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CELL PEN: A study to identify the physico-chemical factors controlling the capacity of nanoparticles to penetrate cells

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EXECUTIVE SUMMARY

One of the major concerns regarding the possible toxic effects of nano-particles is the capacity of these materials to penetrate cells and potentially translocate to other cells, tissues and organs remote from the portal of entry to the body. This is considered to be a necessary step in the movement of particles deposited in the lung, entering the blood, acting upon cells in other tissues, manifesting ultimately in a physiological response. Research on the mechanisms involved in nanoparticles penetrating cells and translocating across the respiratory epithelium is needed.

The objectives of the Cell Pen project were to scope the research required into the mechanisms of translocation across the respiratory epithelium, and the resulting possible toxic effects in and beyond the lung, and advise on the feasibility of achieving the following outcomes:

1. Identifying which features of nano-particles/tubes/fibres are important in particle-cell interactions, considering the potential role of nanoparticle (NP) chemistry, structure, mass, numbers, shape, surface area, surface charge and surface functionalisation;
2. Suggesting how NPs may be modified to enhance or reduce their capacity to enter cells;
3. Suggesting how interactions between NPs and cultured human cells might be studied.

The Institute of Occupational Medicine together with a team of multi-disciplinary experts undertook a review of the available literature, hosted a workshop to discuss the findings, and now present an informed commentary on a prospective research agenda towards elucidating the importance of translocation in nanoparticle toxicology. The project focussed on the target sites of concern for particles that are translocated from their site of deposition on the lung surface, or airspaces, namely: 1) pulmonary interstitium; 2) other lung cells; 3) blood; 4) blood vessel wall; 5) placenta/foetus; and 6) brain.

The ability to enter the interstitium from the airspaces seems to be a fundamental property of nanoparticles. However, the exact role that interstitialisation has in human toxicity is not yet well understood. The blood and the cardiovascular system are significant potential targets for adverse effects of engineered nanoparticles. Combustion-derived nanoparticles appear to be able to enhance atherosclerosis and the possibility that this could be a general property of nanoparticles should be examined. This requires animal studies and could involve susceptible animals such as Apo E mice, which develop atherosclerotic plaques. Size and chemical structure are likely to be important and might act through the effect that they have on lung inflammation and oxidative stress. In the case of translocation to the blood and direct interaction with plaques, size is likely to be important. There are currently no good *in vitro* models for atherosclerosis. There is a lack of data on NP translocation to the placenta, which is a particular concern given the consequences of potential adverse effects on the developing foetus. This information gap should be filled as a matter of urgency. Translocation from the upper respiratory tract or blood to the brain is a concern as several types of nanoparticles have been found to translocate to the brain, including carbon, TiO_2 and manganese. More research is needed, in particular, the role of neural transfer as a pathway versus transfer from the blood needs to be addressed. Size is an important driver of these effects but factors that drive blood access may also affect brain transfer. Brain endothelial cells are used to model the blood brain barrier and measurement of transit across these could help to understand

how particles cross the blood-brain barrier. A particle's ability to enter the blood stream is a pre-requisite factor to be considered, in addition to the role other factors such as size and charge may have in influencing any potential toxicity to the placenta and foetus. The situation here is analogous to both transfer to blood from the lungs and transfer to brain across the blood brain barrier, as the placenta is a special case of an endothelial surface across which nanoparticles would need to pass to enter the foetal circulation.

Programmes of research on the relationships between size, composition etc on interstitialisation are needed. The role of nanoparticle structural features, effects of modifications to the structure and the ability to produce inflammation are likely to be important factors in rendering a particle able to enter the interstitium. Development of cellular models is urgently needed but these will be difficult to validate, given the lack of understanding of the events that occur *in vivo*. Research is needed on the ability of particles to cross the placenta and blood brain barrier. In particular, the role of reactive surfaces in causing inflammation means that surface reactivity might be important and should be the target for research. Studies with nerve cells in culture, measuring transit of particles along them, may illuminate the factors that dictate neural transfer.

