particles are removed from the circulation in a matter of minutes and sequestered into organs of the mononuclear phagocyte system (MPS) (Owens and Peppas, 2006). Most nanoscale and microscale particles, upon contact with biological matrices, are immediately coated by proteins, leading to the formation of a protein ‘corona’. These coronas are complex and variable and of the approximately 3700 proteins in the plasma proteome, about 50 have been identified in contact with various nanoparticles. Their presence creates a molecular signature which is recognized by immune cells and determines the biodistribution (reviewed in Aggarwal et al, 2009).

Significant differences can be observed in the binding of blood proteins and opsonins to nanoscale particles, depending on surface properties of the particle (Moghimi et al, 2005). Whether nanoparticles bind proteins at all depends mainly on the surface characteristics, primarily hydrophobicity and charge. Other factors including core constituents, size, shape and curvature, are reported to mainly influence the amount of protein bound, but not protein identity (Aggarwal et al, 2009). Thus, differential opsonisation may account for differences in clearance rates and macrophage sequestration of particulates (Moghimi et al, 2005).

Shinohara et al, (2013) administered Degussa P25 TiO$_2$ nanoparticles by intravenous injection to rats at a dose of 0.95 mg/kg bodyweight. Blood concentrations of TiO$_2$ were 420 ng/mL and 19 ng/mL at five and 15 minutes after administration (equivalent to only 2.8% and 0.13% of the administered dose, respectively) indicating rapid clearance from the blood. At 6 h, the highest concentrations of TiO$_2$ were found in the liver (94% of administered dose) followed by the spleen, lung, kidney, heart and blood with 2.0%, 0.17%, 0.023%, 0.014% and 0.026% respectively. TiO$_2$ concentrations in the liver and spleen remained relatively constant over the 30 day experimental period. Elimination rates of TiO$_2$ in the faeces and urine did not differ significantly from controls.

Fischer et al, (2006) administered QDs (cadmium-selenium; CdSe core; Zinc Sulfide; ZnS shell; coated with mercaptoundecanoic acid [QD-LM] or bovine serum albumin [QD-BSA]; hydrodynamic diameter (HD) 7-25 nm and 80 nm, respectively) intravenously to rats and measured blood and tissue levels, at time points up to 90 minutes. Faeces and urine were collected for up to 10 days. Plasma levels decayed according to first-order kinetics. Blood clearance of QD-BSA was 1.23 mL/min per kg compared to 0.59 mL/min per kg for QD-LM. The QD-BSA half-life was 39 min versus 58 min for QD-LM. The volume of distribution (Vd) for both QDs was approximately 65 mL/kg. Approximately 40% of the administered dose of QD-LM was taken up by the liver 90 minutes following dosing, while virtually all QD-BSA was found in the liver at the same time point. There was no evidence that particles were excreted in urine or faeces over the 10 day period.

Yang et al, (2007) administered QDs (Cadmium Tellurium; CdTe core; ZnS shell; methoxy-PEG-500 coating; approximately 13 nm diameter) by injection into the tail vein of ICR mice. The mice were killed at 1, 4, 24 h and at 3, 7, 14, and 28 days after dosing and plasma, red blood cells, liver, lungs, kidneys, spleen, muscle, fat, brain, skin and bones were collected for analysis. The plasma half-life of QD705 was approximately 18 h. The concentration of cadmium was highest in the spleen, liver and kidney, with kidney levels increasing over the 28 day study period. At day one, mass balance studies showed overall recovery as a percentage of dose was highest in the liver (29%), blood (10%), spleen (4.8%) and kidney (1.5%). At day 28, recoveries were liver (40%), blood (0%), spleen (5.2%) and kidney (9.1%). QD was not detectable in faeces and only 0.01-0.04% was recovered in urine.

Ballou et al, (2004) administered QDs with four different coatings by intravenous injection to mice and monitored the localization of the QDs by fluorescence imaging, optical and electron microscopy. To determine the long-term stability and retention of QDs, mice were imaged at 15 minutes, one day, three days, seven days, 28 days and 133 days. Notably, fluorescence from QDs was decreased but
visible in the liver, lymph nodes and bone marrow after one month, and persisted at 133 days in the axillary, inguinal, and lumbar lymph nodes.

Choi et al, (2007) intravenously administered a series of $^{99m}$Technetium-labelled QDs with a CdSe core, a ZnS shell and zwitterionic coating (cysteine) ranging from 4.36 to 8.65 nm HD to CD-1 mice. Blood was sampled from the tail vein intermittently and mice were killed 4 h after injection. The β-phase terminal half-life increased from 48 minutes to 20 h as the HD increased from 4.36 to 8.65 nm. QD515 (4.36 nm) was recovered primarily in the bladder at 4 h, with radioactivity also detected in the liver, kidney and intestine. The largest QD (QD574; 8.65 nm) showed increased uptake in the liver (approximately 27% injected dose ID), lung (approximately 9% ID) and spleen (approximately 6% ID), and proportionally lower levels in the bladder. Excretion in urine was highest (approximately 80% ID) for the QD515 and lowest for QD574 (approximately 20% ID). An inverse relationship was observed for retention in the carcass.

5.6.5 Elimination

The renal elimination of particulates from the blood is relatively well understood and can at least partially be explained by renal anatomy and physiology. For particulates, glomerular filtration is dependent on the HD of the particle. For example:

- Inulin, which has an HD of about 3 nm, is completely filtered whereas for IgG, which has an HD of ca 11.0 nm, glomerular filtration is negligible.
- For non-biodegradable or slowly biodegraded materials such as QDs it has been suggested that a final HD ≤ 5.5 nm is needed to permit complete elimination from the body in urine (Choi et al, 2007). Similar findings have been reported for hydrophilic macromolecules such as dendrimer-based MRI contrast agents (Kobayashi and Brechbiel, 2005).

The hepatobiliary system represents the primary route of excretion for particles that do not undergo renal excretion. The weight of evidence to date suggests that elimination of non-biodegradable particles in the bile and faeces may occur at low levels, but this is likely to be a relatively slow and inefficient process. For example, following intravenous administration of 50 nm polystyrene spheres, approximately 4% of the administered dose was excreted in the bile as intact particles in 24 h (Ogawara et al, 1999).

5.6.6 Adequacy of the existing OECD toxicokinetics guideline

The OECD WPMN considered that absorption/distribution studies are of key importance when investigating the likely toxicity of nanomaterials. It was noted that studies will probably need to be designed on a case by case basis, and are likely to be technically challenging due to the analytical difficulty in tracking distribution in vivo using realistic exposure scenarios. The WPMN emphasised the need for care to ensure that the label remains with the particle, and that the label does not alter the toxicity of the particle (OECD, 2009).
5.6.7 Conclusions on studies investigating the toxicokinetics of nanoparticles

- No classical toxicokinetic studies were found on nanoparticles following inhalational, oral and dermal exposure, quantifying bioavailability, metabolism, distribution or excretion; probably due to the generally low absorption and tissue concentrations.

- However, available studies suggest that the systemic absorption of insoluble nanoscale materials following inhalational, oral and dermal exposure is significantly restricted (compared to soluble small molecules) by the physiological barriers which exist to defend the body from particulate invasion.

- In studies where insoluble materials have been administered by intravenous injection, the test material has typically been rapidly removed from the blood and sequestered into macrophage-like cells of the liver, spleen, lungs and bone marrow. Residence times in the blood were generally short which, dependent on particle size, may reflect sequestration into MPS organs, rather than excretion in the urine.

- An important finding from oral ingestion and intravenous studies is that particulates taken up into MPS organs may be retained for extended periods, with little indication of elimination over extended investigation periods. Therefore, it is recommended that special attention be paid to organs of the MPS in toxicokinetic studies, and that extending the length of such studies should be considered where justified.

- Particulate materials should be appropriately labelled and care taken to ensure that the label does not dissociate from the test material. Where appropriate, combination techniques may be required to differentiate dissolved and particulate material in biological matrices.
5.7 Toxicology

5.7.1 General considerations

Inhalational particle and fibre toxicology studies conducted in the 1980s and early 1990s are considered to be the first to examine the toxicity of nanoparticles. For example, it was understood in the 1990s that particle surface area correlated well to toxicity in certain cases, and that increased surface area provides an opportunity for a significant effect on toxicity through, for example, transition metal-mediated Fenton chemistry (Donaldson et al., 1998). Similarly, Fubini (1997) observed that the toxicity of solid particles is not simply predictable from the chemical composition, but that the micromorphology determines the abundance of surface sites, which affects reactivity towards cells and tissues.

Since 2000 the field has expanded rapidly such that the term ‘nanotoxicology’ has been proposed to describe toxicology relating to nanoparticles and journals like Nanotoxicology have appeared to deal specifically with this area. Maynard et al., (2011) noted that in 2005, there were an estimated 179 articles published on the potential environmental health and safety implications of engineered nanomaterials, and that by 2009 the number had risen to 791. A search of Pubmed using the terms ‘nanoparticle’ and ‘toxicity’ for the current (2014) review found more than 7000 results.


5.7.2 Mechanism of action

Many in vitro studies have employed a range of heterogeneous nanoparticles in different cell types and culture conditions to investigate the uptake and toxicity of nanoparticles.

Unfried (2007) considered it impossible to ascribe a unique common mechanism of cellular uptake for nanoparticles and that the mechanism depends upon the physicochemical characteristics of the material (eg size), the cellular environment (eg serum components) and the characteristics of the target cell (eg phagocyte vs non-phagocyte). A recent review by Treuel et al., (2013) further discussed cellular uptake of nanoparticles and the effects of the protein corona on uptake efficiency. The structure and composition of the protein corona is influenced by the physicochemical properties of the nanoparticle, the nature of the physiological environment (eg culture medium) and duration of exposure. It alters both the size and composition of the nanoparticle at the interface, which can influence biological response, including cellular uptake and toxicity.

The generation of reactive oxygen species is a common finding in in vitro assays employing a diverse range of nanoparticles including fullerenes, SWCNTs, QDs and conventional ultrafine particles. (Donaldson et al., 2004; 2009; Oberdorster et al., 2005a; Nel et al., 2006; Balbus et al., 2007; Lewinski et al., 2008; Unfried et al., 2007). It has been proposed that oxidative stress may be due to the direct
effects of particles in the inside of the cell, impacts on mitochondrial respiration, or depletion of antioxidant species within the cell (Donaldson and Seaton, 2012). Increased levels of reactive oxygen species may damage nucleic acid bases and membranes, and activate apoptosis and necrotic pathways.

This has led some investigators to propose that oxidative stress in cellular systems may serve as a relevant endpoint in determining the toxicity of these materials. However, others have cautioned that while the generation of reactive oxygen species may serve as a useful starting point for the toxicological investigation of nanomaterials, it should not be seen as a way to generalise the mechanistic toxicity of a diverse group of materials (Donaldson et al, 2009; Stern and McNeil, 2008; Unfried et al, 2007). For instance, Donaldson et al, (2009) noted that while the conventional particles PM10, asbestos and quartz induce similar oxidative stress effects in cell culture, they induce quite different pathologies in vivo.

Lewinski et al, (2008) comprehensively reviewed the cytotoxicity data for a range of nanoparticles including carbon-based nanoparticles (fullerenes, CNTs), metal-based nanoparticles and semiconductors (QDs). Dose and time-dependent cytotoxicity was observed for all these classes of nanoparticles. While the cytotoxicity of these nanoparticles is related to the core structure, the surface coating of the nanoparticle is an important determinant of cytotoxicity. For instance, surface coating may prevent leaching of the toxic core constituents or increase water solubility. The authors note that a thorough comparison of results between studies is often limited by incomplete physicochemical characterization of nanoparticles, and differences in cell lines and exposure conditions.

Unfortunately, there has been a tendency to over-interpret the results from in vitro studies in the literature, particularly where unrealistic concentrations of nanoparticles have been used. Also, cellular exposure for particulates in in vitro studies requires additional consideration because nanoparticles do not behave like soluble chemicals. Processes including settling, diffusion and aggregation may significantly affect the dose to which cells are exposed (Teeguarden et al, 2007). Furthermore, agglomeration and dispersion of nanoparticles can be expected to vary with each concentration within the test system (OECD, 2012b). These factors should be carefully considered in the design and interpretation of in vitro toxicity studies.

5.7.3 Inhalational toxicity

As mentioned above, the origins of nanotoxicology are derived from inhalation experiments with conventional nanoparticles and fibres. These studies are numerous but generally relate to a limited range of poorly soluble particles such as TiO₂, carbon black, diesel soot, talc and asbestos. Collectively they identified that lung fibrosis and tumours can be formed in the lung of rats due to high particle concentrations of relatively low toxicity materials due to either high exposure levels, or a failure to remove inhaled particles. These studies also provide the basis for an understanding of the importance of dose, dimension and duration, often referred to as the ‘three Ds’ (Oberdorster, 2002), which make up the fibre pathogenicity paradigm. An understanding of this historical work provides a framework for assessing the inhalational toxicity of potentially novel nanoparticles that may be used in agricultural or veterinary chemicals.

a) Nanoparticles

Much of the concern related to the inhalational toxicity of nanoparticles is derived from early studies which demonstrated that ultrafine particles elicited greater toxicity than larger-sized particles of the same material. These initial studies conducted in the early 1990s showed that ultrafine particles of TiO₂ and Al₂O₃ intra-tracheally instilled in rats, resulted in greater pulmonary inflammation and interstitial translocation than fine particles of the same material (Ferin et al, 1990; Oberdorster et al, [missing page number]).
1990; Oberdorster et al, 2007). On the basis of later studies with TiO$_2$ and carbon black, it was suggested that lung toxicity correlates better with surface area than mass, volume or particle number (Oberdorster et al, 1992).

A considerable body of evidence now suggests that lung fibrosis and tumours in rats develop as a consequence of prolonged inflammatory response induced by high concentrations of poorly soluble particles, either due to high exposure levels or an inability to remove inhaled particles from the lung, which leads to rat lung overload. Rat lung overload has been defined as a “consequence of exposure that results in a retained lung burden of particles that is greater than the steady-state burden predicted from the deposition rates and clearance kinetics of particles during exposure” (ILSI, 2000). The sequence of events leading to the development of pathological effects in the lung in rat lung overload is generally considered to involve: failed clearance with accumulation of dose → inflammation and oxidative stress → altered particle kinetics with retention consequent on impaired clearance → fibrosis → proliferation → the development of benign and malignant lung tumours (Donaldson and Poland, 2012).

The mechanistic basis for these effects is believed to involve an overwhelming of the alveolar macrophage-mediated clearance of particles which defend the lungs from particulate invasion and oxidative stress. Chronic recruitment of inflammatory cells into the alveolar compartment and their activation leads to oxidative stress which is believed to result in secondary genotoxicity eventually leading to tumour development. The concept of dose is extremely important because, as for non-genotoxic chemicals, low toxicity particulates in the lung can generally be expected to exhibit a dose-response relationship. That is, there is generally a dose below which (the threshold dose) the number of damaged alveolar macrophages is low enough not to cause appreciable inflammation. Therefore, a high enough dose of any particle may eventually cause lung injury, but it can reasonably be assumed that it will require a lower exposure of a toxic material (Donaldson and Tran, 2002). While increased retention may enhance the potential for local effects and systemic redistribution, data from rats and other rodent species has not indicated adverse effects outside the respiratory tract and associated lymph nodes for poorly soluble particles deposited in the lung (ILSI, 2000). This is consistent with Section 5.1, which notes there is currently no convincing evidence for a significant redistribution of particulates outside the respiratory system and local lymph nodes in the absence of lung injury.

Notably, rats appear much more susceptible to lung overload following exposure to poorly soluble particles than mice or hamsters. Inhalation exposures of hamsters and mice to talc, TiO$_2$, or diesel soot have not resulted in lung tumours, despite similar or greater lung particulate lung burdens than those that produced tumours in rats. As such, the relevance of lung tumours in rats following instillation or inhalation of poorly soluble particles of low toxicity to human health risk assessment has been a subject of debate (ILSI, 2000). Nevertheless, the concept of rat lung overload applies to poorly soluble particles of low toxicity, and other more toxic particles such as crystalline silica and synthetic fibres may actively damage alveolar macrophages. On that basis, impairment of alveolar macrophage clearance should not be viewed as particle overload in all cases (Borm et al, 2004).

For experimental convenience most early studies were conducted using intra-tracheal administration which may lead to artefactual findings due to the formation of heavy, localized deposits of large particles, with ensuing localized inflammatory responses and increased permeability of the epithelium (reviewed in Pauluhn, 2009). This method of administration may also allow non-respirable materials to access the lung by avoiding nasal deposition of larger particles that would usually be filtered by physiological mechanisms (refer to Section 5.1). Furthermore, instillation techniques require dispersion with surfactants and sonication, which may also affect particle size and toxicity. Therefore, studies employing intra-tracheal administration should be viewed cautiously and accompanied by data indicating the type and fate of nanoparticles in the lung, since toxicological effects may reflect the methods of administration rather than the nature of the administered material.
Conversely, while it is recognized that generating stable, homogeneous, reproducible aerosols is technically difficult, inhalation is the physiological process by which nanoparticles are deposited in the respiratory tract and lungs. It has the technical advantage of not requiring carrier systems and the properties of the particles can be characterized and controlled (Pauluhn, 2009). In addition, it provides a more realistic exposure rate and an opportunity for normal clearance processes to occur (OECD, 2012c). The technical aspects of inhalation toxicology studies, including the use of nose-only inhalation systems, are addressed in current OECD guidelines for inhalational toxicity testing. Several investigators have also recently published methods describing the generation and characterization of test atmospheres with nanoparticles (Creutzenberg, 2012; Ma-Hock et al, 2007).

A growing number of more recently conducted inhalation studies on a diverse range of nanoparticles are becoming available in the public literature. For instance, Klein et al, (2012) recently reported on short-term inhalation studies conducted in rats with nanoscale TiO$_2$, amorphous silica (Zeosil 45), silica gel (Sylloid 74) and pyrogenic Cab-O-Sil M5, ZnO, barium sulfate, zirconium dioxide, carbon black and CeO$_2$. The protocol involved a 28 day study period, consisting of a five day inhalation exposure (head-nose) in male Wistar rats and a three week post-exposure period. The available results have been compared against available information for those materials in the public literature to demonstrate the utility of the assay. The authors further note that short-term inhalation study results for more than 20 nanomaterials are available, including representative nanomaterials listed by the OECD WPMN.

b) Nanofibres

Studies with asbestos fibres administered to experimental animals either by inhalation or intracavitary injection identified that the most important parameters to determine the toxicity and carcinogenicity of fibres are the ‘three Ds’. The mechanism of toxicity is generally considered to involve frustrated phagocytosis which occurs when a macrophage fails to engulf a particle larger than itself, leading to the release of reactive oxygen species.

The three Ds describe what is referred to as the fibre pathogenicity paradigm, a criterion which a particle must meet if it is to present a fibre-type hazard. On the basis of this paradigm, it is reasonable to suspect that some high-aspect ratio nanoparticles such as carbon nanotubes (CNT) or nanowires might pose a hazard to the lungs, pleura and peritoneal mesothelium (Donaldson and Poland, 2012).

Indeed, the first published studies investigating the pulmonary toxicity using instillation of CNTs emerged in 2004 (Lam et al, 2004; Warheit et al, 2004). Instilling high doses of short single-walled carbon nanotubes (SWCNT) into the lungs of rats led to the formation of multifocal granulomas with foreign body giant cells typical of a foreign body-induced reaction. However the high doses resulted in non-uniform distribution in the respiratory tract and approximately 15% mortality in instilled rats due to asphyxiation, which limited the utility of the results to risk assessment (Warheit et al, 2004).

Since those early studies, the potential for CNTs to induce inflammation and fibrotic changes has been demonstrated in a number of inhalational studies. For example, Ma-Hock et al, (2009) exposed rats to multi-walled carbon nanotube (MWCNT) in a short term inhalation study, and a 90 day inhalation study conducted according to OECD 412 and OECD 413. No systemic toxicity was observed, but a concentration-dependent increase in lung weights and granulomatous inflammation was observed. Inflammation was observed at the lowest exposure concentration (100 µg/m$^3$), such that a NOAEC could not be established.

In another study Pauluhn (2010) exposed Wistar rats nose-only to 0.1, 0.5, 1.5 and 6 mg/m$^3$ Baytubes for 90 days (6 h/day, 5 days/week). Moderate inflammation with granulomatous appearance was noted only at 6 mg/m$^3$. Goblet cell hyper- and/or metaplasia were also observed. During the recovery
period of six months, the effects were not fully reversible, but there was some evidence of regression over time. The NOAEC was 0.1 mg/m$^3$.

5.7.4 Oral toxicity

Humans have been exposed to nanoparticles and nanostructured food substances throughout evolution without significant adverse findings due to the particulate nature of the material. For example, homogenised milk contains oil droplets of 200-2000 nm, cow and human breast milk contain casein particles of around 50 nm and silicon dioxide is commercially available as a food additive in a number of different sizes. Similarly, ferritin is a naturally occurring nano-particulate of around 12 nm that contains an iron oxide core of 6-8 nm, and is widely ingested in both meat and plant foods (Powell et al, 2010).

These observations demonstrate that being nanoscale does not necessarily imply additional safety concerns following oral ingestion. Soluble nanoscale materials of a conventional substance would be expected to exhibit toxicities largely attributable to the constituent ions and monomers. Conversely, relatively insoluble nanoparticles that remain particulate in nature in the final food, and following oral ingestion, may require additional regulatory consideration.