Underpinning all of the above is a need to examine the role of size within the nanoparticle size range, the requirement to label nanoparticles (fluorescent, radioactive etc) to identify small amounts of translocation, a need to examine the role of surface reactivity /composition on toxicokinetic processes; and a need to develop cell-based assays.

1 INTRODUCTION

Several pathways of nanoparticle uptake into the human body have been identified, with the inhalation pathway of greatest interest due to this pathway presenting a significant route of exposure and routes of access to the cardiovascular and central nervous systems. Following inhalation (or ingestion), nanoparticles must cross cellular barriers to enter the body further. Understanding the specific processes and physico-chemical factors controlling the ability of nanoparticles to cross barriers, in particular epithelial cells, will help inform knowledge of their intracellular fate as well as the potential for distribution of nanoparticles around the body.

This review examines the peer-reviewed literature on translocation and seeks to identify the physico-chemical properties of materials (e.g. size, specific surface area, charge, surface chemistry etc) and evidence of how they influence particle migration through and between cells in various tissue types. Considered first is translocation from the surface of the lung to the interstitium, and subsequently from the airspace to the blood, endothelium, vessel wall and to atherosclerotic plaques. Subsequent parts of the review consider the available evidence for translocation to the placenta / foetus and the brain.

The respiratory system can be divided into upper (nasal cavity, pharynx and larynx) and lower (trachea, primary bronchi and alveoli) sections. Each section of the respiratory system is lined by a barrier of epithelial cells, whose structure and function differ between parts of the pulmonary system. For example, in the upper airways epithelial cells are ciliated and covered in a thick sticky mucus. These cilia continually beat in order to move mucus from the deep lung, up and out of the respiratory system (mucociliary escalator), to be blown from the nose or to be swallowed into the stomach. In contrast, the epithelium of the alveolar regions is not ciliated, nor is it covered in mucus. These epithelial cells form a confluent barrier in healthy tissues and control the movement of substances in both directions across the epithelium. These epithelial cells are held together by a series of tight junctions which regulate the passage of substances between cells, termed paracellular transport. Damage to the epithelium by toxicants or disease can lead to an increase in tight junction permeability and therefore toxicant absorption into the body. Toxicants may also be taken up directly into the cell, and then pass through the epithelial cell into the interstitium or cardiovascular system. This is known as the transcellular route of absorption.

The upper section of the respiratory system presents a potential route of entry to the central nervous system via transport along nerves. In addition, if inhaled nanoparticles cross the lung epithelium and become bloodborne, they may have the potential to gain access to the blood-brain barrier (BBB). The BBB is composed of endothelial cells which are also held together by strong tight junctions and adherens junctions, forming both an anatomical and a physiological barrier that controls the movement of substances into and out of the nervous system. The tight junctions of the BBB are also highly regulated, forming a tight seal so that transport via the transcellular route is very limited. In addition to tight junctions acting to prevent transport in between endothelial cells, there are two mechanisms that act to prevent passive diffusion through the blood-brain barrier: i) a lipid barrier of glial cells called astrocytes surround approximately 85% of the surface of the capillaries, and ii) the low concentration of interstitial proteins in the brain preventing access by hydrophilic molecules. Under normal conditions, exchanges occur through the capillary cells via the paracellular route. Low molecular weight lipid soluble substances (e.g. alcohol) penetrate these cells easily by dissolving in their lipid plasma membrane. All other substances, exchanged between the blood and the brain interstitial fluid, including essential materials such as glucose, amino

acids, and ions are actively transported by highly selective membrane bound carriers. Some molecules that freely enter brain endothelial cells through the luminal membrane undergo rapid metabolic chemical transformations that inhibit them from crossing the antiluminal membrane and reaching the surrounding brain interstitium. In the case of inflammatory diseases, the integrity of the BBB is affected as an indirect consequence of the immune response, which leads to the release of cytokines, chemokines, cellular adhesion molecules, and matrix metalloproteases at the site of inflammation. These molecules alter the structure and function of the BBB and are therefore important potential factors influencing the potential for nanoparticle translocation across the BBB and their interaction with nerve cells. A review of the toxicology and pharmacology literature pertaining to translocation to the brain via the BBB has been undertaken.

1.1 AN OVERVIEW OF THE MECHANISMS OF INTERNALISATION

In order for a substance to enter a cell's interior, it must pass through the diffuse layer surrounding the cell and the plasma membrane which segregates the internal and external environments of a cell and regulates the entry and exit of substances into and out of the cell. The uptake of substances is accomplished via a variety of processes that can be described as active (energy requiring) or passive. There are a number of clearly defined mechanisms for crossing the plasma membrane that include:

(i) diffusion

(ii) facilitated diffusion

(iii) active transport

(iv) endocytosis

(i) Diffusion: The plasma membrane of cells is a lipophyllic environment that results in selective permeability to low molecular weight lipophillic substances by passive diffusion (e.g. alcohol). Such diffusion is usually driven by an electrochemical or concentration gradient. In contrast, hydrophilic or high molecular weight substances must gain access via an alternative route such as channels, transporter proteins or endocytosis, all of which are active (energy requiring) processes.

The immediate extracellular surface of the plasma membrane is negatively charged due to the negative charge of the phospholipid heads, therefore, the surface of the membrane could influence the way in which charged particles interact with the cell membrane. However, superimposed on this is an overall intracellular negative charge, caused by unequal distribution of ions across the plasma membrane driven by the activity of ion channels and pumps. The negative intracellular charge primarily arises due to the action of the sodium / potassium (Na/K ATPase) pump which transports 2 potassium ions into the cell, for every 3 sodium ions pumped out. The activity of other channels and ions also contribute, but to a lesser extent to the overall cell membrane potential. In fact, the cell uses this negative electrical (and chemical) membrane potential to drive the transport of substances, often against a concentration gradient, into or out of the cell. For example, charge influences the uptake of cationic molecules, which are strongly attracted to the cell surface due to the non-specific electrostatic interactions that occur with the negative charge of the plasma membrane interior (Patel et al. 2007), although it is generally accepted that charged molecules cannot pass through the plasma membrane by diffusion. Therefore, this membrane potential could provide a driving force to promote the movement of positively charged particles into the cell. Whether nanoparticles can move into cells via diffusion remains unclear and

much more evidence is required before it is widely accepted that diffusion is a viable uptake route for nanoparticles into or across cells.

(ii) Facilitated diffusion: Another form of passive transport is facilitated diffusion, which allows substances to pass from an area of high to low concentration through selective membrane protein channels, no metabolic energy is required as cell entry is driven by the concentration gradient. The carrier proteins and channels are open or closed depending on the requirements of the cell; for example ligand and voltage gated ion channels are evident. As the entry of substances will be limited by the number of carriers present within the plasma membrane, the process can be described as saturable (King 1996). Movement of nanoparticles into the cell via such means would require the particles to be small enough to move through naturally occurring channels. The pore size of such channels, is often in the region of 10-30 nm suggesting that this may be possible route of passage for some nanoparticles. The effects of NP in modulating ion exchange through ion transporters and in carrying charge into cells to affect cellular electro-physiology should be addressed.

(iii) Active transport: In active transport, substances are moved across the membrane against a concentration gradient, thus energy, in the form of ATP, is required. Active transport is conducted by specific transport proteins located within the membrane. The structures of such transporters are very specific for the molecules that they can actively transport. It may be possible for nanoparticles to use this pathway as a route of entry into the cell if their structure physically and chemically resembled that of a transported molecule.