An understanding of the toxicity of insoluble nano-particulates following oral exposure is in its relative infancy, and mainly related to metals and metal oxides. Several of these studies are of questionable quality and of limited use for risk assessment purposes. Limitations include small group sizes, single and unrealistically high doses, examination of only a small number of biological parameters and limited and occasionally unconvincing histopathology.

Chen et al, (2006) investigated the toxicity of copper nanoparticles, copper microparticles and copper ions. Copper ions appeared to have higher oral toxicity than copper nanoparticles, which in turn had higher toxicity than microparticles. Dose-dependent findings were reported in the liver, kidney and spleen.

Wang et al, (2007) compared the acute toxicity of nano-sized TiO$_2$ particles (25 and 80 nm) and fine TiO$_2$ particles (155 nm) following oral administration. The authors reported no overt signs of toxicity in mice administered doses of 5000 mg/kg for any particle sizes. Relative liver weight increased in female mice for 25 and 80 nm particles. Some changes in clinical chemistry were also reported in the serum of female mice for the 25 and 80 nm particles.

In an OECD guideline compliant study, a single dose of TiO$_2$ particles (approximately 140 nm diameter) suspended in deionized water was administered by oral gavage to one female rat each at a dose of 175, 550, or 1750 mg/kg, and to three fasted female rats at a dose of 5000 mg/kg. No mortality occurred on the study. Grey coloured faeces were observed at the two highest doses. No gross lesions were observed at necropsy (Warheit et al, 2007).

Although the available data investigating the oral toxicity of nano-particulates are limited, findings from toxicokinetic studies indicate that nano-particulates may be retained in GALT, as well as in the liver and spleen for extended periods (refer to Sections 5.2 and 5.4). As such, these tissues should be carefully assessed as a part of oral toxicity studies. Careful attention should also be paid to characterisation of the nano-particulate in the feed since it is likely that aggregation/agglomeration (in the feed) will affect the bioavailability and toxicity of the test material. Where comparing the toxicity of a nanoparticle with a non-nanoscale material, control groups should ideally include the larger form of the particle, and where relevant, the soluble form of the material.
5.7.5 Dermal toxicity

While a large amount of literature is available investigating the potential dermal penetration of nanoparticles through skin (refer to Section 5.3), there is a relative dearth of in vivo studies investigating the systemic toxicity of nanoparticles following repeated dermal administration. The limited in vivo studies that have been conducted primarily address the issue of cutaneous toxicity, and have largely only identified irritation as an adverse effect following topical administration of nanoparticles (Stern and McNeil, 2008).

Where available, most of the literature on the potential dermal toxicity of nanoparticles has been focused on the use of TiO$_2$ and ZnO in sunscreens. This subject has been comprehensively reviewed by the TGA on a number of occasions and will not be considered in further detail here. In August 2013, the TGA reaffirmed its scientific opinion that, based on current evidence, TiO$_2$ and ZnO nanoparticles in sunscreen are unlikely to cause harm. This conclusion was based on the current weight of evidence which suggests that TiO$_2$ and ZnO NPs do not reach viable skin cells or the general circulation, and consideration of available toxicological data (TGA, 2013).

5.7.6 Chronic toxicity and carcinogenicity

There is a large database of existing studies with conventional carcinogenic particles, such as quartz and asbestos, and studies of exposure to diesel exhaust nanoparticles in the workplace (reviewed in Borm et al, 2004; Donaldson and Poland, 2012; Donaldson and Seaton 2012; ILSI 2000; Oberdorster, 2002, Oberdorster et al, 2005a; Pauluhn, 2009).

The existing work on conventional nanoparticles is likely to be useful in guiding investigations into the inhalational toxicity of potentially novel nanoparticles, or nano-particulates with a high-aspect ratio. For instance, it seems reasonable to predict that insoluble nanoparticles, particularly those with a reactive surface, may cause inflammation which could lead to secondary carcinogenesis where there is sufficient prolonged inhalational exposure (Donaldson and Poland, 2012). However, to date there are no published chronic inhalation or carcinogenicity studies investigating whether novel nano-particulates may deviate from the classical oxidative stress and inflammation mechanisms proposed for these conventional substances (refer to Section 6.3).

Similarly, on the basis of the fibre pathogenicity paradigm, it is reasonable to suspect that any insoluble high-aspect nanoparticle of suitable dimensions has the potential to be carcinogenic at the pleura and in the lung, given sufficient doses. Some data are available for MWCNT, however the relevance of these studies is difficult to assess due to limitations in experimental methodology and contrasting results. These issues have been well addressed in the NICNAS 2007–09 review of the literature on toxicological and health-effects relating to six nanomaterials (Priestly, 2009). Specifically, the NICNAS report notes a study by Muller et al, (2009) which showed a clearly positive peritoneal mesothelioma response to asbestos in rats following intraperitoneal injection, but did not show any mesothelioma response following a single administration of two dose levels of MWCNT. In contrast, another study by Sakamoto et al, (2009) found that MWCNTs instilled into the scrotal sac of rats induced a peritoneal mesothelioma response, but asbestos failed to show a similar response. Further work is required to establish the potential for MWCNTs to induce a mesothelioma-type response.
5.7.7 Genotoxicity

Although the mechanisms have not been fully elucidated, it is generally considered that nanoparticles may elicit genotoxic responses through direct interaction with DNA, indirectly as a result of induced intermediates such as reactive oxygen species, or as a result of ions released from soluble nanoparticles. Secondary mechanisms may also be relevant, whereby nanoparticles induce a chronic inflammatory response in tissues in vivo through the recruitment of macrophages and neutrophils to the site and the subsequent release of reactive oxygen species (Donaldson et al, 2010; Gonzalez et al, 2008; Landsiedel et al, 2009; Magdolenova et al, 2013; Oesch and Landsiedel, 2012; Warheit and Donner, 2010).

In a recent review, Magdolenova et al, (2013) identified 94 in vitro and 22 in vivo studies published between 2000 and 2012; 67 genotoxicity studies used the comet assay (58 in vitro, nine in vivo), 44 used the micronucleus assay (31 in vitro, 14 in vivo), 11 used the chromosome aberration test (10 in vitro, one in vivo) and 13 used the bacterial reverse mutation assay. Investigated nanoparticles included TiO$_2$, iron, silver, fullerenes, silica, carbon black, zinc, gold, SWCNT, MWCNT, polymer nanoparticles, QDs, metals and metal oxides. The authors noted that results in the literature are often conflicting and attribute the variability to factors including the source of nanoparticles, the method of preparation and synthesis, the dispersion protocol and variables in experimental conditions (eg cell type, exposure time and concentration).

Doak et al, (2012) observed that of 19 reviewed studies using the Ames test, 17 of the tests were negative for mutagenicity and the remaining two studies reported only weak mutagenic effects, despite several nanoparticles testing positive for genotoxic responses in other in vitro tests in mammalian cells. Considering the utility of bacterial assays, they concluded the Ames test was unsuitable for analysing particulate nanoscale materials, perhaps due to the presence of a cell wall in prokaryotes which prevents uptake into the cell.

EFSA (2011) considered a bacterial reverse mutation test inappropriate for nano-particulates, on the basis that nanoparticles may not be able to penetrate the bacterial cell wall and because bacteria cannot phagocytose particles. EFSA recommend an in vitro test for induction of gene mutations in mammalian cells, and a micronucleus assay. It noted that choosing the appropriate in vivo genotoxicity test(s) requires expert judgment based on all other relevant data, including toxicokinetics. However an in vivo micronucleus test, an in vivo Comet assay or transgenic rodent gene mutation assay may all be suitable.

Where adequate exposure cannot be achieved, conventional genotoxicity studies have little value, so it is essential to give due consideration to the toxicokinetics of the test material before conducting in vivo testing. Modified protocols should be adopted where it is evident that standard protocols will give a false negative result. This is not unique to nanoparticles. There are a number of compounds for which in vivo tests do not provide useful information because data on the toxicokinetics indicate that they are not available to target tissues. Some examples include radio-imaging agents, aluminium-based antacids, some compounds given by inhalation and some dermally, or other topically applied pharmaceuticals.
5.7.8 Reproductive and development toxicity

No adequate reproductive or developmental toxicity studies were identified in the literature, however there is limited evidence that nanoparticles may reach the foetus following dosing of maternal animals by intravenous and subcutaneous injection. Semmler-Behnke et al, (2008) reported that nanoscale gold particles were transferred to the embryos of rats following intravenous injection. Similarly, Yamashita et al, (2011) reported that silica and TiO$_2$ nanoparticles injected intravenously into pregnant mice at gestational day 16 crossed the placental barrier and were distributed to foetal tissues.

Takeda et al, (2009) reported that TiO$_2$ (25-70 nm) administered subcutaneously to pregnant mice on gestation days three, seven, 10 and 14 was found in the brain and testes of six-week-old male offspring. Particles in the testis and brain were identified as TiO$_2$ using transmission electron microscopy (TEM) and field emission SEM. Aggregates of TiO$_2$ nanoparticles (100-200 nm) were present in Leydig cells, Sertoli cells and spermatids in the testes. Testicular morphology in TiO$_2$-exposed mice was abnormal; some seminiferous tubules appeared disorganised and disrupted and the Sertoli cells had large nuclei and nucleoli. Sperm morphology did not differ significantly from controls but daily sperm production and sperm motility were decreased. Nanoparticles of TiO$_2$ were also present in the olfactory bulb and cerebral cortex (frontal and temporal lobes) of the six-week-old mice.

While the utility of these studies is limited by a lack of detail in reporting, there is some evidence that following administration of large doses of nano-particulates intravenously or by subcutaneous injection, some test material may reach the developing foetus.

5.7.9 Adequacy of OECD guidelines for assessing the toxicity of nanoparticles

a) Inhalational toxicity

The OECD WPMN considered whether the current toxicity testing guidelines are adequate for determining the inhalational toxicity of novel nanoparticles (OECD 2009). It noted that Test Guideline 403 (adopted 1981) for acute inhalational toxicity includes only very limited histological examination at autopsy. The WPMN recommended that studies investigating the acute toxicity of nanoparticles by inhalation should include detailed examination of the respiratory tract, with consideration of the addition of broncho-alveolar lavage (BAL) and possibly pulmonary cell proliferation endpoints. Detailed histological examination of the entire respiratory tract would be expected when investigating the effect of nanoparticles following repeated exposure by inhalation, with consideration of the addition of BAL and possibly pulmonary cell proliferation endpoints.

The adequacy of OECD guidelines for inhalation toxicity testing was further considered in an OECD WPMN on inhalation toxicity testing of nanomaterials held in The Hague, the Netherlands in 2011 (OECD, 2012c). The meeting report contained suggestions for some minor technical revisions to the OECD Test Guidelines (TG 403, TG412, TG413 and TG436), the Guidance Document on Acute Inhalation Toxicity Testing [ENV/JM/MONO(2009)28], and the Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials. This guidance should be consulted for further technical discussion related to the design and conduct of inhalation experiments using nanoparticles.
b) Oral toxicity

The OECD WPMN concluded that Test Guidelines 420, 423 or 425 are appropriate for an initial investigation of oral toxicity. It recommended that specific attention should be paid to cardiovascular and inflammatory parameters and the MPS in repeat dose studies. Furthermore, the WPMN anticipated that it might not be possible to strictly adhere to some OECD guidelines for in vivo tests requiring specifically high mass concentrations to be tested due to limited dispersability of some nanomaterials. In such cases it is recommended to test a dose range up to the dispersability limit (OECD, 2012b).

c) Dermal toxicity

OECD (2009) considered it desirable to have enhanced pathology in the current guideline for dermal exposure, Test Guideline 402, when assessing nanoparticles. It further recommended that the local lymph node assay (LLNA), Test Guideline 429, may be the most appropriate method for investigating skin sensitization.

d) Genotoxicity

OECD (2009) considered that Test Guideline 471 bacterial reverse mutation assay, Test Guideline 473 in-vitro mammalian cell gene mutation test and Test guideline 476 in vitro mammalian cell gene mutation assay (with the mouse lymphoma assay being the preferred assay) are suitable for an initial investigation of the mutagenicity of a nanoparticle. It further recommended that positive results in vitro would need to be followed up in vivo using Test Guidelines 474, 475, or 486 if the bone marrow or liver were appropriate target organs.

5.7.10 Conclusions on studies investigating the toxicity of nanoparticles

- The literature on the toxicity of nanoparticles is growing rapidly and there exist numerous in vitro and in vivo studies in the literature investigating the toxicity of various nanoparticles including metals, metal oxides, fullerenes and CNTs.

- There appears to be a general consensus that as a result of an increased surface area, altered surface chemistry and increased potential for dissolution, there is a potential for nanoparticles to exhibit a toxicity profile that deviates from that of conventional materials of the same composition. However, while the available evidence supports that there may be a difference in potency, there is as yet no convincing evidence to indicate that nanoparticles may induce unique or unconventional toxicities not seen with conventional materials.

- Numerous in vitro studies have shown that some nanoparticles can induce the generation of reactive oxygen species, inflammation and cytotoxicity. However, this is not considered to be sufficient evidence to explain the toxicity of all nanoparticles and other mechanisms are likely to be involved. Furthermore, many of these studies should not be over-interpreted, especially where unrealistic exposure conditions have been used.

- Early studies on the inhalational toxicity of nanoparticles and fibres provide a framework for establishing the toxicity of potentially novel nanoparticles that may be deposited in the lung. Although no chronic studies have been conducted, it seems reasonable to predict that:
  - Insoluble nanoparticles, particularly those with a reactive surface, may be able to cause inflammation which could lead to secondary carcinogenesis where there is sufficient prolonged inhalational exposure.
• Any insoluble high-aspect nanoparticle of suitable dimensions has the potential to be carcinogenic at the pleura and in the lung given sufficient doses.

• A large number of genotoxicity studies are available in the literature. Bacterial cell assays are not recommended due to the inability of nanoparticles to penetrate the cell wall and the inability of bacteria to phagocytose particles. Rather, in vitro genotoxicity studies should be conducted in mammalian cells. Available toxicokinetic studies suggest that tissue distribution is likely to be limited compared to small molecules. As such, the choice of an in vivo genotoxicity assay should be carefully considered to ensure the test material is distributed to the target organ.

5.8 Conclusion

It is the APVMA’s view that current risk assessment and testing methodologies are generally appropriate for assessing the toxicity of nanoparticles. However, it is anticipated that some modifications to test methodologies will be required. Adequate characterisation of test materials is critical so that the test material lies within the range of specifications for the material for which approval is sought. It should address the physicochemical properties of the naked particle and the nanoparticle in the final product, as well as any changes that may occur through the life-cycle of the product.

An understanding of the toxicokinetics of nanoparticles is critical to risk assessment as a key determinant of potential novel toxicities. Special attention should be paid to organs of the MPS, since available toxicokinetic studies indicate that nanoparticles are generally rapidly sequestered into these organs and the test material may be stored for extended periods. Detection, quantification and characterisation of nanoparticles in biological media present additional challenges and are likely to require appropriately radiolabelled materials.

Genotoxicity assays in bacterial cells are considered inappropriate because nanoparticles may not be able to penetrate the cell wall and bacteria cannot phagocytose particles. As such, in vitro tests should be conducted in mammalian cells. It is essential that the toxicokinetics of nanoparticles are duly considered before in vivo genotoxicity testing and modified protocols adopted where it is evident that achieving adequate target tissue concentrations is unlikely.

The APVMA recognizes that the state of the science in relation to nanoparticle toxicology has undergone rapid development in recent years so there may be instances where a novel nanoparticulate material has toxicological endpoints that are not addressed through standard guidelines. It is the responsibility of the applicant to develop suitable methods and protocols to address particular toxicological concerns related to the material, based on its novel physicochemical properties. In such cases, the APVMA would generally recommend that the applicant consult early with the Authority in relation to the safety of the end use of the product.

The APVMA notes that most of the accumulated knowledge related to human health risk assessment of nanoparticles relates to relatively simple nanoparticles. It will be important to monitor and periodically revise the validity of the current conclusions as the development of nanotechnologies allows the manufacture of more sophisticated materials.
5.9 References


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OECD (2012) Inhalation toxicity testing : expert meeting on potential revisions to OECD test guidelines and guidance document.


6 POTENTIAL ENVIRONMENTAL RISKS ASSOCIATED WITH THE USE OF NANOTECHNOLOGIES IN AGRICULTURAL AND VETERINARY CHEMICALS

There are no fundamental differences in the general risk assessment paradigms for chemicals and nanomaterials (Canady 2012; OECD 2010). The general categories of information required to carry out a risk assessment for nanomaterials include:

- identity information
- physicochemical properties
- industrial and consumer uses and environmental releases
- environmental fate
- absorption, distribution, metabolism and excretion (ADME) and the potential toxicity of nanomaterials.

The steps in the risk assessment paradigm are the same as for other chemicals and include exposure assessment, effects assessment (hazard identification and classification), and risk characterisation (Figure 6.1).

Figure 6.1: The risk assessment framework for nanoscale agricultural and veterinary chemicals in Australia.
In the case of nanotechnologies, the potential number of nanomaterials, which may each have different properties, uses and exposure pathways, requires that a range of important issues be considered or addressed to enhance their risk assessment. These issues relate principally to the physicochemical properties of nanomaterials and possible exposure pathways.

The problem formulation stage is a problem-scoping exercise. It identifies the relevant sources and targets of suspected harm and guides the remainder of the assessment. It may provide an opportunity to consider the physical and chemical properties of the nanomaterial upstream to reduce or avoid downstream risks. For example, if the problem formulation stage suggested a ‘safe by design’ approach for environmental sustainability reasons, the manufacturing process might be modified accordingly.

The exposure assessment analyses the physicochemical properties of the nanomaterial and possible exposure pathways whereas the effects assessment (hazard identification and classification) evaluates ecotoxicology information. The elements of the risk assessment at these stages include:

- the behaviour of nanomaterials in various media
- the persistence of nanomaterials (both chemical and physical persistence)
- transportation/distribution
- Predicted Environmental Concentrations (PECs)
- transformation products and impurities
- bioaccumulation
- effects/Predicted No Effect Concentration (PNEC).

Risk characterisation draws together the various lines of evidence from the assessment of the nanomaterial. A weight-of-evidence approach is used to determine the potential for harmful effects.

The discussion that follows addresses the environmental risk assessment of potential agricultural and veterinary chemicals. The reader is also referred to Kookana et al (2014), for which the APVMA provided financial support and scientific expertise.
6.1 Nanomaterials in environmental systems

Nanomaterials may have unique properties due to their size and/or shape that make them desirable for application in industry, in consumer goods or in agriculture. There is increasing interest in the use of nanomaterials in agricultural systems as fertilisers, plant protection products and/or for animal husbandry. Nano-sized agricultural and veterinary products introduced (deliberately or unintentionally) into environmental systems may include metals and metal oxides, nanoclays, emulsions, nanopolymers, nanocapsules and nanocages (Table 6.1). This makes regulating nanomaterials a challenge for agencies applying existing frameworks designed to address the environmental safety of soluble chemicals and bulk-sized solids. The issue is complicated further by the range of natural nanoparticles already in the environment, including colloidal clays, iron and manganese hydrous oxides, metals such as silver, dissolved organic matter (comprising fulvic and humic acids), and fibrous colloids (exopolymers).

After dispersion in the environment, nanomaterials may have a very different fate compared to soluble or bulk-sized agricultural and veterinary chemicals (Figure 6.2). Nanomaterials can behave quite differently to soluble chemicals in natural waters, soils and sediments in that they may associate via particle-particle interactions with natural colloids such as clays or organic matter. Nanomaterials are also likely to be more mobile in the environment than the equivalent bulk-sized material due to greater filtration and straining of bulk materials in soils and sediments. The high surface area and small diameter of nanomaterials also means that interactions with biota may be unique so bioaccumulation and biomagnification may be different to soluble or bulk-sized materials.