(iv) Endocytosis: Larger molecules can also enter cells via endocytosis, another example of an active process of uptake. Endocytosis is a collective term that describes the energy-dependent internalisation of substances, and is characterised by vesicle formation, that enables the carriage of the cargo into the cell interior (Johannes et al. 2002; Patel et al. 2007). A number of distinct endocytic pathways have been identified for substances to enter a cell (Medina-Kauwe 2007). These multiple endocytic routes all originate at the plasma membrane and include phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (Conner & Schmid 2003). The size of the vesicles formed is different for each pathway, for example clathrin coated pits are approximately 120 nm in diameter, caveolae generally 50-80 nm and micropinosomes 1 to 5 µm (Patel et al. 2007). Although the size of the vesicles is not definitive and there are exceptions to this general statement made, it is thought that the size limits that exist act to restrict the size of the cargo that is internalised, thus introducing a form of selectivity to the process (Patel et al. 2007). Such pathways therefore form a likely route of uptake of nanoparticles, and even nanoparticle aggregates, into cells.

2 OBJECTIVES

The objectives of this project, as specified by Defra, were to scope the existing and required research into the mechanisms of translocation across the respiratory epithelium and the resulting possible toxic effects in and beyond the lung, and advise on the feasibility of achieving the following outcomes:

1. Identifying which features of nanoparticles are important in particle-cell interactions, considering the potential role of surface chemistry, structure, mass, numbers, shape, surface area, surface charge and surface functionalisation;
2. Suggesting how nanoparticles may be modified to enhance or reduce their capacity to enter cells;
3. Suggesting how interactions between nanoparticles and cultured human cells might be studied.

3 METHODS AND PLAN OF WORK

An extensive review of the issues identified above was performed. This was achieved through a comprehensive survey of published, web based and grey literature, including conference monographs, reports and the internet, to examine the state of the science.

In the first stage of the project, information was collected from the public domain and the grey literature on translocation of nanoparticles using hosts such as Dialog-Datastar, STN, Web of Science, and PubMed amongst others. The collated information was assessed to determine its scientific quality and relevance to the objectives of the review.

The review process was structured according to the target sites of concern for particles that may translocate from their site of deposition on the lung surface, or airspaces, to: 1) pulmonary interstitium; 2) other lung cells; 3) blood; 4) blood vessel wall; 5) placenta/foetus; and 6) brain.

In the course of reviewing the literature, consideration was given to the quality of the evidence, the potential for different interpretations of the evidence, key assumptions underpinning the evidence, levels of certainty in the evidence, what is known or is currently under investigation, research gaps and identifying future priorities.

Presentation and discussion of the principal findings at a facilitated workshop provided additional input towards achieving a consensus on the project's conclusions and recommendations. Stakeholders included key members of the toxicology, regulatory and other appropriate communities. A number of participants from the US and EU were invited to attend to reflect the international dimension of this activity. The workshop was informed by draft review document which was provided to all participants prior to the workshop. The workshop was structured as follows:

- Facilitators introduced the workshop aims, objectives and protocol;
- An overview of the key components of the study was presented;
- Chaired round table discussion, with break out groups were held;
- Discussion of recommendations / comments contributed to finalising the report.

The workshop was held at the Central Science Laboratory in Yorkshire. A record of the discussion was made and used to inform the finalisation of the project report.

The component reviews and input from the workshop have been collated into an overall review document. This document provides an overview of the current state of the knowledge concerning translocation of nanoparticles. Recommendations were developed on the areas in which further research is needed to understand the influence of physicochemical features on particle translocation.

4 REVIEW OF THE EVIDENCE

Presentation of the evidence review is structured according to the target sites of concern for particles that may translocate from their site of deposition on the lung surface, or airspaces, to: 1) pulmonary interstitium; 2) other lung cells; 3) blood; 4) blood vessel wall; 5) placenta/foetus; and 6) brain.

4.1 PULMONARY INTERSTITIUM

Although the lung consists of many cell types, the epithelial and macrophage cells are the focus of this section due to their importance in controlling the entry of substances into the body via the lung.

Lung epithelial cells provide a selectively permeable barrier to the exchange of gas or other molecules between the lumen and the underlying tissue. Epithelial cells in the airways and in the centriacinar region of the acinar units receive the highest deposited dose and therefore at a higher risk of exhibiting a toxic response to toxic dusts and the products of phagocytic cells. Epithelial hyperplasia, metaplasia and neoplasia occur in response to various inhaled dusts and it has been deduced that epithelial cells give rise to the tumours seen in some particle exposure studies (Levy 1996; Nikula et al. 1995).

A number of studies have demonstrated ability for nanoparticles to cross the lung epithelium. Probably the first study to do so was published by Oberdorster's group and demonstrated that following instillation or inhalation of TiO_2, nanoparticles (25 nm) to a greater extent than fine particles (250 nm), could be found within the lung interstitium (Ferin et al. 1990b; Ferin et al. 1992; Ferin et al. 1990a). The paper published by Ferin et al. 1992 identified that 'particles not phagocytosed by alveolar macrophages in the alveoli were taken up by alveolar type I epithelial cells, which was probably the first step for interstitial access of particles'. This interstitialisation and reduced clearance was associated with an elevated lung inflammatory response. It is worth noting that the exposure concentrations used in this study were very high (500 μg instilled and up to 5 mg/rat lung burden following inhalation), however a number of *in vivo* studies conducted by different groups using different particles have also identified similar results. For example, using electron microscopy (EM), it has been demonstrated that carbon black particles instilled into the mouse lung were accumulated in gaps between the cytoplasmic processes of alveolar epithelial cells, allowing transfer across the epithelial barrier into blood (Shimadu et al. 2006).

In a recent study exposing rats to iridium-192 nanoparticles (Semmler-Behnke et al. 2007), it was suggested that nanoparticles are much less actively phagocytosed by alveolar macrophages than larger particles, but that they are effectively removed from the lung epithelium into the interstitium. This is at odds with *in vitro* studies which suggest that nanoparticles are taken up by macrophages (Clift et al., manuscript submitted), although it has been suggested that nanoparticles decrease the subsequent phagocytosis of micron sized particles (Renwick et al. 2001).

In an inhalation study using TiO_2 particles (4-22 nm; 1 hour and 24 hour; deposited dose estimated as 4-5 μg/rat) (Geiser et al. 2005), electron microscopy revealed TiO_2 within many of the lung cell types (endothelial, epithelial, fibroblasts and within connective tissue). Within cells, they were localised in the cytoplasm, and rarely within the nucleus. This clearly demonstrates that exposure to low concentrations of TiO_2 nanoparticles via inhalation, results in rapid penetration of particles across the epithelial barrier of the lung. On the basis of the EM images used to detect the TiO_2, the authors suggested that for intracellular particles, there was no clear plasma membrane around

the nanoparticles, from which it was concluded that uptake must be via a non-endocytic pathway such as diffusion. However, much more evidence is required before it is widely accepted that diffusion is a viable uptake route for nanoparticles into or across cells.

When the alveolar macrophages' ability to phagocytose becomes impaired, the integrity of the epithelium can also become compromised. Unphagocytosed particles interact with epithelial cells leading to necrosis or apoptosis (Iyer et al. 1996) as well as activation leading to the release of pro-inflammatory cytokines (Driscoll et al. 1995 and 1996; Finkelstein et al. 1997). Necrotic type I cells are replaced by the division and differentiation of Type II cells to type I cells, however, this is not instantaneous (Evans et al. 1975). Therefore, on exposure to high particle concentrations, used in many toxicology studies, lung overload is induced resulting in compromised epithelial cells, Type I cell hyperplasia and inflammation. The continuing presence of non-phagocytosed particles on the injured, activated and partially denuded epithelial surface will enhance their likelihood of being transferred to the interstitium (i.e. an increase in the rate of dust translocation to the interstitium). As these particles become interstitialised, they are likely to interact with interstitial macrophages which reside in close contact with fibroblasts, epithelial and endothelial cells (Adamson & Hedgecock 1995). The proximity of the macrophages to these cells means that any mediators released by the interstitial macrophage can have a detrimental impact on the interstitial basement membrane and other interstitial cells leading to interstitial fibrosis. These interstitialised particles, either free or phagocytosed, may eventually be transported to the lymph nodes (Lehnert 1993). Current evidence suggests that the transportation of inhaled particles to the lymph nodes is also size dependent (Oberdorster et al. 1988). The increase in the rate of dust interstitialisation is reflected in an increase in the rate of dust translocation to the lymph nodes.