Figure 6.2: Potential reactions and fate of agricultural and veterinary nanomaterials in the environment (modified from Batley et al. 2012). With permission from CSIRO, Copyright CSIRO 2013
Table 6.1: Examples of nanomaterial products on overseas markets for use in the agricultural and veterinary medicines sector

<table>
<thead>
<tr>
<th>Function</th>
<th>How this can be achieved</th>
<th>Current examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enhance apparent solubility of active ingredient (a.i.)</strong></td>
<td>Nano- and micro-emulsions</td>
<td>Emulsion based registered pesticides Banner MAXX® of Syngenta (Observatory NANO 2010)</td>
</tr>
<tr>
<td><strong>Enhance uptake /efficacy of a.i.</strong></td>
<td>Nano and micro-emulsions, nanospheres</td>
<td>Nanopermethrin (Anjali et al. 2010); nanosphere insecticides (Boehm et al. 2003)</td>
</tr>
<tr>
<td><strong>Targeted delivery of a.i.</strong></td>
<td>Nanocapsules</td>
<td>Nanocapsules glyphosate or sulfonylurea herbicide (Perez-de-Luque and Rubiales 2009); Nano-liposomal drug formulation</td>
</tr>
<tr>
<td></td>
<td>Nano-liposomal drug formulation</td>
<td>Delivery of adriamycin (anticancer agent) via hyaluron-bound liposome to target hyaluron receptors on tumour cells (Yoo and Park 2004); PLGA-loaded drugs</td>
</tr>
<tr>
<td></td>
<td>PLGA-loaded drugs</td>
<td>PLGA-loaded growth hormones for pigs (Wang et al. 2011)</td>
</tr>
<tr>
<td><strong>Controlled release of a.i.</strong></td>
<td>Nanocapsules, nanospheres</td>
<td>Polymeric stabilised bifenthrin (Liu et al. 2008); Nanocomposite 2:4:D (bin Hussein et al. 2005); Porous hollow Si-encaged validamycin (Liu et al. 2006)</td>
</tr>
<tr>
<td><strong>Enhance toxicity of a.i. to target organism (lower dose)</strong></td>
<td>Nanodispersion, nanosusensions</td>
<td>Nanodispersed triclosan (Zhang et al. 2008)</td>
</tr>
<tr>
<td><strong>Protect a.i. from immediate metabolism and excretion</strong></td>
<td>Nanospheres</td>
<td>Nano spheres using PLGA for controlled delivery of porcine somatotropin (pST) hormone(Kuzma 2010)</td>
</tr>
<tr>
<td><strong>Protect a.i. against premature degradation</strong></td>
<td>Nanocapsules with catalyst a.i. conjugate</td>
<td>TiO₂-M262 polymer metaflumizone (Ishaque et al. 2009); Porous hollow Si-encaged validamycin (Liu et al. 2006);</td>
</tr>
<tr>
<td><strong>Enhance decomposition of a.i. in soil and/or plant</strong></td>
<td>Nanocatalyst conjugated a.i. in microcapsules</td>
<td>SDS-modified TiO₂/Ag conjugated with a.i. such as dimethomorph (Yan et al. 2005), imidacloprid and avermectin (Guan et al. 2011; Guan et al. 2008).</td>
</tr>
<tr>
<td><strong>Nanoparticle as a.i.</strong></td>
<td>Nanometals and nanoclays</td>
<td>Nano-Ag biocide (USEPA 2011); Nano-Si.(Nair et al. 2010)</td>
</tr>
<tr>
<td><strong>Early disease detection</strong></td>
<td>Quantum dots</td>
<td>Quantum dots with specific surface coatings to target cancer cell (Mattoussi et al. 2012)</td>
</tr>
</tbody>
</table>
The fate of nanomaterials in the environment is governed by both their route of entry into the environment (i.e. whether applied to land or water) and on their behaviour in environmental media (soils, sediments or waters) (Figure 6.3). A primary consideration for some nanomaterials will be solubility, as this dictates whether the material can be evaluated as a soluble chemical or as a particulate chemical. Transformation and degradation products of the nanomaterial need to be similarly evaluated. Transformation/degradation of the nanomaterial in the environment may markedly affect its bioavailability, bioaccumulation and potential toxicity (Figure 6.3). Distribution of the nanomaterial (and its transformation/degradation products) between air, water and soil/sediment will depend on physico-chemical properties of the nanomaterial as well as the physico-chemical properties of the receiving media (Figure 6.3).

This document reviews the intrinsic properties of nanomaterials that need to be considered for evaluating the risk of agriculture and veterinary nanomaterial products to the environment, as well as properties leading to unique interactions with environmental media (Table 6.2), and with biota that need to be considered when evaluating environmental risk.
Figure 6.3: Framework for environmental risk characterisation (exposure, fate/transport behaviour and effects) of potential agriculture and veterinary products containing nanomaterials. Note that degradation and transformation products will require re-evaluation based on solubility and aggregation considerations. For nanomaterials that reach the atmosphere and/or accumulate in edible plants, assessment of human risks must be undertaken.
Table 6.2: Important environmental considerations for some potential nanomaterials in agricultural and veterinary products based on mechanism of action.

<table>
<thead>
<tr>
<th>Product Category</th>
<th>Function</th>
<th>Forms</th>
<th>Details</th>
<th>Environmental consideration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery systems</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nanoformulation of</td>
<td>Enhance solubility (loading capacity) of otherwise insoluble substances</td>
<td>Oil/water nanostructure</td>
<td>O/w emulsions are labile</td>
<td>May be instantly destroyed in the environment</td>
</tr>
<tr>
<td>existing product</td>
<td></td>
<td>emulsions</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biodegradable polymers</td>
<td>Degradability is influenced by the type of polymer used, formulation and mechanism of degradation (bio, o xo- bio, and hydro-bio- degradation)</td>
<td>Degradation process may take a few weeks to years</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(biopolymers)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controlled release</td>
<td>nanomaterials are used as template for loading large amounts of substances</td>
<td>Mesoporous silica</td>
<td>Good chemical and thermal stability</td>
<td>Will likely persist and can become sorbents for other contaminants</td>
</tr>
<tr>
<td>carrier</td>
<td></td>
<td></td>
<td>High sorbing capacity</td>
<td>Movement through soil will be dependent on the hydrophilicity of the silica surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Biocompatible</td>
<td>Low toxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mesoporous silica</td>
<td>Good chemical and thermal stability</td>
<td>Can act as sorbents for other contaminants</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High sorbing capacity</td>
<td>Movement through soil will be dependent on the hydrophilicity of the silica surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biocompatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mesoporous silica</td>
<td>Good chemical and thermal stability</td>
<td>Will likely persist and can act as sorbents for other contaminants</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High sorbing capacity</td>
<td>Movement through soil will be dependent on the hydrophilicity of the silica surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biocompatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polymer-clay composites</td>
<td>Excellent absorbents – clay is known for their swelling properties</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nanoclays</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mesoporous silica</td>
<td>Good chemical and thermal stability</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High sorbing capacity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biocompatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mesoporous silica</td>
<td>Good chemical and thermal stability</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High sorbing capacity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biocompatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product Additives</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalyst</td>
<td>Enhance degradation of pesticide to reduce pesticide residues</td>
<td>TiO&lt;sub&gt;2&lt;/sub&gt; + pesticides</td>
<td>TiO&lt;sub&gt;2&lt;/sub&gt; is a good photocatalyst; anatase form considered more superior than the rutile form</td>
<td>Persistence could be a threat as it can facilitate the generation of toxic reactive oxygen species</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Likelihood for transformation of the TiO&lt;sub&gt;2&lt;/sub&gt; core is low as uncoated TiO&lt;sub&gt;2&lt;/sub&gt; has poor solubility in water.</td>
</tr>
<tr>
<td><strong>Protective material</strong></td>
<td>Protect active ingredients that are prone to degradation</td>
<td>TiO$_2$ or AlO$_2$-silicon + a.i.</td>
<td>For composites like Al$_2$O$_3$-silicon, though Al$_2$O$_3$ is typically presumed to be insoluble, dissolution and release of Al$^{3+}$ is still possible</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------------------------------------------------</td>
<td>-----------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Nanomaterial as Active material</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fungicide</strong></td>
<td>To kill and inhibit growth of fungi or fungal spores; i.e. control downy blight disease occurring on plants and powdery mildew</td>
<td>TiO$_2$</td>
<td>TiO$_2$ is a good photocatalyst; anatase form considered more superior than the rutile form</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>Persistence could be a threat as it can facilitate the generation of toxic reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>Likelihood for transformation of the TiO$_2$ core is low as uncoated TiO$_2$ has poor solubility in water</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>Agnanoparticles (and Ag$^+$) are known antibactericidal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>Persistence of intact Agnanoparticles may be low since it is vulnerable to transformation – it oxidises in O$_2$ and forms more stable silver salts (sulphides, chlorides, oxides, etc.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>Antibacterial property of Agnanoparticles (and Ag$^+$) could pose threat to microbial communities</td>
<td></td>
</tr>
<tr>
<td><strong>Photoprotective Material</strong></td>
<td>As a superspreading material to protect leaves from UV light – almost like a plant sunscreen</td>
<td>TiO$_2$/ZnO</td>
<td>TiO$_2$ is a good photocatalyst; anatase form considered more superior than the rutile form</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$/ZnO</td>
<td>Persistence could be a threat as it can facilitate the generation of toxic reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$/ZnO</td>
<td>Likelihood for transformation of the TiO$_2$ core is low as TiO$_2$ bulk has poor solubility in water</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$/ZnO</td>
<td>Persistence of intact ZnO nanoparticles will be low as uncoated ZnO nanoparticles have high water solubility and is susceptible to dissolution</td>
<td></td>
</tr>
<tr>
<td><strong>Plant Growth Improver</strong></td>
<td>Enhance plant growth *enhanced growth in spinach was related to photocatalytic reduction of N$_2$ to NH$_3$ in leaves</td>
<td>TiO$_2$</td>
<td>TiO$_2$ is a good photocatalyst; anatase form considered more superior than the rutile form.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>Persistence could be a threat as it can facilitate the generation of toxic reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>Likelihood for transformation of the TiO$_2$ core is low as TiO$_2$ bulk has poor solubility in water</td>
<td></td>
</tr>
</tbody>
</table>
### Enhance germination

- **Carbon nanotubes**
  - Excellent sorbents; can come in different lengths and surface functionalisation
  - Can act as sorbents and carriers for other contaminants
  - Mobility will be dependent on the surface functionalisation
  - Preparations of carbon nanotubes often contain metallic impurities (i.e. Fe)
  - Long pristine CNTs have been shown to be more toxic than shorter and functionalised CNTs
  - Metallic impurities have been reported to contribute to the toxicity of CNTs due to its redox activity

### Dust insecticides

- **SiO$_2$, Al$_2$O$_3$, ZnO**
  - SiO$_2$ is typically considered inert
  - Al$_2$O$_3$ is typically presumed poorly water soluble
  - Persistence of uncoated SiO$_2$ and Al$_2$O$_3$ nanoparticless is high since both have poor solubilities in water - some dissolution of Al$_2$O$_3$ can occur under highly acidic conditions
  - ZnO is photocatalytically active
  - Persistence of intact ZnO nanoparticless will be low as uncoated ZnO nanoparticless have high water solubility and is susceptible to dissolution

### Devices

<table>
<thead>
<tr>
<th>Precision farming and residue determination</th>
<th>Enhance productivity in agriculture by providing accurate information (i.e. on soil conditions)</th>
<th>Monitoring systems</th>
<th>Typically in solid-state form</th>
<th>Release of nanoparticless in devices will be slower</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nanochips</td>
<td></td>
<td></td>
<td>Persistence in the environment may be high, but would likely be in embedded forms.</td>
</tr>
</tbody>
</table>

### Genetics

| Nanoparticle-mediated gene transfer | Crop improvement and developing new insect resistant varieties | This process will be concentrated in the laboratory – not in the field |
6.2 Exposure to nanomaterials derived from nano AgVet products

While the unique properties of nanomaterials offer a range of potential benefits for agricultural and veterinary (AgVet) products, the same properties could lead to unintentional or un-targeted release to the environment and expose non-target biota to these materials or their transformed or degraded products.

Using AgVet products containing nanomaterials may expose terrestrial and aquatic environments to materials that behave differently from their normal-sized counterparts. The majority of AgVet applications will involve products being directly discharged to land, in the form of fertilisers and plant protection products for agriculture, to soil through manure, or to water in the form of pharmaceuticals for aquaculture or animal husbandry (Gogos et al. 2012). It is expected that through their use and weathering, nanomaterials will eventually be released from these products (Nowack et al. 2012). Their intentional use will inevitably increase the concentration of nanomaterials in the environment up to levels that may be significantly higher than those initially predicted (Table 6.3).

Environmental risks associated with exposure to nanoparticles from AgVet products will be governed by processes that control fate (form and chemistry), transport and consequent concentrations of nanomaterials in the environment (Figure 6.2). Given the dynamic nature of nanoparticles, the first forms that will likely enter the terrestrial and aquatic environments will be dictated by the AgVet product formulation, such as whether they are solid or non-solid, their particle/aggregate size and their surface composition/modification – all of which will be different from the pristine nanoparticles used in manufacturing.

Proper nanomaterial characterisation in the product mix, for example size/size distribution and morphology, mass concentration in the applied dose, charge characteristics, water dispersibility and reactivities, will be instrumental in providing a preliminary assessment of their potential fate and behaviour.

Table 6.3: Modelled fluxes of different manufactured nanomaterials and application rates of plant protection products or fertilisers from scientific literature and patents. Reproduced with permission from Gogos et al. 2012. Copyright 2012 American Chemical Society.

<table>
<thead>
<tr>
<th>nanomaterial</th>
<th>Modeled flux into soila (high exposure and realistic exposure scenarios)</th>
<th>Application rate and calculated flux from plant protection products and fertilisersab</th>
<th>Flux ratioac</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂</td>
<td>HES: 4.8 µg kg⁻¹ y⁻¹</td>
<td>For AR of 4.5 kg ha⁻¹, CF: 1607-5357 µg kg⁻¹ y⁻¹</td>
<td>334-1116</td>
</tr>
<tr>
<td></td>
<td>RES: 0.4 µg kg⁻¹ y⁻¹</td>
<td>For AR of 7.5 g ha⁻¹, CF: 2.7 µg kg⁻¹ y⁻¹</td>
<td>0.56</td>
</tr>
<tr>
<td>Ag</td>
<td>HES: 0.1 µg kg⁻¹ y⁻¹</td>
<td>For AR of 15 g ha⁻¹, CF: 5.4 µg kg⁻¹ y⁻¹</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>RES: 0.02 µg kg⁻¹ y⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon Nanotubes</td>
<td>HES: 0.02 µg kg⁻¹ y⁻¹</td>
<td>For AR of 3-12 g ha⁻¹, CF: 1.1-4.3 µg kg⁻¹ y⁻¹</td>
<td>55-215</td>
</tr>
<tr>
<td></td>
<td>RES: 0.01 µg kg⁻¹ y⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sources for each data could be found in the review by Gogos et al. (Gogos et al. 2012). Assumes an application volume of 300 L ha⁻¹, 20 cm plough depth, a soil bulk density of 1.4 g cm⁻³ and 1 application per year. Calculated as flux from plant protection products/fertilizers divided by the value of the highest modeled flux. HES = high exposure scenario; RES = realistic exposure scenario and AR = application rate.
6.2.1 Physico-chemical properties and nanomaterial behaviour

The physical and chemical properties of nanomaterials are important factors influencing their fate and ecotoxicity in aquatic and terrestrial environments (Batley et al. 2012). The properties of nanomaterials recognised as relevant to toxicological testing have been compiled by a number of organisations, including the Organisation for Economic Co-operation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) and the International Organization for Standardization (ISO; OECD 2010). Recently, a tiered approach for reporting nanomaterial properties in different nanotoxicology studies, for example in vitro and in vivo, was proposed by members of the Australian consortium which participated in the OECD Sponsorship Programme on Safety Testing of Manufactured nanomaterials (McCall et al. 2013). Table 6.4 lists the properties of nanomaterials identified as important in their fate and toxicity and the multiple techniques available for characterisation. McCall et al highlighted the importance of the characterisation of nanomaterials throughout their lifecycle from the pristine state through to formulations, coatings and ecotoxicity test media (McCall et al. 2013).

Table 6.4: nanomaterial properties most commonly identified as being important in fate and toxicity.

<table>
<thead>
<tr>
<th>Nanomaterial property</th>
<th>Characterisation technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size, size distribution</td>
<td>Transmission electron microscopy, scanning electron microscopy, x-ray diffraction</td>
</tr>
<tr>
<td>Shape and aspect ratio</td>
<td>Transmission electron microscopy, scanning electron microscopy</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>Brenauer-Emmett-Teller (N₂-BET) adsorption method</td>
</tr>
<tr>
<td>Solubility</td>
<td>Dialysis, centrifugation, membrane filtration</td>
</tr>
<tr>
<td>Aggregation and agglomeration</td>
<td>Dynamic light scattering, cryo-transmission electron microscopy, field flow fractionation, disc centrifugation</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>Spectroscopy, Thermogravimetric Analysis</td>
</tr>
<tr>
<td>Surface Charge</td>
<td>Electrophoretic mobility</td>
</tr>
<tr>
<td>Elemental and chemical composition</td>
<td>X-ray diffraction, inductively-coupled plasma mass spectrometry and inductively-coupled plasma optical emission spectroscopy, gas and liquid tandem mass spectrometry, liquid chromatography-ultra violet and liquid chromatography-fluorescence detection</td>
</tr>
</tbody>
</table>

6.2.1.1 Size

The size of nanoparticles will significantly affect their dissolution, aggregation, surface area and reactivity (Batley et al. 2012). Nanomaterials with smaller size/dimensions could potentially have enhanced mobility through soil pores, reactivity (ie increased surface area), biological uptake via passive transport and toxicity. Kim et al found in zebra fish (Danio rerio) embryos that 20 nm Ag nanoparticles were more toxic than 110 nm Ag nanoparticles (Kim et al. 2013). Ag nanoparticles coated with polyvinylpyrrolidone (PVP) that remain unagglomerated in suspension were also observed to be more toxic than Ag nanoparticles coated with citrate, despite similar sizes of the core nanoparticles. Soil retention of Ag, CeO₂, and ZnO nanoparticles has been shown to be dependent on particle size with significantly higher numbers of nanoparticles retained compared to micron-sized (bulk) particles (Cornelis et al. 2012; Cornelis et al. 2010; Cornelis et al. 2011; Milani et al. 2012).

Smaller sized nanomaterials are more likely to have unique properties and therefore require more careful evaluation.
6.2.1.2 Morphology/shape/surface area

The morphology/shape of nanomaterials is recognised as an important property that can significantly affect the surface area and reactivity of the nanomaterials (Batley et al. 2012). Higher surface area-to-volume ratios suggest an increased number of available sites for interaction that may increase adsorption and enhance reactivity, cellular uptake and toxicity. Pal et al investigated the effect of nanomaterial shape on the antibacterial properties of Ag nanoparticles against the gram-negative bacterium *Escherichia coli* (Pal et al. 2007). The authors found that truncated triangular silver nanoplates with a (111) lattice crystallographic plane as the basal plane displayed the strongest biocidal action, compared with spherical and rod-shaped nanoparticles or with soluble Ag (in the form of AgNO₃) – emphasising the importance of the nanoparticle surface. Xiong et al investigated the effect of surface area on the photoactivity of TiO₂ particles and found those with a larger specific surface area induce higher cytotoxicity (UV absent) and phototoxicity (UV-activated) to cells after 24 h incubation (Xiong et al. 2013). A large number of hydroxyl radicals were detected from TiO₂ particles with larger surface areas. The authors suggested this was due to their increased interaction with biomolecules.

In the case of inert and porous materials used in controlled-release applications, their high pore volumes could enable sorption and facilitate transport of other contaminants that enter and are retained in their pores, especially once the active ingredient (ie pesticide) has been delivered.

| High aspect ratios are more likely to lead to greater retention in soils and sediments | Effects of shape on toxicity are not well understood at this stage | High surface areas are likely to lead to greater reactivity with environmental media and biota |

6.2.1.3 Solubility

‘Solubility’ is often loosely applied in nanomaterial studies, especially in relation to carbon-based nanomaterials, often meaning ‘forming stabilised suspensions’ rather than truly dissolving (Batley et al. 2012). Hydrophobic nanomaterials are virtually insoluble, for example fullerene solubility has been calculated as 10⁻¹⁸ mol L⁻¹ (Abraham et al. 2000), and require the addition of organic solvents to form stabilised dispersions.

Interactions of hydrophobic nanomaterial with different aqueous/soil components, such as dissolved organic matter, may result in a variety of nanoparticle transformations, including partial/complete degradation, chemical transformation, and surface modification that can promote nanomaterial solubility/dispersibility. Hydrophobic fullerenes (C₆₀) show increased dispersion in the presence of dissolved organic matter (possibly due to changes in surface chemistry via hydrophobic or pi-pi interactions) and increased solubility through transformation into more water soluble poly-oxygenated/hydroxylated C₆₀ derivatives (ie oxygenation and hydroxylation via photoactivation or ozonation) (Hwang and Li 2010; Klavins and Ansone 2010; Pycke et al. 2012).

Most metal-based nanoparticles are hydrophilic with solubility dependent on size, shape, composition and reactivity. In many studies solubility is not measured, despite the soluble ionic metal fraction being the most toxic to aquatic and terrestrial biota. Franklin et al, while investigating the biological impacts of ZnO nanoparticles, found nanoparticulate ZnO rapidly dissolved to produce 6 mg L⁻¹ of dissolved Zn within 6 h and 16 mg L⁻¹ in 72 h in a buffered pH 7.5 soft water, in excess of the 5 mg Zn L⁻¹ that would be toxic to most aquatic biota (Franklin et al. 2007). By contrast, nanoparticulate CeO₂ has a very low solubility (ng L⁻¹), so the effects of nanoparticle versus micron-sized particle toxicity could be readily investigated (in the absence of the confounding influence of dissolution). Rogers et al observed higher toxicity to algae exposed to nanoparticulate CeO₂ compared to its micron-sized equivalent (Rogers et al. 2010).
6.2.1.4 Aggregation and agglomeration

Aggregation (homo- and hetero-) and agglomeration are important properties that determine the fate and toxicity of nanomaterials in the environment (Batley et al. 2012). Aggregation results when the repulsive forces that keep colliding particles apart are weakened (or when the attractive forces are enhanced). This process is heavily influenced by the properties of the suspension such as pH, ionic strength, and the presence of other colloids. Because nanoparticles can aggregate with each other (homoaggregation) and with other natural particles (heteroaggregation), the main route for the removal of most nanoparticles in the environment will be through aggregation, followed by sedimentation. Aquatic and terrestrial environments contain a wide range of natural particles, including colloidal clays, iron and manganese hydrous oxides, and dissolved organic matter (comprising fulvic and humic acids) and fibrillar colloids (exo-polymers) that are exudates from algae and other microorganisms (the exudates are largely polysaccharides and some proteins) (Wilkinson and Lead 2007). Heteroaggregation of artificial nanomaterials with natural colloids is therefore likely to control the fate of most nanoparticles. Despite this, most studies have focused on investigations of homoaaggregation (Batley et al. 2012). The aggregation process may change the overall size of the nanomaterials, surface area and reactivity that will affect their mobility and ecotoxicity in the environment.

In a recent study by Quik et al, CeO$_2$ nanoparticle (20 nm) aggregation in river water containing natural organic matter (NOM) followed first-order kinetics towards a residual concentration of the nanoparticles in the water phase (Quik et al. 2012). The authors suggested heteroaggregation with, or deposition onto, the solid fraction of natural colloids was the main mechanism causing sedimentation. Cornelis et al, in an investigation of the retention of CeO$_2$ nanoparticles (nominal particle size 20 nm) in soils, found a positive correlation with clay content and not with parameters that increase the homoaaggregation rate of CeO$_2$ nanoparticles (Cornelis et al. 2011). This finding in natural soils suggests the negatively charged CeO$_2$ nanoparticles were adsorbed preferentially by clay surfaces at positively charged sites.

The uptake sites in/on organisms such as fish, invertebrates, plants and microorganisms consist of cell surfaces (eg gill epithelial cells and root epidermis cells) that contain polysaccharides, proteins, and other ligands with an overall slight negative charge. Nanomaterials with a positive charge can be attracted through electrostatic attractions to negatively charged cell surfaces that can result in shear forces causing either particle aggregation or higher concentrations of the nanoparticles at the surface of the organism. In contrast, negatively charged nanoparticles are electrostatically repelled from the cell surfaces/membrane and are less likely to be taken up.

| Homoaggregation is less important than heteroaggregation for environmental fate of AgVet products | Heteroaggregation is likely to be a key property controlling environmental fate and effects |

6.2.1.5 Surface Chemistry

The surface chemistry of a nanomaterial has implications for its dissolution, aggregation, reactivity and toxicity. In AgVet products, the nanomaterial surface could be expected to be coated/modified with multiple organic/inorganic coating combinations in the different formulations. These modifications are typically done for the following reasons: to passivate the surface of the nanoparticles (remove free electrons and dangling bonds), control the size of the nanoparticles, render the nanoparticles dispersible in a particular solvent/formulation, and, in some cases, to improve specificity (ie plant uptake via biologically recognisable ligands).

Nanomaterials with hydrophobic coatings can render the nanoparticles dispersible in hydrophobic formulations, while hydrophilic (charged) coatings make nanomaterial dispersible in hydrophilic formulations.
In the same manner, the hydrophobicity/hydrophilicity of the nanoparticle surface can influence its overall soil retention, where transport and mobility within soil may be promoted by more hydrophilic (typically charged) surface chemistries. This property is exploited in some AgVet products, where oil-water-based emulsions are used to contain active ingredients that are otherwise poorly water soluble. Loss of surface coatings (ie biodegradation, hydrolysis etc) may lead to nanoparticle aggregation and/or dissolution (Kirschling et al. 2011).

In water, a practical measure of the potential stability of a colloidal system can be obtained from the zeta potential. By convention, it has been used as a measure of surface charge. This parameter is affected by surface chemistry as well as solution properties and composition, such as pH, ionic strength and dissolved organic matter. It provides an indication of the degree of repulsion between adjacent, similarly charged particles, ie low values indicating poor stability and potential aggregation of particles. Zeta potential also has implications for other environmental processes such as, adsorption, complexation (eg with organic matter), and interaction with biological systems such as cell membranes. In aquatic and soil environments (Milani et al. 2012; Stebounova et al. 2011), nanoparticle interaction with other materials is more likely. For heteroaggregation, the polarity of the zeta potential will be more important than the magnitude of the charge. Nanomaterials having negative zeta potentials are less likely to heteroaggregate than those with positive zeta potentials and hence will likely have greater mobility in the environment.

**Zeta potential is an important property of nanomaterials as it governs interactions with environmental media and biota**

**High zeta potential impacts greater stability of particles to remain in suspension and to resist homoaggregation BUT may lead to stronger interactions by heteroaggregation with clays and organic matter**

The influence of surface chemistry on uptake and toxicity has been demonstrated in a number of studies. For example, Chompoosor et al investigated the effect of surface hydrophobicity on toxicity, such as cytotoxicity and consequently DNA damage, using gold (Au) nanoparticles (2 nm core) featuring quaternary ammonium functionality with varied hydrophobic alkyl chain (Chompoosor et al. 2010). In this study, the group found increasing hydrophobicity on the surface of the nanoparticles resulted in higher cytotoxicity with concomitant ROS production. In a study by Kim et al, Agnanoparticles coated with PVP were found to be more toxic to zebra fish (*Danio rerio*) embryos than Agnanoparticles coated with citrate at the same particle core size (20 nm or 110 nm) (Kim et al. 2013). The effect of surface charge (as dictated by surface coatings) on the uptake and dissolution of Au nanoparticles by four plant species (*rice, Oryza sativa*; *ryegrass, Lolium perenne L*; *radish, Raphanus sativus*; *pumpkin, Cucurbita mixta cv. white cushaw*) was investigated by Zhu et al (Zhu et al. 2012). In this study, the group found that positively charged Alannoparticles were most readily taken up by plant roots which are predominantly negatively charged. On the other hand, negatively charged Alannoparticles were more efficiently translocated into plant shoots, including stems and leaves, from the roots, indicating surface chemistry-controlled behaviour. In a study by Feswick et al, the authors found that carboxyl-modified (negatively charged) cadmium selenide/zinc sulfide quantum dots (QDs) were taken up to a greater extent by *Daphnia magna* than either the amino-terminated (positively charged) or polyethylene glycol (uncharged) QDs (Feswick et al. 2013).

**Surface coatings control the behaviour of nanomaterials through changing hydrodynamic size, surface charge, and hydrophobicity**
6.2.1.6 Concentration and Composition

The concentration and composition of manufactured nanomaterials (and their impurities) can affect their fate (eg dissolution, adsorption and aggregation) and ecotoxicity in environments. Xia et al found changing the composition of ZnO nanoparticles by doping with iron (1-10 wt%) decreased ZnO nanoparticle dissolution and reduced toxicity to zebrafish embryos (Xia et al. 2011). Quantum dots, such as CdS, CdSe, Cd/Te and CdSe/ZnS, contain toxic components (eg cadmium and zinc) that can adversely affect organisms and biological processes (Bottrill and Green 2011; Gagne et al. 2008). The observed toxicity may be directly related to the nanoparticles or release of the toxic component ions, such as Cd$^{2+}$. Quantum dots containing Cd are more toxic than those containing only Zn due to the higher toxicity of Cd$^{2+}$ (Li et al. 2011). Metallic impurities in CNTs (resulting from synthesis) may lead to toxicity which is unrelated to the CNT (Hull et al. 2009). Nanomaterials have been shown to display dose-response relationships to aquatic and terrestrial organisms (Handy et al. 2012; Handy et al. 2008b; Tourinho et al. 2012). However, ecotoxicity testing of nanomaterials can be complicated and often difficult to interpret due to changes in the actual dose (dissolution, aggregation/agglomeration, precipitation, adsorption, and complexation, for example, onto gill tissue surfaces) during the test period [46]. Cornelis et al found the retention ($K_r$) of CeO$_2$ nanoparticles onto solid phases in soils to significantly increase with the rate of CeO$_2$ nanoparticle addition (Cornelis et al. 2011). The authors suggested higher CeO$_2$ nanoparticle concentrations may have increased the collision efficiency and thus the fraction of larger aggregates.

6.2.1.7 Reactivity

Evaluating nanomaterial reactivities—catalytic activity, redox potential, radical formation potential—is important, particularly when considering potential ecotoxicity (i.e., nanomaterials facilitating the formation of toxic ROS). Reactivity is closely associated to other fundamental properties such as size, surface area, composition, and crystalline structure. For example, TiO$_2$ in its anatase form, is known for its photocatalytic activity (i.e., TiO$_2$ nanoparticles used to enhance pesticide degradation (Khot et al. 2012), while it is photostable in rutile form (i.e., TiO$_2$ nanoparticles used to protect a system from photodegradation (Gogos et al. 2012). When doped with different metals (i.e., Ce-doped TiO$_2$ used as a fungicide), the photocatalytic response of TiO$_2$-anatase is changed significantly, shifting from UV to the visible region. When surface coated with other materials (i.e., Al(OH)$_3$ or SiO$_2$ as in sunscreen formulations (Auffan et al. 2010), surface reactions, such as generation of ROS, are diminished. Nanomaterials known to produce these toxic species would then be of environmental concern, especially if fate and transport data suggest them to be unintentionally active and available to non-target organisms.
6.2.2 Preliminary Considerations for Risk Characterisation

A detailed framework of stages required for the environmental risk characterisation (exposure, fate/transport and effects) of agricultural and veterinary nanomaterials can be found in Figure 6.4. The first step toward the environmental risk assessment of an AgVet product that contains nanoparticles is to determine whether or not a special nanomaterial risk assessment must be applied (Figure 6.4, A). Aside from the size range (1-100 nm) that initially separates nano AgVet products from traditional products, another important consideration for preliminary assessment would be nanomaterial stability – does the nanomaterial remain in the nano-scale in the formulation and when delivered? Nanomaterial dissolution and aggregation are two processes that would directly affect nanomaterial size and can significantly affect the nanomaterial activity.

Dissolution refers to the release of individual ions and/or component molecules that are soluble in water. Homoaggregation refers to the aggregation/attachment of two similar particles forming larger units. Homoaggregation of nanoparticles would be important in areas such as formulation stability and ecotoxicity testing. However, heteroaggregation of nanoparticles with natural colloids is likely to control their fate and ecotoxicity in aquatic and terrestrial environments.
Figure 6.4: Framework of different stages toward environmental risk characterisation of potential agricultural and veterinary nanomaterials highlighting different processes that can control their exposure, fate, transport and effects. Note that degradation and transformation products will require re-evaluation based on solubility and aggregation considerations. For nanomaterials that reach the atmosphere and/or accumulate in edible plants, assessment of human risks must be undertaken.
NP dissolution must be evaluated using the AgVet formulation and in the relevant application scenario. Due to the dynamic character of nanoparticulate systems, information on dissolution rates would be more instructive than dissolution state. Dissolution rates may have to be established in both abiotic and biotic conditions (or where relevant), depending on product usage. This must not be confused with “nanopesticide degradation” as this terminology often refers to the degradation of the pesticide a.i. and not the nano-delivery medium that encapsulates the pesticide.
6.2.2.2 **Aggregation of primary particles**

Aggregation of particles in aqueous suspensions may occur either with other nanoparticles (homoaggregation) or with natural nanoparticles (colloids) (heteroaggregation). Aggregation is controlled by the nanoparticles' characteristics such as size, shape, and surface charge, and by the solution/formulation conditions such as pH, ionic strength and cation composition. Nanomaterials that acquire a near-neutral charge homoaggregate rapidly, such as when pH and ionic concentrations are close to or at the point of zero charge (PZC) and critical coagulation concentration (CCC) respectively. Consideration of nanoparticle aggregation in the preliminary assessment is significant to AgVet products that would be used as dispersions, including products where powder formulations are redispersed for application. This preliminary screening also assumes that nano-specific activity/behaviour is lost with the formation of larger and closely-packed aggregates. For example, the ability of C_{60} to produce ROS is significantly reduced when in the form of closely-packed aggregates (Kong and Zepp 2012). Nano-Agvet products whose components readily aggregate (homoaggregation rates are high, thus nano-lifetime is low) may be amenable to traditional risk assessment. However, manufacturers are likely to ensure aqueous suspensions of nanoparticles remain dispersed in the formulation and that little or no homoaggregation takes place (otherwise nano-specific properties would be lost before use). It must be noted that once dispersed in the environment, heteroaggregation with natural colloids is much more important, though homoaggregation is unlikely due to the very low concentrations of nanoparticles expected in aqueous environmental media.

6.3 **Environmental fate and transport**

Nanomaterials that are released from AgVet products are likely to go through the different environmental processes highlighted in Figure 6.3. These include:

- physical and chemical transformation during use (B)
- retention/partitioning between soil and water (C)
- transport and remobilisation from soil to water (D)
- abiotic/biotic degradation/transformation of nanoparticles in soil and water (E)
- uptake by terrestrial and aquatic organisms (F).

While these processes are not unique, because of their dynamic nature the responses of nanomaterials to these processes may differ from those of traditional chemicals.

6.3.1 **Physical and chemical transformations**

Release of nanoparticles from matrix

The use and eventual weathering of an AgVet product will result in the release of nanoparticles from the originally introduced product matrix. Following design principles that are dictated by the desired AgVet function, such as delivery systems and additives, nanoparticle release rate will depend on the complexity of the matrix and how the nanoparticle is incorporated, that is whether or not the nanoparticles are loosely or tightly bound to the product matrix (Table 6.5). For example, it could be expected that nanomaterial delivery systems (ie mesoporous nano-SiO_2 emulsions) in AgVet products will be released readily from the product matrix via simple hydrolysis. On the other hand, nanoparticles that are contained in cage, capsule or devices may be released only after exposure to the environment (continued exposure to light, air, and water) via photochemical, oxidation-reduction, and/or dissolution reactions, resulting in a controlled release rate that
occurs over an extended period. During this release process, it could be expected that the physicochemical properties of the nanoparticles would have been modified. For example, in other types of products, TiO$_2$ nanoparticles released from paints via natural weathering, and ZnO nanoparticles released from surface coatings via abrasion, could still be surrounded by the matrix. On the other hand, Ag nanoparticles released from socks (detected in washings as AgCl) and TiO$_2$ nanoparticles released from sunscreens could undergo considerable transformation. In particular, for the TiO$_2$-based nanocomposite used in sunscreens (Figure 6.5), the surface coatings that are designed to reduce ROS formation and impart hydrophobicity have reportedly altered with aging (as a function of light and time). The observed desorption of the outer hydrophobic layer (PDMS) could lead to increased dispersion of this material in aqueous environments. These examples were described in a review paper by Nowack et al. (2012).

Product-weathered nanoparticles that are released from the main product matrix will then undergo further physical and chemical transformations which will be controlled by the receiving matrix (plant or soil). These are alterations that become increasingly significant over time.

Transformation processes

The different transformation processes that can occur before and after the release of nanoparticles from a product matrix, and affect (or are affected by) nanoparticle form, are described below. These changes may involve transformation of the nanoparticle core, nanoparticle surface, or both. The likelihood of each type will depend on the application protocol (initial form – as solid, dispersion, device or receiving matrix), agricultural practices employed (soil tilling, irrigation or addition of other chemicals) and natural weathering (wind, rain or heat).
Table 6.5: Some nano-enabled products illustrating release behavior of nanomaterial components as described by Nowack et al. (2012)

<table>
<thead>
<tr>
<th>nanomaterial components</th>
<th>Release potential</th>
<th>Examples</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solid-bound</strong></td>
<td>Nanomaterials incorporated in the solid matrix will be released from the product at a much slower rate than if they were in liquid dispersed form. Nanomaterials that would leach from this product may be altered significantly in the product even before it reaches the environment.</td>
<td>TiO₂ in paints (Kaegi et al. 2008) CNTs in composite materials</td>
<td>Use of the product involves nanomaterial embedded onto surfaces. Release of nanomaterials may occur with weathering, and as particles that are heteroaggregated with the paint matrix. Nanomaterials in the product are directly embedded in a composite material. These composites are typically durable and can withstand physical and chemical transformations. Hence, release and alteration of nanomaterials may occur over longer periods.</td>
</tr>
<tr>
<td><strong>Loosely-bound</strong></td>
<td>Nanomaterials that are loosely-bound will be readily released as nanomaterials with very few modifications.</td>
<td>TiO₂ in cosmetics (Auffan et al. 2010) Ag in textiles (Benn and Westerhoff 2008)</td>
<td>Nanomaterials could be released with washing of skin. Nanoparticles could be readily released as matrix-modified materials. Change in composition is not expected given the stability and insolubility of TiO₂. Use of the product involves addition of a copious amount of water which will potentially facilitate release of component nanomaterials. Application (washing) results in significant alteration of the nanoparticles (ie detergents, water).</td>
</tr>
</tbody>
</table>
Figure 6.5: An example of a TiO$_2$-based nanocomposite used in sunscreens, characterised by: (a) XRD, (b) TEM, and (c) High Resolution TEM. (d) A schematic view of the formulation highlights the TiO$_2$ core, as well as the Al(OH)$_3$ and polydimethylsiloxane (PDMS) layers that surround the TiO$_2$ nanoparticles. Reproduced with permission from Labille et al. (2010). Copyright 2010 Elsevier.

6.3.1.1 Photochemical Transformation

Description: Photoexcitation facilitates in situ generation of (highly reactive) ROS via transfer of excited electrons from the nanoparticles to H$_2$O, O$_2$ and OH$^-$ ions, or other nearby electron acceptors. The ROS generated can then oxidise compounds adsorbed onto the nanoparticle surface.

Relevant Materials: Photochemical transformation is a process that is most applicable to products which will be exposed to significant levels of light, such as AgVet products sprayed directly onto plants or soil surfaces, and/or are generally photoreactive, including nanoAgVet products containing TiO$_2$-anatase, ZnO and Al-SiO$_2$.

- Ubiquitous NOM could also act as a photosensitiser and could absorb light to initiate a photochemical reaction.

Reaction Specifics: The extent of the photochemical transformation will be influenced by (a) the wavelength of incident light (ie TiO$_2$-anatase is active in the UV, while Ce-doped TiO$_2$ is active in the visible region), (b) the capacity of the incident light to penetrate and reach the photoreactive nanoparticles – past surface modifications and aggregation, (c) reactive surface area of the nanoparticles and (d) product composition.

- Other components in the product formulation, such as antioxidants, fillers, and solvents, could also participate in photochemical reactions by forming or neutralising ROS (see Figure 6.5). Products of
photoexcitation could also participate in other oxidation-reduction reactions, such as degradation of other organic species in soil (a process similar to how photoactivated nanoparticles are used to degrade pesticides) (Thomas et al. 2011).

**Nanoparticle Transformation:** This process may or may not result in alteration of the nanoparticle core. Coating ligands could be oxidised by ROS, such as disulfides $\leftrightarrow$ thiols, alcohols $\leftrightarrow$ carboxylic acids, changing the surface properties of the nanoparticles.

**Examples:** Photoreactive nanoparticles like TiO$_2$ (anatase) facilitate generation of ROS. While ROS may not be sufficient to further oxidise Ti$^{4+}$, ROS generation can facilitate oxidation (or degradation) of other species, such as nanoparticle organic coatings, and change the overall surface properties of the nanoparticles. This is the same principle as used with TiO$_2$ for degradation of pesticides (Carp et al. 2004; Thomas et al. 2011). On the other hand, Ag nanoparticles are susceptible to photochemically-induced changes. ROS generation could oxidise Ag to Ag$^+$ (Gorham et al. 2012). For nanoparticles like fullerenes (C$_{60}$), photochemical transformation may lead to formation of hydroxylated/oxygenated products (Pycke et al. 2012) that have been reported to be more toxic, that is (ie generate more ROS - Chae et al. 2010).

### 6.3.1.2 Oxidation and reduction

**Description:** Oxidation and reduction are reactions that involve transfer of (valence) electrons and are typically manifested by changes in oxidation states. This type of transformation requires the presence of oxidising and reducing agents, whose rate is determined by the reduction potential ($E^{0}_{\text{red}}$) of the component ions.

- $E^{0}_{\text{red}}$ constant is influenced by pH (determines favourability of reaction), temperature (kinetics of the reaction), the presence of other species essential for reaction to occur (increases the likelihood of the reaction occurring), and the presence of ligands adsorbed onto the nanoparticles surface (reduces the rate of reaction).

The majority of data from the literature have reported oxidation of nanoparticles. Excluding reports of formation of nano-sized particles resulting by reduction, that is Ag nanoparticles formed by reduction of Ag$^+$ by humic acids (Akaighe et al. 2011), data on the reduction of nanoparticles in the environment has yet to be reported.

**Relevant Materials:** Nanoparticles in AgVet products are susceptible to oxidation or reduction as long as the reaction is thermodynamically favourable. Some of the most common oxidising and reducing agents in soil are:

(a) Oxidising agents: O$_2$ (present in air and could be dissolved in a water/soil-solution; it could also facilitate the formation of ROS), Fe$^{3+}$ and Mn$^{4+}$ (present in soil, part of clay), and SO$_4^{2-}$ and NO$_3^-$ (present in soils/soil solution).

(b) Reducing agents: NOM (present in soil and in soil solution).

- In soil, nanomaterials will be mostly exposed to an oxidising environment as a result of agricultural practices, such as soil tilling or the adding of H$_2$O$_2$, which keeps the soil well-aerated.

**Reaction Specifics:** The extent of this process relies on the availability of the nanoparticle surface, the capacity of oxidising/reducing agent to move through surface modifications and the reactive surface area. Nanoparticle coatings reduce the rate of nanoparticle oxidative/reductive transformation. Poorly passivated/coated nanoparticles that often have partially exposed surfaces will be susceptible to oxidation/reduction of the core nanoparticle.
Nanoparticle Transformation: Oxidation/reduction significantly alters the nanoparticle core. Coating ligands could also undergo oxidation/reduction, such as disulfides $\leftrightarrow$ thiols or alcohols $\leftrightarrow$ carboxylic acids, changing the surface properties of the nanoparticles.

Example: As described in a review by Levard et al. (2012), the most commonly used Ag nanoparticles will not persist under relevant environmental conditions due to oxidation. Surfaces of Ag nanoparticles (oxidation state = 0) can be readily oxidised by O$_2$ to form a layer of Ag$_2$O (oxidation state = +1). Note that other nanoparticles, such as the ultra-stable Ag nanoparticles synthesised by Desireddy et al. (2013) have been suggested to be more stable against oxidation.

- Depending on the surrounding conditions, partially oxidised Ag$_2$O-Ag species may completely convert to Ag$_2$O, form other stable silver compounds (Ag$_2$S in the presence of sulphides) or remain partially oxidised and acquire new surface coatings, such as organic matter.

6.3.1.3 Dissolution and precipitation (for metallic nanomaterials)

Description: Dissolution refers to the release of component ions in solution. The dissolution process starts either with a hydrolysis or oxidation reaction at the nanoparticle surface resulting in a complete/incomplete release of its component ions/molecules. Precipitation refers to the formation of an insoluble material from dissolved ions.

- Dissolution is governed by the solubility product constant ($K_{sp}$) and the standard reduction potential ($E_{red}^0$) of its components (nanoparticle and surface coatings).
- Precipitation is governed by the solubility product constant ($K_{sp}$) and complex formation constant ($K_f$).

Relevant Materials: Dissolution and precipitation will mostly apply to AgVet products based on metallic nanoparticles that are highly water soluble and/or are easily oxidised. In soil, precipitates could be formed with ions such as Cl$^-$, CO$_3^{2-}$, NO$_3^-$, SO$_4^{2-}$ and natural colloids such as clay, organic acids, organic thiols, and NOM.

Reaction Specifics: Dissolution relies on the availability of the nanoparticle surface and the reactive surface area. Nanoparticle coatings reduce the rate of nanoparticle dissolution. Precipitation relies on the availability of dissolved ions. Concentration gradients can also drive or inhibit dissolution and precipitation (hydrolysis/precipitation: NP-X $\leftrightarrow$ NP + X; oxidation: NP$^0$ $\leftrightarrow$ NOP$^+$ + e$^-$).

Nanoparticle Transformation: Dissolution and precipitation directly affects the core nanoparticle. In the absence of precipitation, slow dissolution of metallic nanoparticles would result in them gradually becoming smaller in size, whereas fast dissolution of metallic nanoparticles would result in their rapid disappearance. Otherwise, dissolved ions could form insoluble ionic compounds (ie free Ag$^+$ forming Ag$_2$O), complexes (ie $M^{n+}$-dissolved organic matter) or attach to suspended material and promote settling of nanoparticles out of solution. The loss of ions also changes the nanoparticle surface charge, which can influence its aggregation state and concomitant reactivity.

Examples: Some nanoparticles that have been shown to release component ions/molecules even under mild conditions include ZnO nanoparticles (via hydrolysis), Ag nanoparticles (via oxidation) and polymeric nanoparticles (via hydrolysis). For ZnO and Ag nanoparticles, toxicity has often been associated with released ions. Work by Franklin et al on aquatic toxicity of ZnO nanoparticles has demonstrated that toxic levels of Zn could be readily released (6 mg L$^{-1}$ within 6h) from suspensions in soft water (pH 7.5) (Franklin et al. 2007). Some nanoparticles which are relatively resistant to dissolution of ionic components include TiO$_2$, CeO$_2$, and SiO$_2$ nanoparticles. For these nanoparticles, toxicity is related to properties apparent at the nanoscale – CeO$_2$ nanoparticles was observed to be more toxic to algae compared to CeO$_2$ bulk (Rogers et al. 2010).
6.3.2 Phase partitioning processes (adsorption/desorption or attachment/retention)

One of the important processes that determine the fate of an active ingredient (a.i.) in conventional AgVet chemicals is the (ad)sorption/desorption on the solid phases in soil and water environments. The extent of partitioning of mass between the solid (sediment, soil) and water phases is governed by these processes which take effect as soon as the contact between the a.i. and the solid phase occurs. Phase partitioning processes determine if the a.i. would predominantly reside in solid or solution phases as well as the pathways of exposure to the a.i. (via sediment, soil or water). In addition, processes such as transformations, degradation, bioavailability and transport are dependent upon the partitioning (adsorption/desorption) processes. The phase partitioning into air may also be important for AgVet chemicals, such as through drift. For example, if the nanoparticles get airborne, there may be implications for human health. However, this aspect has not been covered here (see Figure 6.4).

For nanoparticles the phase partitioning between soil/sediment and water may not strictly depend on adsorption/desorption processes but on other physico-chemical interactions such as homoaggregation or heteroaggregation (as described later in this section). However, for nanoformulations of AgVet chemicals that are likely to contain conventional a.i. together with a nano-delivery mechanism such as pesticides, these processes still remain relevant, especially in terms of interaction of a.i. with the nano-delivery system. Adsorption/desorption processes have been well understood for conventional a.i. and are not discussed here. For further details on these, the reader is referred to the book by Cheng et al (1990). However, the effect of adsorption/desorption of nano-AgVet products may also arise through transformations and changes in speciation in nanoparticles in the receiving environment, these interactions in the context of transformations that could influence partitioning behaviour of nanoparticles are discussed below.

6.3.2.1 Adsorption of substances onto nanomaterials

Description: Adsorption involves attachment of substances onto the nanoparticle surface (and/or nanoparticle surface coating, adsorbents) via Van der Waals attraction forces (physisorption), electrostatic interactions (ionic), and/or chemical bonding (chemisorption). Adsorbing species (adsorbates) can attach to the nanoparticles surface by (a) ligand exchange, following desorption of existing ligands, (b) Van der Waals interaction with the partially exposed nanoparticles surface and/or other ligands that surround the nanoparticles, or (c) by both mechanisms.

- This is the same process that applies to nanoparticles that acquire coatings during their synthesis and formulation.
- Compared to precipitation, adsorption does not require dissolution of the nanoparticle surface and does not necessarily involve formation of an insoluble solid.

Relevant Materials: All nanoparticles will be susceptible to this transformation. In the environment, soil components: Cl\(^-\), CO\(_3\)\(^2-\), NO\(_3\)\(^-\), SO\(_4\)\(^2-\), clay, NOM and other naturally occurring colloids, biological secretions (ie root exudates) organic acids, organic thiols and enzymes could sorb onto nanoparticle surfaces. Soil solutions typically have high concentrations of these natural colloids (especially in comparison with the concentration of nanoparticles in soil) that can form (hetero)aggregates with the nanoparticles (Batley et al. 2012). Porous SiO\(_2\) nanoparticles and carbon nanotubes that are known to have very large pore volumes and adsorptive properties could serve as adsorbents for other contaminants (Pan and Xing 2008).
Humic substances have highly complex molecular structures and composition which vary depending on origin. They can further be subdivided into fulvic acids, humic acids (HA), humins – different fractions that vary in their molecular weights. These fractions could be expected to adsorb onto nanoparticles (and vice versa) and influence nanoparticle surface chemistry to varying degrees.

**Reaction Specifics:** The extent of adsorption will be highly dependent on the surface area available for interaction. For nano-agricultural products that will mostly be applied to soil, the nanoparticles released from the formulations will be readily subjected to this adsorption process.

**Nanoparticle Transformation:** This transformation will result in nanoparticles that are heteroaggregated – which have implications for nanoparticle dispersibility (ie charge). While adsorption with small colloids may or may not result in nanoparticles stable in suspension, adsorption onto charged surfaces or large molecules (ie humic substances) will essentially immobilise the nanoparticle (formation of larger aggregates, and sedimentation) (Stebounova et al. 2011).

**Example:** Interaction between nanoparticles (Fe$_2$O$_3$, Al$_2$O$_3$, TiO$_2$, Au, Ag, C$_{60}$ and CNTs) and NOM (specifically HA) have been shown to facilitate dispersion of the nanoparticles (Batley et al. 2012). Stabilisation of the nanoparticles was attributable to both the steric separation and electrostatic repulsion imparted by the natural colloids. The amphiphilic characteristic of HA allows for both coordinative and Van der Waals interactions. Note that these heteroaggregated species (NP-HA) can further adsorb onto other natural colloids (a heterogeneous mixture of NOM and inorganic binding phases) and form unstable clusters.

### 6.3.2.2 Desorption of substances from nanomaterials

**Description:** Desorption involves the detachment of substances adsorbed onto the nanoparticle surface and/or nanoparticle surface coating.

**Relevant Materials:** All nanoparticles will be susceptible to this transformation.

**Reaction Specifics:** The process relies on the nature of the surface binding (type and strength of the interaction) between the adsorbent and the adsorbate. Weakly bound species (ie bound by Van der Waals interaction) could be expected to desorb easily compared to those that are more strongly bound (ie bound by chemical bonds). For chemisorbed species, desorption can occur when a substance that has higher affinity for the adsorbent (nanomaterial) surface (related to $K_{sp}$, $K_f$ or bond strength), promotes displacement of the original coating, then attaches to the nanoparticle surface (ligand exchange). Concentration gradients can drive or inhibit this process (nanoparticle-adsorbate $\leftrightarrow$ nanoparticle + adsorbate).

- Ligand exchange is a common procedure in preparing water-dispersible nanoparticles from hydrophobically-coated nanoparticles.

**Nanoparticle Transformation:** This process does not necessarily cause significant alteration of the nanoparticle core composition but directly affects nanoparticle surface properties.

**Example:** For nanoparticle formulations dispersed in water, loss of nanoparticle coatings (desorption following hydrolysis process) may result in destabilisation of nanoparticles in the formulation itself. Desorption of nanoparticle coatings has been demonstrated for CdSe QDs (coated with hydrophobic ligands) upon interaction with HA; the process facilitated stabilisation of the hydrophobic nanoparticles in aqueous solution (Navarro et al. 2009).
6.3.2.3 Nanoparticle retention (soil/sediment-water distribution)

Given the different types of transformations that can occur within and outside the formulation matrix (before and after AgVet application), nanoparticles in the environment can then exist in many different forms. One of the main drivers for transport of these nanoparticles will be how they partition between soil and water (NP\textsubscript{water} $\leftrightarrow$ NP\textsubscript{soil}) — in relation to their retention in soil/sediment. Conventional parameters such as partitioning coefficients (K\textsubscript{d} or K\textsubscript{oc}) may not be directly applicable to nanoparticles. Instead, other surrogate parameters such as attachment coefficients are being proposed (Westerhoff and Nowack 2013). Others have proposed retention coefficient (K\textsubscript{r}), defined as the ratio of the concentration of the nanoparticle in the solid phase and the aqueous phase, which provides an estimation of the nanoparticle’s mobility (Cornelis et al. 2010; Cornelis et al. 2011; Milani et al. 2012).

- K\textsubscript{r} is distinguished from sorption coefficients (K\textsubscript{d}) that are traditionally used to describe partitioning of solutes as it accounts for potential dissolution of metal-based nanoparticles.

Examples of processes that may be involved in the retention of nanoparticles onto soil include adsorption, precipitation, and solid-state diffusion, which are all dependent on the characteristics of the nanoparticle surface and of the receiving matrix. Hence, K\textsubscript{r} values would be expected to vary for different soil (sorbent) types, water chemistry conditions, as well as different nanoparticle surface chemistry. These factors are described in some of the examples given below.

- Note that, overall, studies on the behaviour of nanoparticles in soil have been significantly hampered by the limited techniques used to distinguish nanoparticles in complex systems. The influence of these factors (soil and nanoparticle properties) on nanoparticle retention has not been studied systematically. In most cases, comparisons were made between nanoparticles of different composition, which may intrinsically already have different retention behaviours, and without control of nanoparticle surface chemistry, such as size, shape and coatings. Examples below have been derived from both batch and column studies, with reported deposition from column studies treated as examples of retention.

Soil composition:

- High K\textsubscript{r} would generally be expected for soils or sediments that have the capacity to retain the nanoparticles and/or promote conditions that facilitate nanoparticle destabilisation. These retaining soils would typically have high clay, and high organic matter contents.

**Example:** Significant retention of nanoparticles in soils rich in organic matter and/or in clay content has been consistently observed for many of the metallic nanoparticles (uncoated or coated) — Ag nanoparticles (Cornelis et al. 2010; Coutris et al. 2012), CeO\textsubscript{2} nanoparticles (Cornelis et al. 2011) and TiO\textsubscript{2} nanoparticles (Fang et al. 2009), compared to sandy soils (Cornelis et al. 2010; Cornelis et al. 2011). Interaction of C\textsubscript{60} with different clay types has also been investigated and was found to be a function of the available surface charge on the clay relative to the net negative surface charge of C\textsubscript{60}. A
synthetically produced sorbent (layered double hydroxide) exhibited faster association with C$_{60}$ than to montmorillonite and kaolinite (Fortner et al. 2012). Zhang et al noted greater retention of C$_{60}$ in freshwater sediments than model porous media (Zhang et al. 2012). Some interactions also serve as important retention mechanisms to specific nanoparticles, ie formation of insoluble AgCl upon oxidation of Ag nanoparticles when Ag nanoparticles are subjected to high levels of Cl$^{-}$ in the soil (Sagee et al. 2012).

- Note that while different retention behaviours have been observed for different soil types, very few have compared retention behaviour of one nanoparticle in several soils in a single study.

Water chemistry:

- High K$_r$ would be observed during long term heteroaggregation of nanoparticles’ natural colloids.

**Example:** Deposition as a result of changes in ionic strength of the leaching medium has been reported for (uncoated) Fe$_3$O$_4$, TiO$_2$, CuO and ZnO nanoparticles in column experiments using porous glass beads, and for CNTs in a sandy loam soil (Ben-Moshe et al. 2010; Jaisi and Elimelech 2009). Suspensions with higher ionic strengths and valency of constituent ions exhibited faster deposition rates. This aggregation and sedimentation behaviour has also been observed independent of nanoparticle surface modification for Ag nanoparticles in high ionic strength media (Stebounova et al. 2011). When compared to fullerenes, effective retention of CNTs (carboxyl-functionalised) by the soil matrix was associated with large aspect ratios and its highly bundled aggregated state in aqueous solutions (Jaisi and Elimelech 2009). Clay (montmorillonite) has also been shown to destabilise nanoparticles of varying surface charges (negatively-charged Ag and positively-charged TiO$_2$) at relevant environmental conditions (pH 5-8) (Zhou et al. 2012). Indeed, heteroaggregation with suspended particulate matter could facilitate efficient removal of nanoparticles with sedimentation, as demonstrated for TiO$_2$ nanoparticles in a river system (Praetorius et al. 2012).

- No data are available on the combined effects of ionic strength and natural colloids on retention and deposition.

Nanoparticle surface chemistry:

(Nanoparticles of different surface characteristics but the same core composition and soil type)

- High K$_r$ would be expected for nanoparticles that have highly hydrophobic (less charged) surfaces such as nanoparticles coated with long chain alkyl groups, such as octylamines and phosphonic acids. Nanoparticles that have hydrophilic coatings with high positive zeta potentials may also result in high K$_r$ following removal or desorption of bound ligands.

**Example:** The retention behaviour of Ag nanoparticles coated with citrate, PVP and HA varies with respect to the size and hydrophilic/hydrophobic characteristics of the coating material (Navarro et al unpublished data)—Ag nanoparticles K$_r$—citrate<PVP<HA—though the contribution from potential particle dissolution cannot be excluded. A similar observation was reported for CdSe QDs that were coated with mercaptopropionic acid and hydrophilic polymers, where the polymer-coated nanoparticles exhibited less retention, and was suggested to be potentially due to the higher density of hydrophilic (OH) groups on its surface (Navarro et al. 2011). In the case of fullerene nanoparticles, retention was also significant for C$_{60}$, which is intrinsically hydrophobic, compared with its hydroxylated form (fullerol) (Lecoanet et al. 2004). The effects of surface charge have also been demonstrated, where positively-charged Al nanoparticles were preferentially retained compared to negatively-charged nanoparticles (Darlington et al. 2009).
Indeed, when compared to uncoated nanoparticles, nanoparticles coated with hydrophilic ligands are poorly retained in soil. This was observed by Coutris et al. for Ag nanoparticles (Coutris et al. 2012).

Studies that specifically investigate the effect of size or shape on nanoparticles retention were not found.

**Nano vs Bulk forms:**

**Example:** When compared to ionic (soluble) controls, retention behaviours vary for different nanoparticles. $K_r$ values for Ag nanoparticles and Ag$^+$ (performed in five soils) were reported to be in the same order of magnitude, whereas $K_r$ values for coated CeO$_2$ nanoparticles dispersed in citrate were significantly lower than Ce$^{3+}$ and Ce$^{4+}$. When compared to bulk controls, retention of nanoparticles is still lower (Cornelis et al. 2010; Cornelis et al. 2011).

6.3.2.4 **Measurement of retention parameters**

The applicability of batch sorption tests used for conventional active ingredients of AgVet chemicals for nanomaterials is currently being debated in the literature. For some carbon-based nanomaterials (CNTs) as well as metal-oxide nanomaterials, batch sorption tests have been successfully used to predict behaviour under soil and wastewater treatment plant conditions (Westerhoff and Nowack 2013). However, since the nanoparticles do not behave like dissolved chemicals, their properties may change based on the conditions used in the protocol. Size, surface charge, particle density or other properties such as the capping agent used or interactions with NOM need to be taken into consideration. Some workers suggest that since the nanoparticles form thermodynamically unstable suspensions in the aqueous phase, their retention should be considered in terms of heteroaggregation. Hence the attachment efficiency of heteroaggregation would be a suitable measure of retention (Praetorius et al. 2012). Column tests may be more appropriate for measuring the retention of nanoparticles under conditions more realistic of the receiving environment. Certain parameters used for conventional active ingredients such as $K_{oc}$ are not suitable for use with nanoparticles. Indeed, the search for global parameters that are appropriate for nanoparticles requires much more research (Westerhoff and Nowack 2013).

6.3.3 **Transport and remobilization**

Though environmental inputs of nanoparticles from AgVet products may be heavily concentrated to soil, AgVet nanoparticles can indirectly enter water bodies through soil pores. Nanomaterial surface chemistry will influence this transport process. In general, transport through porous media will be likely for the nanoparticles that are poorly retained in soil, ie have low $K_r$. Enhanced soil mobility of nanoparticles may be observed with increased flow rates, and in the presence of species that could stabilise nanoparticles. Conversely, soil mobility could be limited when the nanoparticles enter small pore spaces (straining), and/or as a result of destabilising interactions between the nanoparticles and soil components (ie clays and soil organic matter) (Mcdowellboyer et al. 1986). The mobility of nanoparticles has been observed to be reduced with increasing aggregate size (not primary nanoparticles size - Darlington et al. 2009). For the heteroaggregated nanoparticles in the environment, this suggests that temporal changes in nanoparticle surface characteristics will likely impede their transport (Batley et al. 2012).

Studies on the remobilisation of retained nanoparticles have been fairly limited. Nonetheless, in theory, nanoparticles that are initially retained in soil could be remobilised and redispersed when interactions that facilitate retention are weakened or disturbed, often resulting in some surface transformation. For AgVet products, the process could be promoted as a consequence of agricultural practices, such as adding biosolids, fertilisers, treatments with hydrogen peroxide, tilling, and intermittent wet-drying cycles. In addition,
the sodicity of soils, prevalent in Australia, could also be a factor leading to dispersion of nanoparticles from the soil matrix.

- Mixing biosolids that are rich in organic and inorganic compounds with soil could release species that potentially restabilise retained nanoparticles. The ability of dissolved organic matter to stabilise nanoparticles could essentially enhance nanoparticle mobility (Fang et al. 2009). In a recent study on the behaviour of \( C_{60} \)-spiked biosolids in soil, the levels of \( C_{60} \) released following water leaching correlated with the levels of dissolved organic carbon (DOC) released from the system (Navarro et al. 2013).
- Adding phosphate (from fertilisers) has also been reported to decrease retention (which potentially increases mobilisation) of (uncoated) CeO\(_2\) nanoparticles in soils that have low colloid concentration. Poor retention was attributed to attachment of negatively charged phosphate onto the nanoparticles resulting in nanoparticle stabilisation (Cornelis et al. 2011). Hence, for retained nanoparticles, remobilisation via this route is possible.

Processes such as soil erosion and surface run-off could also facilitate the transport of soil-adsorbed nanoparticles to water bodies where it is subjected to further transformation and potential remobilisation. In water, soil-adsorbed nanoparticles will again be subjected to a change in surrounding conditions. High surface area properties of the nanoparticles could make them effective sinks for some organic compounds and the nanoparticles may act as vectors for certain contaminants.

### 6.3.4 Abiotically and biotically-mediated processes

The abiotic and biotic processes may involve degradation (and/or transformation) of the nanoparticles leading to breakdown of the chemical or nanoparticles to non-toxic elements, compounds or building blocks such as C, H and O. Degradation is one of the major processes that determines the persistence and fate of a product in the environment. For conventional active ingredients a measure of persistence, such as half-life, would be a crucial parameter determining their risk in the environment. However, the situation with nanomaterials is slightly more complicated in that they undergo a range of transformations that may have a bearing on the toxicity profile of the nano-product. These are discussed under abiotic and biotic categories of processes below.

#### 6.3.4.1 Abiotic processes

Abiotic processes include photochemical transformations, oxidation-reduction reactions, dissolution/precipitation and hydrolytic transformations. All of these processes have the potential to transform and/or breakdown the active ingredient or other constituents in the nano-AgVet products to render them non-toxic and contribute to their loss from the environment. The details of such processes were provided earlier in this report and need not be discussed again. However, these processes are being harnessed to enhance the environmental friendliness of nano-AgVet products. For example, nano-formulations of conventional AgVet chemicals have already been developed, such as imidacloprid, avermectin and chlorfenapyr incorporating different proportions of nano-Ag and nano-TiO\(_2\) (see review by Kah et al. (2012); Gogos et al. 2012). Due to the presence of these photo-catalysts in the formulations, the persistence of these AgVet products on plant surfaces and in soil were found to be shorter than that of conventional products (see reviews by Kah et al. (2012) and Gogos et al. (2012)).
6.3.4.2 Biotic processes

Biodegradation and biotransformation are critical processes that determine the persistence of toxicants such as pesticides and other organic compounds in soils. During the last forty years, much research has been published on conventional active ingredients of pesticides, demonstrating the ability of a wide range of microorganisms in soils to mineralise these compounds and use them as a source of carbon and energy (see reviews in Cheng et al. (1990). Repeated applications of pesticides and other organic molecules to soils have been reported to result in adaptation of microbial populations leading to accelerated degradation or much shorter persistence of the same compound. For nano-AgVet products, the biological processes leading to transformations or mineralisation of active ingredients or other constituents of formulated product remain highly relevant. Biotic transformations of nano-AgVet products may occur at several fronts. For example, the carrier, such as a nanocapsule, may be broken down by biological processes in the receiving environment and thus influence the rate of release of the active ingredient. The active ingredient released may be transformed and/or mineralised by microbial processes. Similarly, nanoparticles in the formulation, such as an active ingredient or a catalyst, may undergo biotransformation through biological processes, as described earlier. Some of the processes discussed above under abiotic processes, such as oxidation/reduction, hydrolysis and other transformations may indeed be biologically-mediated, as has been observed in the case of conventional chemicals (Bollag 1990).

6.3.4.3 Differences between conventional and nano agvet products

Several aspects of nano-AgVet products are expected to be different in comparison with the conventional AgVet chemicals.

1. The stability of the nano-active ingredient complex, such a polymer-active ingredient capsule, may be a significant rate-limiting step that may determine the rate of biotransformation/biodegradation of the active ingredient.
2. Similarly, the capping agent used in the case of a nanoparticle as an active ingredient may be biologically removed and the properties of the particle may change.
3. A pristine nanoparticle may be modified through interactions with organisms by adsorption of organic ligands and biological materials, as discussed before.
4. Microorganisms may transform a nanoparticle into species that is insoluble, such as the conversion of AgNO$_3$ to Ag$_2$S.
5. Unlike conventional organic compounds, metal oxide and other novel actives such as Si in AgVet chemicals may not be able to support microbial processes through the supply of C or energy.
6. The nano constituents of the formulation may inhibit biological activity in the receiving environment. Conversely, the targeted delivery of the active ingredient through nano-formulations may protect the beneficial functions of the microorganisms.

Currently there is limited understanding of the role of microbial processes in determining the fate of nanoformulation constituents or nano-AgVet chemicals, or the effect of these on microorganisms in the environments. However, studies on metal oxide and carbon-based nanoparticles have shown some effects on soil microbial communities Dinesh et al. (2012). The wide recognition of the antibacterial properties of metallic nanoparticles has led to development of metal-based nanopesticides such as Ag-based biocides). In terms of nanoproduct biodegradation, Fukushima et al. (2010) studied the biodegradation of poly($\varepsilon$-caprolactone) and its nanocomposites and reported that the polymer was effectively degraded in composts but that nanoclay delayed the process. In a study using an OECD protocol on the ready biodegradability of C$_{60}$, Hartmann et al noted negligible transformation in 28 days (Hartmann et al. 2011). However, it has been reported that fungi can biodegrade the photolytic transformation product of C$_{60}$ (fullerol) but not the parent...
6.3.4.4 Transformation products

The biotic and abiotic processes discussed above may lead to the production of transformation products or metabolites that may retain toxic properties. This has been recognised for conventional AgVet chemicals and therefore for several products the parent and the transformation products are generally considered together as a total toxic residue in the environment, for example atrazine and its transformation products and organophosphates such as aldicarb and fenamiphos and their thioxidation products. For nanomaterials the transformation products need to be considered for their environmental fate and toxicological properties (as shown in the conceptual diagram – Figure 6.3).

For example, transformation products of the nano-Ag biocide Ag₂S are known to have very different toxicological properties and environmental fate. Ag₂S, which is produced when nano-Ag undergoes oxidative sulfidation, has very limited solubility and thus its mobility in the environment is expected to be very different from the AgNO₃ biocide. Peterson et al. (2012) studied the adsorption and breakdown of penicillin in the presence of TiO₂ (anatase) nanoparticles in water. They found that a range of products such as penicilloic acid, penilloic acid and related de-ammoniated by-products were formed by degradation of ampicillin on TiO₂ surface. The degradation process was found to be pH dependent. Nano-AgVet products may be deliberately designed to have nanoparticles such as TiO₂ in the formulation. It is therefore important to understand the transformations that may be induced by the coexistence of nanoparticles and conventional active ingredients and the potential fate and effects of the by-products in the environment.

6.4 Ecotoxicological Effects

6.4.1 General considerations

For conventional AgVet products there is a clear and broadly accepted approach for ecotoxicological effects assessment. However, this is based on assessing the effect of the active ingredient, and where it exerts its toxicological effect through its molecular interactions with the organism as a toxicant rather than as a particle. The ability of nanomaterials to enhance penetration or bioavailability and toxicity is among the main drivers for some of the emerging nano-formulations of AgVet chemicals. Therefore it is crucial that such properties of nanoformulations are given adequate consideration in effects assessments on non-target organisms. Also the nano-constituent of the formulation (active ingredient or the excipient) may in some cases have potential to exert a toxicological effect in its own right. However, other formulations may be designed to reduce the toxicological impact or risk of the product in the environment. Regardless of the toxicity profile of the active ingredient in nano-AgVet products, the effects assessment may need to be on a formulation basis rather than on an active ingredient basis.

The toxicity of nano-AgVet chemicals may arise either individually or from a combination of the active ingredient (either as a nanomaterial or a conventional molecule) and/or from the carrier or the nano-active ingredient complex and/or the nanoparticles themselves that have been added to the formulation. In the case of nanomaterials an understanding of particle chemistry and interactions in the receiving environment is crucial in the context of bioavailability and ecotoxicology (Handy et al. 2012; Handy et al. 2008b; Klaine et al. 2008).

Consequently, methods of assessing the ecotoxicological impact of engineered nanoparticles may require considerable adaptation and careful control during the tests (e.g. (Crane et al. 2008; Handy et al. 2012).
Indeed, the challenges that ecotoxicologists face during effect assessment of nano-AgVet products are significant. The next section identifies some of the differences between conventional approaches and those that may be suitable for nano-AgVet chemicals. It also identifies some of the aspects deserving extra care or caution.

6.4.2 Chemistry considerations for uptake and toxicology

6.4.2.1 Lipid solubility

For a conventional active ingredient the aqueous and/or lipid solubility (often measured by octanol:water partition coefficient $K_{ow}$) is one of the fundamental properties that determines the fate, bioavailability, uptake, bioaccumulation and effects of the chemical in organisms. However, depending on the nature of the nano-active ingredient complex for nano-AgVet chemicals, lipid solubility alone may not be an appropriate indicator of nanoparticle phase transfer potential and the presence of nanoparticles can influence the partitioning behavior of organic compounds. For hydrophobic organic compounds (HOCs), conventionally $K_{ow}$ has been a robust predictor of bioaccumulation. A number of studies examining nanoparticle accumulation in organisms (Hou et al. 2013) have shown that partitioning processes as measured by $K_{ow}$ may not be relevant for predicting nanoparticle bioaccumulation. The uptake and accumulation of nanoparticles has been noted to be independent of factors influencing partitioning processes, such as the lipid content of organisms and organic carbon content of receiving environments. Indeed, the accumulation of nanoparticles in daphnids was found to be higher than that in fish, reflecting more their feeding mechanism than a partitioning process, unlike HOCs (Hou et al. 2013). For example, the accumulation of particles at the organ surfaces may be more important than their solubility (aqueous or lipid) for uptake and toxicity. Similarly, due to the tendency of particles to accumulate at the interface of octanol:water phases, depending on the pH of the system, measuring the $K_{ow}$ of nanomaterials is fraught with difficulty. A suitable adaptation of the conventional approach may be desirable to identify a surrogate parameter suitable for predicting the interaction of nanoparticles with biological or environmental interfaces, such as attachment/deposition efficiency (Westerhoff and Nowack 2013).

6.4.2.2 Speciation

Interactions and transformations, such as dissolution, functionalisation, redox process, and photo-transformation that nanoparticles can undergo in the environment (waters, sediments, soils, and in or on biological organisms) may change their speciation, charge and other surface characteristics substantially, thus altering their fate, transport, bioavailability and ecotoxicology.

Hydrophobic fullerenes ($C_{60}$) have been shown to have increased dispersion in the presence of dissolved organic matter and their solubility may also increase due to the transformation into more water soluble poly-oxygenated/hydroxylated products such as fullerols (Hwang and Li 2010; Klavins and Ansone 2010; Pycke et al. 2012). Adding Suwannee River HA has been shown to greatly enhance the dispersion of multi-walled CNTs (Hyung et al. 2007). HAs have also been demonstrated to stabilise iron oxide, alumina, titanium dioxide, gold, and Ag nanoparticles (largely dependent on surface charge) (Akaighe et al. 2011; Baalousha 2009; Diegoli et al. 2008).

Metallic nanomaterials can undergo dissolution and transformation processes in the environment as has been reported for Ag nanoparticles and ZnO nanoparticles (Kaegi et al. 2011; Lombi et al. 2012). The major pathway for Ag nanoparticles into the environment, such as through their use as an antibacterial agent, is believed to occur through the application of sewage sludge/biosolids to land. Kaegi et al reported the
majority of Ag nanoparticles added into a pilot waste water treatment plant were converted to silver sulfide (Ag$_2$S) (Kaegi et al. 2011). The authors concluded that physical and chemical transformations of Ag nanoparticles in waste water treatment processes would control the fate and toxicity of Ag nanoparticles and therefore need to be considered in future risk assessments.

Assessing the fate and ecotoxicity of ‘as-supplied’ nanomaterials may not provide a true understanding of the potential risks of AgVet products. A life cycle history of AgVet products is needed for a true understanding of their potential risk, such as primary and secondary species, that may undergo speciation/transformations changes in the environment. For nano-AgVet products, changes in the characteristics of an active ingredient or nano-active ingredient complex may occur due to interactions with the constituents of the formulation. From an operational standpoint, the stability of the formulated product may govern the solubility and reactivity of the active ingredient. As mentioned earlier, nanoparticles are expected to behave very differently than the soluble molecules of the active ingredient. Speciation would be particularly relevant for those formulated products designed to enhance efficacy by facilitating their solubilisation through nanoparticulate size, such as the controlled particle size of bifenthrin by Liu et al. (2008).

### 6.4.2.3 Dispersion and aggregation

What makes nano AgVet chemicals different to conventional active ingredients is their dispersion (in natural waters, sediments or soils), their surface chemistry and reactivity, and especially their aggregation and colloidal chemistry. The physico-chemical properties of the receiving environment are likely to play an even greater role for nanoparticles than appreciated for conventional chemicals. Environmental factors such as pH, salinity, divalent ions, and the presence of NOM, have been identified as important factors that alter the ecotoxicological impact of (Handy et al. 2008b; Klaine et al. 2008). The different nature of interactions of nanoparticles, such as aggregation chemistry, in various environmental matrices (air, fresh water, seawater, sediment or soil) may result in different ecotoxicological effects. Dispersion and aggregation are very important among different processes impacting ecotoxicology of nanoparticles (Handy et al. 2008b), namely:

- Manufactured nanoparticles may aggregate or form stable dispersions.
- Aggregation chemistry and ecotoxicity may be affected by the particle and surface properties such as size, shape, surface area and surface charge.
- Manufactured nanoparticles may sorb on surfaces in soil, suspended sediment and on organism cell walls.
- Environmental conditions such as pH, salinity, water hardness, and the presence of NOM, may affect nanoparticle chemistry.

### 6.4.3 Uptake of Nanoparticles by organisms

#### 6.4.3.1 Bioavailability

Not only the molecular chemistry of the active ingredient (as in the case of conventional AgVet chemicals), but also its particle chemistry, or the carrier, or active ingredient nano complex all assume major importance in relation to the bioavailability and ecotoxicology of nanoparticles. The ecotoxicological effects of nanoparticles may either follow the same exposure pathways as conventional AgVet chemicals or, in some cases, may expose the non-target organisms through unconventional routes. The nature of effects may also be different, such as the particulate nature of the active ingredient, or the excipient may elicit different forms
of toxicological impact. For example, fullerene nanoparticles can potentially inflame and injure the gut walls of fish or earthworms (on microvilli) leading to poor feeding efficiency (Pakarinen et al. 2011).

**Fish and vertebrates**

The mechanisms and processes of uptake and toxicology are likely to be different for nano AgVet product active ingredients or excipients compared to conventional active ingredient molecules. For example, the mechanisms of absorption, distribution, metabolism and excretion (ADME) for nanoparticles in comparison with conventional chemicals in fish were considered by Handy et al. (2008a). They noted that adsorption of nanoparticles on the gill surfaces of fish are likely to be similar for nanoparticles as other chemicals but their uptake in epithelial cells are more likely to occur via vesicular processes, such as endocytosis, than via diffusion or membrane transporters. This may make fish more vulnerable than mammals since fish guts are able to take up much larger materials across the cell membrane (indeed the oral delivery of fish vaccines has exploited this phenomenon (Handy et al. 2008b). The inflammation or injury caused by nanoparticles to the gut wall may facilitate direct uptake of nanoparticles in the blood, if nanoparticles do not aggregate in high ionic strength body fluids oozing out of injured tissue. Even low concentrations of nanoparticles such as TiO$_2$ and CNTs have been reported to cause inflammation of gills and injury to gut walls (Handy et al. 2008b). Similarly in terms of metabolism and excretion processes, they observed that rather than renal or bronchial excretion, the hepatic excretion into bile is likely to be the main mechanism of efflux for nanoparticles (Figure 6.6).

**Invertebrates**

In invertebrates also, the major route of entry for nanoparticles may be through endocytosis, ie penetrating through the semi-permeable cell wall (consisting of cellulose in algae and plants and chitin in fungi) and then through the bilayer lipid plasma membrane through active (ion channels or protein carriers) or passive mechanisms (Handy et al. 2008b). The nanoparticles may subsequently bind to organelles in the cell, produce toxic ions or ROS and interfere with the metabolic processes.

**Plants**

Plants have also been observed to uptake nanoparticles via endocytosis (Ovecka et al. 2005). The surface area (leaf area index) as well as the nature of plant surfaces, such as waxy leaves, may have an effect on the accumulation, bioavailability and uptake of nanoparticles by plants. The accumulation of nanoparticles and blocking of stomata may influence gas and heat exchange in plants and consequently change their physiology (Da Silva et al. 2006). Photo-induced electron transfer caused by nanoparticles may affect photosynthetic surfaces. Adsorption to cell surfaces in organisms can interfere with the uptake of essential nutrients.

It is well established that root exudates can enhance the bioavailability of metals through organo-metal complexes. However, it is not clear if these can influence the distribution, diffusion and bioavailability of nanoparticles to plants. Soil properties such as pH, salinity, nature of cations, clay mineralogy, and organic matter content are likely to affect the nanoparticle chemistry and thus are expected to affect the bioavailability of nanoparticles to soil organisms. It is therefore imperative that these soil properties are adequately considered in terrestrial ecotoxicological assessments.
Figure 6.6: An illustration of the different routes for uptake, excretion and metabolism of TiO$_2$ nanoparticles compared to C$_{60}$. Reproduced with permission from Handy et al. (2008a). Copyright (2008) Springer.
Soil organisms

The type of soil matrix may have profound effects on the fate, behavior, and bioavailability of the test material. Although this is also well known for traditional hydrophobic organic compounds (HOCs), the effect of soil type on nanoparticle bioavailability may be through different, little known, mechanisms. However, recent studies on the bioavailability of nanoparticles to earthworms indicate that in contrast to HOCs (where soil properties such as organic carbon content determine the partitioning to soil and moderate the bioavailability of the contaminants to organisms) the uptake of CNTs was found to be independent of soil OC content (Petersen et al. 2011b). The likely soil factors that would affect particle chemistry directly (and thereby the bioavailability of nanoparticles to organisms depending on the exposure route) include soil pH, salinity, dissolved organic matter and other factors affecting fate in soil. Given the antimicrobial properties of nano Ag and TiO$_2$, it is expected that soil microbial processes may be sensitive to certain nanoparticles. However, there is limited information in the literature about this.

6.4.3.2 Bioaccumulation and biomagnification (trophic transfer)

Bioconcentration of HOCs (conventional AgVet chemical active ingredient) is well studied and represents the absorption (passive uptake) of chemicals by organisms from the environment, such as water or air phases, through dermal or respiratory pathways, excluding dietary uptake. The bioconcentration factor (BCF) is simply the ratio of chemical concentration in the organism to that in the environmental media — water, soil and sediment. The term bioaccumulation is used where food as an exposure pathway contributes to the accumulation of a chemical in organisms. Where the chemical gets concentrated in organisms of higher trophic level in the food chain, the term biomagnification is used. The accumulation through these processes has been the subject of recent studies on nanoparticles and has highlighted how nanoparticle behaviour can be very different to that expected of HOCs.

A recent review (Hou et al. 2013) of ecotoxicological studies and compilation of data on the accumulation of nanoparticles in aquatic (mainly daphnia and fish) and terrestrial organisms (mainly earthworms) have shown that mechanisms explaining accumulation behaviour of conventional HOCs do not hold good for nanoparticles. For example, BCF values for accumulation of a range of nanoparticles, such as TiO$_2$, Ag nanoparticles and nC$_{60}$, daphnids were generally large enough (log BCF ranging from 3.16 to 5.64) to fall into the category 'very bioaccumulative substances', for example USEPA criteria of $\geq 3.7$. In contrast, the log BCF values for fish nanoparticle accumulations were much lower, (ranging from 1.27 to 2.87) converse to that expected of HOCs, implying that the underlying mechanism of nanoparticle accumulation is different. The filter feeding behaviour of daphnids, and their ability to filter particles with size 0.4 to 40 μm (including nanoparticle aggregates), may be responsible for this. Furthermore, a lack of dependence on nanoparticle composition, particle size, aspect ratio and surface coating was noted. The uptake of CNTs by daphnids was reported by Petersen et al to be unaffected by their surface modification with polymers with different charge characteristics (positive, negative or neutral) (Petersen et al. 2011a). The current literature suggests that the bioaccumulation potential of nanoparticles in fish is relatively low and the major route of uptake may be oral (direct ingestion of nanoparticles) or via food (Hou et al. 2013). Bioaccumulation of several nanoparticles in earthworms has been found to be relatively lower than traditional HOCs such as polyaromatic hydrocarbons. The organic carbon content of soil (from 1.6 to 5.7%) was found to have little effect on bioaccumulation in earthworms, which is in sharp contrast with HOCs (Petersen et al. 2011b). Similar to daphnids, the surface properties (coating with polyethyleneimine polymer) had a minimal effect on bioaccumulation.

Trophic transfer, such as from ‘algae to daphnid’ and ‘daphnids to fish’, has been reported for QDs and TiO$_2$ nanoparticles (Hou et al. 2013). Similarly, biomagnification of QDs from contaminated bacteria to ciliated protozoa has been reported by Werlin et al (2011). In terms of terrestrial food chain effects, Judy et al reported tobacco hornworm ca
terpillars (*Manduca sexta*) bioaccumulating Au nanoparticles after ingesting plant tissue surfaces contaminated with nanoparticles, and found biomagnifications factors ranging from 6.2 to 11.6 (Judy et al. 2011). However, they found no dependency on the Au particle size (from 5-15 nm). Clearly the plant uptake of nanoparticles also raises the possibility of human health exposure to nanoparticles through the food chain.

In summary, the bioaccumulation of nanoparticles in organisms has been demonstrated through several studies now. It is apparent mechanisms and routes of exposures are different for nanomaterials than for conventional HOC AgVet chemicals. Currently no robust measure of the potential bioaccumulation potential of nanoparticles exists (such as $K_{ow}$ for HOCs), although some attempts are being made to adapt conventional approaches in search of a suitable surrogate parameter (Hou et al. 2013).

### 6.4.4 Ecotoxicological test systems and their characterisation

#### 6.4.4.1 Characterisation of formulated product and associated nanoparticles

One of the major weaknesses in several of the published studies on the effects of nanomaterials on organisms thus far has been poor control on the exposure to, and inadequate characterisation of, nanoparticles during the ecotoxicity testing. Nano AgVet formulations are likely to range from nanoemulsions (with essentially no particle chemistry involved) to nano-active ingredient complexes doped with a nano catalyst, TiO$_2$ for example, and pristine metal oxide nanoparticles such as Nano Ag and Nano ZnO). It is therefore crucial to have a very clear understanding of the constituents of the formulation so that an appropriate test protocol can be designed. In this regard, even the minor constituents of the formulations, such as nanoparticles, or the impurities present in the formulation and/or indeed used in delivering the nanoparticles in test systems, may become a source of toxicity (Table 6.6). Some of the early work on ecotoxicity of nanoparticles such as fullerenes was seriously compromised by the presence of solvent impurities, for example tetrahydrofuran used in stabilising the suspension, as highlighted by Fortner et al. 2005. Appropriate nomenclature and details of manufacturing processes can help establish the validity of the comparison with published work. Here particle size, shape and surface area to volume ratio for nanoparticles must be included, given their important role in determining particle chemistry, as discussed earlier.

Considering the currently available techniques and their practicality, a minimum set of characteristics for nano AgVet products used in ecotoxicity tests have been suggested (Table 6.6). Further details on some of the aspects may be found in Crane et al. (2008) and Handy et al. (2008b).

#### 6.4.4.2 Test organisms, organs and endpoints

Ecotoxicological tests on conventional AgVet active ingredients have been conducted on aquatic organisms, mostly in aqueous media. However, for nanoparticles toxicity may arise from particles and therefore different organisms, such as mammals, as well as different endpoints, respiratory health and inflammation for example, may be necessary, depending on the environmental compartment being studied. Fairly well developed experience on particle toxicity in mammals could be valuable in guiding nanoparticle ecotoxicology. Lethal toxicity endpoints commonly used for conventional active ingredients for fish may be difficult to achieve for nanoparticles as they may aggregate more readily at high concentrations (Handy et al. 2008b). Therefore, sub-lethal effects, especially on target organs for nanoparticles, may be more appropriate. Target organs where nanoparticles are likely to adsorb, aggregate and accumulate, such as on gill surfaces, gut tissues, liver and brain in fish, may be the focus of ecotoxicological investigations (Kashiwada 2006; Smith et al. 2007). Oxidative stress in the developmental toxicity of fish is relevant for nanoparticles (Smith et al. 2007). Improved understanding of ADME of nanoparticles is required and the research and development on this in coming years may guide the selection of test organs and endpoints. For
aquatic invertebrates, the water flea (*Daphnia magna*) and scud (*Hyallela azecta*) have been studied for carbon-based nanoparticles and lethal and sub-lethal effects such as moulting, mobility or feeding behaviour have been reported (Handy et al. 2008b). There is a lack of information on terrestrial invertebrates, though some studies on earthworms are available in the literature. For example, as stated above, inflammation and injury to the gut wall of earthworms could lead to poorer feeding efficiency (Pakarinen et al. 2011). Given the antimicrobial properties of nano Ag and TiO$_2$, it is possible that soil microbial processes may be sensitive to certain nanoparticles, however, there is limited information in the literature on this aspect.

### 6.4.4.3 Exposure conditions and their environmental relevance

Substantial uncertainty in current ecotoxicological studies of nanoparticles arises from the lack of control on nanoparticle exposure during the tests. This is because the particle chemistry and particle stability may be changing during the test period. Even nanoparticles in a stable suspension may aggregate with time. Also, often it is not clear if a toxicological response is due to the particles, or the dissolved form, or both. Nanomaterials tend to aggregate and settle out of the water column depending on the test conditions imposed. For example, particle chemistry in seawater and freshwater is very different because the aggregation behaviour of nanoparticles is strongly influenced by changes in salinity (Stolpe and Hasselov 2007). It is therefore crucial that during test exposures, factors affecting the particle dispersion and aggregation (such as pH, salinity, water hardness, and the presence of NOM) are controlled and are maintained at environmentally relevant levels.

### 6.4.4.4 Use of reference materials, positive and negative controls

The availability of reference materials for nanoparticles is improving and their use in ecotoxicological studies should facilitate greater clarity on sources of toxicity. For example, in mammalian respiratory toxicological studies particles with known toxicity to rodents, such as quartz or carbon black, have been used both as a reference material and as a positive control (Oberdorster et al. 1992). Reference materials of a known inert nature could be used as a negative control but such materials are currently lacking for aquatic and terrestrial ecotoxicological assessments. However, experience with suspended particles in the aquaculture literature may provide a starting point (Crane et al. 2008). Simultaneous measurements of reference material may provide a crucial insight as to the suitability of the ecotoxicological test method employed for the nanoparticles.
Table 6.6: Key characterisation and other details required for ecotoxicological tests; adapted from Crane et al. (2008); Handy et al. (2008b)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Property</th>
<th>Reasons</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear nomenclature and manufacturers information</td>
<td>Formulation make up of individual component (especially nano)</td>
<td>To design the test method appropriately and to know what to expect in terms of impurities</td>
<td>–</td>
</tr>
<tr>
<td>Surface charge</td>
<td>Zeta potential</td>
<td>To assess electrokinetic potential and stability of the suspension</td>
<td>Surface charge is often dependent on pH of the system and hence a good control on pH is needed</td>
</tr>
<tr>
<td>Particle concentration or surface area by volume</td>
<td>Number or specific surface area by volume</td>
<td>To capture particle chemistry-driven toxicological effect</td>
<td>–</td>
</tr>
<tr>
<td>Particle size</td>
<td>Mean particle size + SD</td>
<td>To understand nano-particle related effects</td>
<td>Measure particle size in both the stock and the test solutions. Use at least two different methods</td>
</tr>
<tr>
<td>Aggregation in test solution</td>
<td>Mean particle size + SD</td>
<td>To take into account any aggregation during the test</td>
<td>–</td>
</tr>
<tr>
<td>Particle stability (eg dispersion) at various dilutions</td>
<td>A spectroscopic image</td>
<td>Visual evidence of dispersion/aggregation</td>
<td>Cover various dilutions used in the test system</td>
</tr>
<tr>
<td>Particle shape</td>
<td>Aspect ratio</td>
<td>Particle shape may affect toxicological impact</td>
<td>–</td>
</tr>
<tr>
<td>Presence of the impurity in the formulation</td>
<td>Concentration</td>
<td>To assess potential contribution of toxicity due to impurities</td>
<td>–</td>
</tr>
<tr>
<td>Impurity profile</td>
<td>Residual concentrations of impurity</td>
<td>To eliminate potential contribution of toxicity due to impurity</td>
<td>Details of washing procedure employed</td>
</tr>
<tr>
<td>Adsorption to vessels</td>
<td>Loss of particles or decreased concentration in control</td>
<td>To eliminate detoxification due to adsorption on test containers</td>
<td>Details of how the loss on test containers was eliminated</td>
</tr>
<tr>
<td>Appropriate controls</td>
<td>Effect assessment (eg EC50)</td>
<td>To isolate any experimental artifacts and clearly identify the toxicity source</td>
<td>Both positive or negative control may be useful</td>
</tr>
<tr>
<td>Relevance of test organism</td>
<td>Effect assessment (eg EC50)</td>
<td>To ensure an appropriate test organism is chosen for the target environmental compartment</td>
<td>–</td>
</tr>
<tr>
<td>Relevance of test endpoint</td>
<td>Effect assessment (eg EC50)</td>
<td>To ensure an appropriate endpoint is chosen for the effect of nanoparticles</td>
<td>–</td>
</tr>
<tr>
<td>Environmentally relevant conditions</td>
<td>Effect assessment (eg EC50)</td>
<td>The test condition should reflect the environmental conditions</td>
<td>For example, the environmentally relevant concentrations of DOC, salinity, pH can influence aggregation of nanoparticles</td>
</tr>
<tr>
<td>Comparison of formulation versus a.i.</td>
<td>Effect assessment (e.g. EC50)</td>
<td>To compare the contribution of formulation constituent with that of a.i.</td>
<td>–</td>
</tr>
</tbody>
</table>
6.5 Concluding Remarks

The application of nanotechnology to AgVet chemicals may span a wide range of products considered to be nanoformulations (some examples are given in Table 6.1). These may vary from nanoemulsions, nanoencapsulations, such as polymer-active ingredient complex, to pristine nanoparticles like nano metals and nanoclays. The literature discussed in this chapter is generally more relevant to nanoparticles as active ingredients because most of the literature is based on metal oxide or fullerenes particles. Since nanoemulsion formulations are metastable and do not contain nanoparticles, they may not need to be treated any differently than the conventional AgVet chemicals. The category of nano-active ingredient complex (nano encapsulation with TiO$_2$ catalyst) is perhaps the most complex formulation where the approach to be adopted is quite different. For this category a pragmatic approach, based on the durability of formulated product, may be the way forward.

6.5.1 Need for a pragmatic approach

For nano-metal oxides or nano clays, the nanoparticles are expected to be persistent. However, the changes in their surface characteristics (either during product formulation or in the environment) may have implications for their fate and potential effects. For the nano-active ingredient complex involving complex formulations, information on the durability of a product, ie the fate of the product in soil or water, may become crucial in making decisions as to what material (conventional active ingredient, nano-active ingredient complex or free nanoparticles) should be tested and analysed in aquatic/terrestrial fate studies and in aquatic/terrestrial ecotoxicity studies. For example, if the polymer cage or capsule does not persist in the environment, and readily releases the active ingredient without releasing any persistent nanoparticles, the conventional risk assessment approach may be appropriate. Conversely, if the formulation results in the release of a significant number of nanoparticles, such as TiO$_2$ catalyst, the product has to be treated differently and assessed for risk as a nanomaterial.

6.5.2 Special considerations for fate and effect assessment

There are some major challenges that nanoparticles present during fate and effect assessment and they require a very different approach to that for conventional AgVet chemicals. The key considerations for fate and effect studies are as follows:

- The fate and effects of nanoparticles are likely to be governed by particle chemistry and their interactions, heteroaggregation for example, rather than the traditional molecular interactions with the environmental media and organism surfaces used for solutes.
- The aggregation chemistry and ecotoxicity of nanoparticles may be related to particle and surface properties such as size, shape, surface area and surface charge.
- Environmental conditions such as pH, salinity, water-hardness and the presence of NOM may modify the physico-chemistry of nanoparticles and consequently their fate and effects.
- Conventional measures of fate and bioaccumulation potential such as sorption coefficient ($K_{oc}$) or octanol:water partition coefficient ($K_{ow}$) may not be directly relevant to nanoparticles. New measures such as attachment efficiency and retention coefficient ($K_r$) may be more relevant. However, these new indices are still under development and require further testing and standardisation.
- A critical weakness in current ecotoxicological studies in the literature is the lack of characterisation of nanoparticles during the test exposure time. Due to continuously changing properties
(aggregation, deposition) with time, the exposure conditions must be controlled for a proper assessment of ecotoxicological effects.

**6.5.3 Descriptors for fate and transport of nanomaterials**

As discussed earlier, some of the global parameters commonly used by regulatory agencies for the assessment of fate and transport may not be directly applicable to nanomaterials. Recently, some good discussion papers have considered the issue of suitability or adaptability of these parameters. For example, Westerhoff and Nowack (2013) considered the utility of a set of commonly used global parameters, such as $K_{ow}$, $K_d$ and $K_{oc}$ to predict the distribution of nanoparticles between environmental compartments. They suggested that while a number of the existing parameters have considerable potential to be adapted for nanomaterials, the crucial need is the measurement techniques that are appropriate for nanomaterials and colloids. They presented a set of testing schemes that may be considered as a potential strategy for predicting the fate and transport of nanomaterials (Table 6.7).

**6.5.4 Recommended requirements for characterisation of nano agvet chemicals**

Given the above discussion and the importance of certain properties for fate and effects assessment, a minimum set of characterisation criteria are essential for nano-AgVet chemicals. These have been listed in Table 6.8 for both environmental fate and effect studies. Generally speaking, the properties that determine the stability of particles in suspension or their aggregation behaviour, namely, size, shape, zeta potential and specific surface are a minimum set that is needed for both fate and effects assessments. Aggregation state is a crucial parameter that determines the fate, as it provides an indicator of partitioning into different environmental compartments and the extent of exposure in, for example, the sediment or the water column in aquatic systems. However, the aggregation state is a function of ambient conditions such as pH, salinity, the presence of DOC and other complexing agents. Therefore, characteristics such as zeta potential and aggregation should be presented as a function of environmental parameters. Where possible more than two methods based on different scientific principles should be used in the characterisation of nanoparticles.

<table>
<thead>
<tr>
<th>Testing Approach</th>
<th>Example Schemes</th>
<th>Potential Fate and Transport Outcomes</th>
<th>Global Descriptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent exchange</td>
<td>Measuring the distribution of nanomaterials between water containing nanomaterials and a solvent phase (octanol)</td>
<td>Single coefficient ($K_{ow}$, % hydrophobicity) indicating potential to interact with hydrophobic phases (e.g., soil, lipids, tissue)</td>
<td>$K_{ow}$</td>
</tr>
<tr>
<td>Surface affinity</td>
<td>Dynamic column tests using media coated with different surfaces (e.g., silica, iron oxide, hydrophobic material)</td>
<td>Single coefficient ($\alpha$) from 0 to 1 indicating tendency to interact with different environmental surfaces (soils, suspended sediment)</td>
<td>$\alpha$</td>
</tr>
<tr>
<td>Sorption</td>
<td>Interaction of ions or NOM with nanomaterials</td>
<td>Changes in zeta potential, sorptive capacity factors</td>
<td>Freundlich isotherm K and $1/n$ values OR Ligand binding constants ($L_1$) and conditional stability constants ($K_1$)</td>
</tr>
<tr>
<td>Sediment retention</td>
<td>Measuring the retention of nanomaterials from water or natural soils or sediment</td>
<td>Single coefficient ($K_R$ or $K_D$) indicating tendency to interact with environmental surfaces</td>
<td>$K_R$ or $K_D$</td>
</tr>
<tr>
<td>Self-aggregation</td>
<td>Measuring aggregation kinetics of nanomaterials with themselves in water matrices with different ionic composition or NOM</td>
<td>Single coefficient ($\alpha$) from 0 to 1 indicating the effect of water composition on the tendency of nanomaterial to aggregate into larger particles that could settle out of a water column</td>
<td>$\alpha$</td>
</tr>
<tr>
<td>Electrostatic repulsion</td>
<td>Measuring the zeta potential as an indicator of particle stability</td>
<td>Zeta potential of nanomaterials can be binned into likely to be stable or likely to aggregate</td>
<td>$E_{NET}$ (net energy barrier)</td>
</tr>
<tr>
<td>Multidimensional parameter or high-throughput testing</td>
<td>Automated chemical addition of salts, organics, acids, or suspended sediment to quickly assess nanomaterial stability</td>
<td>3D contour plots of key parameters (zeta potential, turbidity) to understand nanomaterial stability in a series of water chemistries</td>
<td>STIFF diagram plotting parameters</td>
</tr>
<tr>
<td>Dissolution kinetics</td>
<td>Measuring dissolution of metallic nanomaterials as a function of dissolved oxygen, pH, and redox conditions</td>
<td>Thermodynamic conditional stability coefficients and surface area-dependent kinetic rate constants; solubility limits</td>
<td>$k_{dissolution}$</td>
</tr>
<tr>
<td>Weathering</td>
<td>Simulated photolysis, dissolution, and biodegradation of nanomaterials and/or their coatings</td>
<td>Changes in size, zeta potential, composition, coatings, etc.</td>
<td>$k_i$ (multiple rate constants for different “i” mechanisms)</td>
</tr>
</tbody>
</table>
Table 6.8: Recommended minimum characterisation requirements for AgVet chemicals.

<table>
<thead>
<tr>
<th>Property/parameter</th>
<th>Environmental fate</th>
<th>Effects</th>
<th>Techniques available</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical and/or elemental composition</td>
<td>Capping and functionality may have a major impact on fate.</td>
<td>Essential to assess the potential toxic impact on non-target organisms, especially of the nano component and any impurities</td>
<td>Combination of conventional (eg chromatography) and nano-specific techniques listed below in this column</td>
<td>Appropriateness and robustness of method should be demonstrated</td>
</tr>
<tr>
<td>including capping agents, impurities (especially nano)</td>
<td>Fate of impurity only important if shown to be toxic in nature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubility</td>
<td>Determines mobility and phase transfer</td>
<td>Conventionally toxicity is linked with solubility</td>
<td>Dialysis membrane, centrifugation</td>
<td>Toxicity may arise from both soluble and particulate fractions</td>
</tr>
<tr>
<td>Primary particle size, size distribution</td>
<td>Fate and transport is dependent on particle size</td>
<td>Essential for ecotoxicological effects assessment where size is seen as an important determinant of toxicity</td>
<td>TEM, SEM, Cryo-TEM, XRD</td>
<td>Size distribution is more appropriate. At least two methods based on different principles to be used</td>
</tr>
<tr>
<td>Aspect ratio</td>
<td>May impact fate and transport (desirable but not essential)</td>
<td>Shape may have a major impact on toxicology</td>
<td>TEM, SEM, cryo-TEM</td>
<td>Morphology may elicit unique toxicity responses for inhalation exposures, eg asbestos</td>
</tr>
<tr>
<td>Specific surface area (SSA)</td>
<td>Particle chemistry-driven fate processes are dependent on SSA</td>
<td>High specific surface area may induce toxic effects</td>
<td>BET</td>
<td>Surface interactions with organisms and in environment are influenced by SSA</td>
</tr>
<tr>
<td>Surface charge</td>
<td>Determines the stability of suspension and mobility of nanoparticles; will determine mobility in the environment</td>
<td>Stability of suspensions during ecotoxicity testing is linked to zeta potential</td>
<td>Electrophoretic mobility</td>
<td>Surface charge distribution at different environmentally-relevant pH values is needed</td>
</tr>
<tr>
<td>Aggregation behaviour</td>
<td>Governs fate and behaviour but heteroaggregation may be more important in this case</td>
<td>Homoaggregation may be more important for ecotoxicity testing. It is crucial to maintain suspension stability during the ecotoxicity testing.</td>
<td>Light scattering, cryo-TEM, FFF, disc centrifugation</td>
<td>Data relating to important environmental parameters, eg pH, salinity, dissolved organic matter, is needed</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>Any product-modified transformations as well as those due to weathering in environment</td>
<td>Toxicity depends on surface chemistry (eg capping on nanoparticles)</td>
<td>Spectroscopy, TGA</td>
<td>Including any transformations that are expected to occur in the formulated product or in the environment</td>
</tr>
<tr>
<td>Speciation</td>
<td>Fate of nanoparticles is markedly influenced by speciation (eg. AgNO3 versus Ag2S)</td>
<td>Different species (including transformation products) may have different toxicity than</td>
<td>Synchrotron, FFF, Chromatography</td>
<td>Speciation may change with time in product or in the environment</td>
</tr>
<tr>
<td>Stability of formulation</td>
<td>The information about the stability of nano-a.i. complex is crucial for appropriate assessment of environmental fate</td>
<td>The decision about appropriate ecotoxicity assessment can only be made based on the stability of the product</td>
<td>Conventional plus light scattering, TEM, Cryo-TEM</td>
<td>The fate and ecotoxicity assessment of the product needs to be compared with a.i. to ensure there is no added toxicity due to the formulation</td>
</tr>
</tbody>
</table>
6.6 References


ISO Nanotechnologies - Terminology and definitions for nano-objects - Nanoparticle, nanofibre and nanoplate.


APPENDIX 1

The information presented below relates to the instruments and techniques used to measure the size and shape of nanoparticles. Accordingly, microscopy, scattering, separation, aerosol characterization and surface area measurement are addressed.

- Microscopy

**Electron Microscopy (EM)**

Electron microscopes use electrons as illumination rather than visible light. This is because the maximum resolution (ability to discriminate features) of a microscope is approximately equal to the wavelength of the illumination used. This is 300 to 600 nm for visible light but for electrons the wavelength, and hence the potential resolution, is 0.002 to 0.1 nm depending on the electron energy.

The transmission electron microscope (TEM) produces magnified images or diffraction patterns of the sample by passing the electron beam through a very thin sample and interacting with it. The spatial variation in this image is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light-sensitive sensor such as a charge-coupled device (CCD) camera. The image detected by the CCD may be displayed in real-time on a monitor or computer.

New generation TEMs have overcome spherical and chromatic aberration and produce images with very high sufficient resolution. The ability to determine the positions of atoms within materials has made high resolution TEMs an indispensable tool for nanotechnology research and development in many fields.

Unlike the TEM, where electrons are detected by beam transmission, the scanning electron microscope (SEM) examines and analyses the physical information (such as secondary electrons, backscattered electrons, absorbed electrons and X-ray radiation) obtained by generating electron beams and scanning the surface of a sample in order to determine the structure, composition and topography of the sample. Generally, the TEM resolution is about an order of magnitude better than the SEM resolution. However, because the SEM image relies on surface processes rather than transmission it is able to image bulk samples and has a much greater depth of field, and so produces images that are a good representation of the three-dimensional structure of the sample.

EMs are very useful tools in nanoparticle characterization, though they are very expensive to purchase and require great expertise to operate and maintain properly. They can provide representative images of nanoparticles, as well as measurements on a single particle basis. For particle sizing, the measurand in EM is a diameter such as a projected area diameter, \( x_a \) or a Feret's diameter, \( x_F \). The number-weighted particle size distribution (PSD) can be constructed by analysing a large number of particles. However, it can be very time consuming to generate results that are statistically relevant and representative of the entire sample.

The TEM requires an ultra-high vacuum and a high voltage. To be imaged the sample needs to be transparent to the electron beam, small enough to be placed on a copper support grid (about 3 mm in diameter) and inserted into a suitable holder. The electron optical column in the SEM is shorter than in the TEM, as there are fewer lenses involved in generating the beam. The column typically houses gun alignment coils, lenses that condition the beam into a fine spot on the sample surface and the scan coils. In the SEM,
the focused electron beam is scanned in a raster pattern across the sample using a set of scan coils. As the beam scans across the specimens, different interactions between the beam and the sample occur. A range of detectors in the chamber above the specimen detects the signals from these interactions. The most commonly used detectors pick up the signal from secondary electrons; that is, electrons that have been knocked out from their positions by the scanning focused beam. Different interactions of the beam and sample give images based on topography, elemental composition (x-rays), density variation or crystalline structure of the sample.

TEMs can have resolution limits as low as sub-nm, and can be used to analyse both large numbers of nanoparticles at lower magnifications and individual particles at high magnifications. The technique can be suitable for characterization by image analysis, as the darker areas in the formed image represent areas where fewer electrons have passed through the sample due to higher electron density, such as nanoparticles.

The TEM beam is quite wide in normal imaging mode but, due to the sophisticated system of lenses, it can also be formed into a very fine (sub-nm) focused electron probe. Combined with the thin, electron-transparent sample it enables the generation of diffraction patterns for crystallographic information.

The resolution of an SEM is dependent on the size of the beam, but ranges from a few nm up to several mm. The SEM can be used to study almost any kind of sample, as long as it is conductive. This makes sample preparation for SEM much less complicated than for TEM. For particle sizing, the measurand in SEM is a number weighted distribution of diameters such as a projected area diameter, $x_a$ or a Feret’s diameter, $x_F$.

The accelerating voltages used in SEM are much lower than in TEM since the beam does not need to penetrate the specimen. As the imaging is not dependent on the density of the sample, it is possible to image low-density samples. The sample chamber in an SEM is considerably larger than in a TEM and often capable of housing many different samples at the same time. The SEM has a large depth of field, which allows a large amount of the sample to be in focus at one time and produces an image that is a good representation of the three-dimensional sample. Using EDS (Energy-dispersive x-ray spectroscopy) allows for elemental detection, but as the beam-sample interaction volume in SEM is quite large the element detection is less sensitive than for TEM.

The sample stage for a TEM is nearly always capable of holding only one sample grid at a time, so if multiple samples are to be examined it lengthens the process considerably. Sample preparation for TEM can be very time consuming and it requires expertise to get good and reproducible results. For nanoparticle suspensions, the sample has to be thoroughly dried and this may alter the appearance of the particles. The drying process itself can generate artefacts such as apparent aggregation. In addition, for nanoparticle suspensions, the particle concentration needs to be low which may lead to a poor sampling ratio. It can also be very time consuming to generate results that are statistically relevant and representative of the entire sample.

An SEM usually requires a conducting sample. If the sample is non-conducting, a conductive coating may be applied to prevent charge from being built up in the sample, but if carrying out dimensional measurements, this has to be done carefully, as any measurement will now show both the feature of interest plus the applied coating. Particles suspended in a liquid have to be deposited onto a suitable substrate and thoroughly dried before inserting into the vacuum chamber. This may cause significant changes to the sample. Also, as the scanning motion of the electron beam in an SEM gives a topographical image, this often leads to some edge distortion caused by strong beam interaction.
To ensure the best possible measurement results, electron microscopes should be checked, verified and calibrated as required. For TEM a typical particle measurement is usually based on image analysis, making the image calibration crucial. There is an ISO standard (International Organization for Standardization 2004b) on image/instrument calibration at a range of magnifications using a purpose-made artefact. There are also ranges of reference materials of different composition (Au, SiO$_2$, PS latex) that can be used for image calibration.

**Atomic force microscopy**

Scanning probe microscopy (SPM) is a method of imaging surfaces by mechanically scanning a probe over the surface under study and measuring the response of a detector. This generic term includes many methods such as atomic force microscopy (AFM), scanning near field optical microscopy (SNOM), scanning ion conductance microscopy (SICM) and scanning tunnelling microscopy (STM). AFM is one of the most common techniques where a solid tip measures the van der Waals forces between the tip and the surface. The tip is on the end of a flexible beam or cantilever whose displacement is measured using a laser beam.

The instrument can be operated in three modes: contact mode, non-contact mode and tapping mode. The first involves the tip staying in contact with the surface at all times. This can result in the tip scratching the surface for very soft materials and excessive wear of the tip for hard materials, changing its original shape. The non-contact mode uses an oscillating cantilever with high stiffness. The tip is brought into such close proximity with the surface that the oscillation frequency changes. The change in frequency is measured during scanning and produces a map of the surface. The tapping mode also uses an oscillating cantilever but the tip is brought closer to the surface than in non-contact mode, so that the tip touches (taps) the surface intermittently. At the point of contact, the oscillation of the cantilever is reduced and this change can be used to detect features of the surface.

AFM has several advantages over the EM. Samples viewed by an EM require special treatment that is often destructive and need an expensive vacuum environment for proper operation. AFMs work perfectly well in an ambient or even liquid environment.

The resolution of an AFM is very high. Features down to 0.1 nm can easily be detected in the z-direction. The resolution in x- and y-direction is directly linked to the sharpness and shape of the tip, but features as small as a few nm can be imaged with a standard tip. The AFM can generate three-dimensional maps of surfaces and image individual nanoparticles. Some information about size and shape can be deducted from AFM images and it is possible to make particle size measurements based on height measurements.

The main disadvantage of the AFM is the image size. The SEM can show an area in the order of millimetres by millimetres and a depth of field in the order of millimetres. The AFM can only show a maximum height in the order of micrometres and a maximum area of around 150 by 150 μm. Also, the AFM cannot scan images as fast as an SEM. It may take several minutes to scan a typical region with the AFM, whereas an SEM is capable of scanning at near real-time.

The information generated using AFM is strongly dependent on the tip shape (Villarrubia 2004). The illustration in Figure 3.1 shows a nanoparticle on a substrate being scanned with a) an ideally thin and sharp tip, b) a more realistic AFM tip and c) the result from a 100 nm polystyrene particle scanned with a typical AFM tip. The image illustrates the complexity in using AFM for nanoparticle sizing, where the tip may cause severe distortion to the shape of a feature in the x and/or y directions.
Appendix 1 - Figure 1: A schematic on a substrate being scanned with a) an ideally thin and sharp tip, b) a more realistic AFM tip and c) the result from a 100 nm polystyrene particle scanned with a typical AFM tip (Jamting and Miles, 2013).

The sample preparation for AFM measurement can be complex and the process of placing a particle suspension onto a substrate may change the properties of the particles. Like the other microscopy techniques it can also be very time consuming to generate results that are statistically relevant and representative of the entire sample.

To calibrate an AFM, the preferred method is to perform a set of measurements on an appropriate physical standard, which should be chosen according to the requirements. There are ranges of suitable artefacts with regular periodic structures of well-known dimensions in one, two or three dimensions, a comprehensive list of which can be found on the German NMI website (Physikalisch-Technische Bundesanstalt – PTB). There are also ranges of nanoparticle reference materials available with different composition (Au, SiO$_2$, PS latex) that can be used to evaluate performance.

- **Scattering**

**Dynamic light scattering**

Dynamic Light Scattering (DLS), also known as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS), is a method in which particles undergoing Brownian motion in a liquid suspension are illuminated by a laser. Analysing the time-dependent intensity of the scattered light yields the translational diffusion coefficient and hence the particle size as the hydrodynamic diameter via the Stokes-Einstein relationship (see Figure 3.2).
Appendix 1 - Figure 2: Typical DLS configuration: the laser illumination source, the sample under measurement in the cuvette and the detector (Jamtting and Miles 2013).

The time dependence of the intensity fluctuation is most commonly analysed using a digital correlator. Such a device determines an intensity autocorrelation function that can be described as the ensemble average of the product of the signal with a delayed version of itself as a function of the delay time. The ‘signal’ in this case is the number of photons counted in one sampling interval. At short delay times, correlation is high and, over time as particles diffuse, correlation diminishes to zero and the exponential decay of the correlation function is characteristic of the diffusion coefficient of the particles. Data are typically collected over a delay range of 100 nanoseconds to several seconds depending upon the particle size and viscosity of the medium.

Analysis of the autocorrelation function in terms of particle size distribution is done by numerically fitting the data with calculations based on assumed distributions (Morrison et al. 1985) (Ruf 1993). A monodisperse sample would give rise to a single exponential decay, to which fitting a calculated particle size distribution is relatively straightforward. In practice, polydisperse samples give rise to a series of exponentials and several quite complex schemes have been devised for the fitting process.

DLS can provide accurate PSDs for samples that approximate a monodispersed system. The technique is fast and easy to use, and can measure particles with diameters in the range from approximately 1 nm to 5 μm, depending on particle density. The sample concentration is typically limited to $10^7$ – $10^{11}$ particles/mL. DLS requires only small sample volumes (typically less than 1 mL of suspension at ~0.1 % particle mass fraction). A range of suspendants can be used, keeping in mind that the viscosity and the refractive index of the suspension medium, as well as the temperature of the system, have to be known. Due to its ease of use and quick turnaround, DLS is commonly used in research and for quality control purposes. This technique is non-invasive and the samples can be fully recovered after analysis.

DLS measurements are sensitive to the quality of the suspension, as the intensity of the scattered light is proportional to the sixth power of the particle size. Thus the scattering signal due to the presence of dust or
agglomerated or aggregated particles will obscure the scattering signal from smaller particles. This also makes DLS less suitable for accurate measurements of broad PSDs. For low particle concentrations, there is a risk of number fluctuations due to a low number of particles in the measurement volume. If the particle concentration is too high, errors can occur due to multiple scattering events. For analysis, it is assumed that the particles are spherical and that the sample composition is homogenous. Information on particle shape can only be obtained in instruments equipped with multi-angle or goniometric detector configuration.

To ensure the best possible measurement results the DLS instrument performance should be checked regularly. The ISO standard (International Organization for Standardization 2008b) recommends that a verification procedure be performed by measuring 100 nm polystyrene latex spheres with a narrow size distribution. Intermittent checks should be performed as required using suitable reference materials.

**Laser diffraction**

Laser diffraction (LD) is a well-established ensemble technique that allows measurements of particle suspensions over a wide range of particle sizes from 100 nm up to several mm. The technique is easy to use and fast.

A collimated laser beam is passed through a sample suspension of particles and an array of detectors is located at different angles to the transmitted beam. The particles in the sample scatter the light at angles related to their size. The larger particles in the sample scatter light with strong intensity at small angles and smaller particles scatter light with lower intensity at larger angles. Assuming that the particles are spherical, and that the refractive index (both real and imaginary) of the particles and the optical properties of the suspendant are known, Mie theory can be used to convert the scattering pattern from all sizes of particles into a volume-weighted PSD. For large particles (diameter $\gtrsim 50 \mu m$) the Fraunhofer approximation can be used, which assumes that the particles are opaque, that particles of all sizes scatter light with the same efficiency and that the particle size is much larger than the wavelength of the light (Bohren and Huffman 1983). Particles with diameters much less than the laser wavelength will scatter light uniformly in all directions and LD systems are often equipped with a backscattering detector to capture the signal from the smaller particles.

The detection range of the LD technique excludes size measurements of very small particles but it can be a very useful tool when assessing complex particle systems containing agglomerates and/or aggregates. In most cases it is possible to recover the samples after analysis.

LD measurements require the sample concentration to be optimized to avoid multiple scattering effects or particle-particle interactions. Since LD is an ensemble technique, the sample is assumed to be of homogenous composition. One of the requirements when applying Mie theory to model the results is that the optical properties of the particles and of the suspension medium must be known, and that assumptions about the particle shape have to be incorporated into the model. The technique is limited in its ability to discriminate between different particle populations with closely spaced mean diameters.

To ensure the best possible measurement results the instrument performance should be checked regularly. The ISO standard (International Organization for Standardization 2009) recommends performing measurements on traceable, spherical CRMs to ensure accuracy.
Small Angle X-ray Scattering

Small Angle X-ray Scattering (SAXS) is a method which measures the elastically scattered intensity of X-rays for small-angle deflections. The angular scattering is usually measured within the range 0.1° to 10°, providing structural information on macromolecules as well as periodicity on length scales typically larger than 5 nm and less than 200 nm for ordered or partially ordered systems.

The technique is non-destructive for most materials and does not require complex sample preparation. The instruments use a collimated, monochromatic X-ray beam, and the sample is rotated through a range of angles generating a scan of scattering intensities versus angle. The resulting scattering signal is modelled and the results can provide specific information about the sample, such as the radius of gyration, particle shape, and size and shape distributions. By using advanced data analysis software, it is possible to resolve complex multimodal size distributions.

Traditional SAXS instruments are very expensive and even more so when considering the synchrotron sources that are now providing SAXS beam lines. Measurements and data analysis can be very complex. Sample preparation is important as this is a scattering technique and the presence of large particles or aggregates may suppress the signal from smaller particles. Both the experiments and the analysis can be time consuming.

The ISO standard (International Organization for Standardization 2001c) recommends verifying instrument performance at regular intervals using certified reference materials or particles with a known size distribution. There are nanoparticle reference materials available that can be used for instrument verification.

Particle tracking analysis

Particle Tracking Analysis (PTA) is a method where a laser illuminates particles undergoing Brownian motion in a liquid suspension and the change in position of individual particles is used to determine particle size.

The instrument dynamically tracks individual particle positions in real time, and records the resulting length of the particle track as well as the scattered intensity. The particle size is derived from an analysis of the track length and time by determining the diffusion coefficient, which can then be related to the hydrodynamic diameter via the Einstein-Stokes equation (International Organization for Standardization 2008b).

The detectable size range is ~20 nm–1000 nm, the absolute limits of which depend on the scattering properties of the particles, their size, and the instrument configuration. A PSD measured using PTA is a number-weighted distribution of the hydrodynamic diameters. Different laser wavelengths allow studies of small-sized particles and/or fluorescent particles.

As the particle size measurement is based on the tracking of individual particles, the results are not greatly affected by size-dependent scattering intensity. It is possible to resolve particle sizes in multimodal mixes to a moderate degree. The technique can be used for in situ studies of particle aggregation (Montes-Burgos et al. 2010). The technique is suitable for use with a range of suspending agents, although the viscosity and temperature of the liquid must be known. Being a single-particle measuring technique, PTA can be used to determine particle number concentration.
The PTA analysis model for hydrodynamic diameter assumes that the particles are spherical. The suspendant needs to be optically transparent and the viscosity and measurement temperature must be known. The particle motion is tracked in a two-dimensional focal plane, and an approximation is made to fit the diffusion behaviour for a particle undergoing three-dimensional Brownian motion. Only a limited number of particles are analysed, providing only limited statistical relevance. Even if analysis is carried out for extended periods of time to generate more data, there is a chance that the same particles will be analysed repeatedly. The data analysis is susceptible to user interpretation and requires a high level of understanding to interpret correctly.

PTA is a newly developed technique and thus no standards for this type of instrument currently exist. However, there is a range of reference materials available of different composition that can be used for instrument verification.

- Separation

**Differential Centrifugal Sedimentation**

Differential Centrifugal Sedimentation (DCS) is a method in which a sample is separated based on size and density using a rotating disc filled with a fluid containing a density gradient. The instrument consists of a hollow spinning disk into the centre of which a sample is injected. The disk is mounted on a drive shaft that rotates at a known speed. The particles sediment radially from the centre of the disk through the fluid after injection. A detector beam (usually a laser beam) passes through the liquid near the outside edge of the disk, and as the particles pass through the beam, the intensity is reduced as the particles obscure the beam proportionally to the concentration and particle size. The measurand is the sedimentation time, which can be converted to diameter using Stokes’ Law and to a volume-weighted PSD using Mie theory, if the optical properties of the particles are known (Jamting and Miles 2013).

The disk centrifuge is capable of measuring a wide range of particle sizes, from 5 nm to 30 μm, depending on the rotation speed and the density of the particles and density gradient fluid. It is possible to achieve very good size discrimination, as illustrated in Figure 3.3, which shows the results from a typical DCS measurement of a 6-modal gold nanoparticle suspension, with nominal particle diameters ranging from 5 nm to 50 nm.
Appendix 1 - Figure 3: Volume-weighted PSD of a 6 modal mix of Au nanoparticles, measured by DCS. The plot illustrates the ability of the technique to clearly separate each of the particle populations in the 6 modal Au suspension (nominal diameters: 5 nm, 10 nm, 20 nm, 30 nm, 40 nm and 50 nm) (Jamtling and Miles 2013).

Typical gradients can be created using sucrose and water, which can easily be adjusted to change the density of the gradient. Other suspendants, such as oil or cell culture media can be used to create the gradient, so particles can be measured in an environment representative of typical applications such as cosmetics or toxicological studies.

The requirement that the particles have a higher density than the gradient is the most limiting factor of the technique. The DCS analysis for the Stokes diameter assumes that the particles are spherical and that the sample is of homogeneous composition. The densities and optical properties of both the particle material and the gradient fluid have to be known. Only dilute samples can be measured. Low-density particles require a low-density gradient and high rotational speed. The measurement duration may be long for complex samples and it may not be possible to keep measurement conditions constant during the experiment.

The sedimentation velocity can be calibrated directly preceding each measurement run using reference particles of known diameter and density. A range of reference materials of different composition is available, though currently there is a lack of these materials for such calibration. Several ISO standards provide information about best practices in DCS measurements (International Organization for Standardization 2001a; International Organization for Standardization 2004a; International Organization for Standardization 2007a).
Field Flow Fractionation

Field Flow Fractionation (FFF) is a separation technique where a field is applied to a liquid suspension passing along a narrow channel in order to separate the particles present in the liquid, depending on their differing mobility under the force exerted by the field. The field can be, for example, gravitational, centrifugal, a liquid flow, electrical or magnetic. Using a suitable detector after or during separation allows determination of the size and size distribution of nano-objects.

Once the external field is applied, the particles in the suspension are forced into a narrow layer along one wall of the flow channel. The particles in this compressed layer interact with both the axial channel flow and the external field, and separation occurs. This depends on the particle size, density, diffusion coefficient or thermal diffusion coefficient, and on which type of field is applied.

The mode of fractionation depends on the size of the particles in the suspension, as shown in Figure 3.4a. For small particles undergoing Brownian motion, the particle diffusion coefficient determines the elution sequence. The smaller particles diffuse at a higher rate and are eluted first, followed by the larger-sized particle fractions.

Appendix 1 - Figure 4: Schematic diagram illustrating the typical operation of FFF, showing the mechanism of separation for particles of different size. a) shows the separation sequence based on particle diffusion coefficients, b) shows particle separation in steric mode and c) illustrates the particle separation in hyperlayer mode (Jamting and Miles 2013).
For larger particle systems with diameters above ~1 μm a different principle occurs, often denoted by the steric mode (see Figure 3.4b). In this mode the particle sizes are large enough to no longer interact with the channel wall by diffusion but instead are forced by the external field into a thin layer close to the opposite channel wall. The parabolic channel flow now interacts more directly with the particles, and the elution sequence is reversed: larger particles elute earlier than smaller particles.

Another wall interaction mode is also possible, called the hyperlayer mode, where the particles are subjected to flow-induced hydrodynamic forces and form thin layers some distance away from the wall (see Figure 3.4c).

The dynamic range of FFF is very broad allowing particle sizes from nanometres up to several micrometres to be measured. FFF is capable of continuously separating out PSDs with diameters spanning the range from 1 to 1000 nm. The particle size discrimination is very good; narrow peaks in the PSD with as little as 5% difference in mean diameter can be resolved. The technique can be used stand-alone or integrated with auxiliary methods for further characterization. It is possible to collect the eluted fractions for further analysis, for example by AFM or EM.

The technique requires extensive method development. Without external detection systems, the algorithms for determining the size separation are very complicated. Some of the FFF techniques such as Sedimentation Field Flow Fractionation, may require very long experimental run times to separate PSDs of very small particles or broad distributions (Jamting and Miles 2013).

- **Aerosol Characterisation**

The characterisation of nanoparticles in an aerosol is not particularly relevant to nanomaterials in pesticides or veterinary medicine so this field of characterisation will only be briefly addressed.

**Condensation Particle Counter**

A Condensation Particle Counter (CPC) is an instrument that measures the particle number concentration of an aerosol. Aerosol particles in the nanoscale range are grown by condensation to a size of 10-12 μm allowing easy detection and counting using laser scattering, normally by counting individual pulses of scattered light.

**Differential Electrical Mobility Classifier**

A Differential Electrical Mobility Classifier (DEMC) is able to select aerosol particles according to their electrical mobility and pass them to its exit. This is accomplished by balancing the electrical force on each aerosol particle with its aerodynamic drag force in an electrical field. Classified particles are in a narrow range of electrical mobility determined by the operating conditions and physical dimensions of the DEMC, while they can have different sizes due to differences in the number of charges that they have.

**Differential Mobility Analysing System**

A Differential Mobility Analysing System (DMAS) is a system used to measure the size distribution of submicrometre aerosol particles consisting of a DEMC interfaced with a detection and analysis system. The DEMC transmits particles within a narrow size range and a detector, often a CPC, counts the number of particles within that differential size interval.
• Surface Area Measurement

Brunauer, Emmet, Teller method

The Brunauer, Emmet, Teller method (Brunauer et al. 1938) determines the total specific external and internal surface area of disperse powders and/or porous solids by measuring the amount of physically adsorbed gas, using the model developed by Brunauer, Emmet and Teller for interpreting gas adsorption isotherms. The method is only available for dry powders.
# ABBREVIATIONS

[This list should be modified to include all the acronyms and abbreviations that actually appear in the publication.]

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A glossary explains technical and unfamiliar terms—but not shortened forms—used in the publication.

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