It is anticipated that modifying the specific surface area and reactivity of the particles, such as their ability to cause inflammation, would have effects on their ability to become interstitialised. That is, the more inflammogenic the particles, the more they are likely to interstitialise. An inhalation study using differently sized poorly soluble particles, TiO_2 and $BaSO_4$ (Tran et al. 1999) demonstrated a linear relationship between the particle burden expressed in units of surface area and the number of neutrophil cells (indicative of inflammation) found in the bronchoalveolar lavage (BAL) fluid, suggesting that high specific surface area of particles enhances inflammation (Duffin et al. 2007; Stoeger et al. 2006) leading to interstitialisation. Since surface area is inversely related to size it could be hypothesised that increased surface area results in increased uptake into cells and penetration of cell barriers, but it is not possible at this time to assess whether such an observation is size or surface area driven. To date, there are no reports of studies using a range of nanoparticle sizes (e.g. 100 nm vs 50 nm vs 10 nm) to investigate this hypothesis. However, the literature on the effects of derivatising (i.e. adding ligands or pendant molecular groups) the surface of carbon-based nanoparticles (C_{60} and CNT) suggests that changing the surface changes the toxicity (Sayes et al. 2004; Sayes et al. 2006) and this would be expected to impact on inflammation and therefore interstitialisation. However, care must be taken in extrapolating between *in vitro* and *in vivo* findings (Sayes et al. 2007).

Nanoparticles may also translocate to the interstitium in the absence of inflammation. A study involving a 1 hour duration inhalation exposure of rats to Palas-generated TiO_2 reported translocation to the interstitium (Muhlfeld et al. 2007). Although not recorded, it is highly unlikely that there was inflammation after such a short exposure. However, in one study mild inflammation was detected after a low exposure, for 7 hours, to

carbon black nanoparticles suggesting that low exposure to low toxicity, low solubility nanoparticles such as carbon can be inflammogenic (Gilmour et al. 2004).

High aspect ratio particles (e.g. asbestos fibres) are well-known to translocate from the airspaces to the pleura and the peritoneal cavity and this raises questions about high aspect ratio nanoparticles. If size and shape determine the possibility of this translocation then the obvious inference is that high aspect ratio nanoparticles would be likely to translocate to the pleura and peritoneal cavity. However, no data are currently available to confirm whether that is the case. Studies with asbestos and mineral fibres have shown that longer fibre length is associated with greater inflammogenicity (Davis et al. 1986; Donaldson et al. 1989; Moalli et al. 1987; Ye et al. 1999) and evidence is emerging that certain multi-walled carbon nanotubes exhibit length dependent inflammogenic/pathogenic effects (Poland et al. 2008).

4.2 EPITHELIAL CELL STUDIES

It is known that size plays a role in determining the route of cellular uptake of liposomes, viruses and polymer nanoparticles (Chithrani et al. 2007). The size of many nanoparticles is similar to biological macromolecules such as proteins and DNA, as well as biological structures such as bacteria and viruses, all of which are readily taken up by cells, including lung cells. The studies described below consist of a range of epithelial cell types, including the lung, since collectively these are likely to be relevant to informing how nanoparticles interact with epithelial cells in general. Specific studies will be required in the future to assess whether all of these findings are relevant to the lung.

Vesicular transport exists in lung epithelial cells, since the existence of large numbers of membrane vesicles within the type I alveolar epithelial cells has been recognised for many years (reviewed in Gumbleton 2001). However the function of these vesicles remains unclear. It has been hypothesised that vesicular transport may provide a route of transport for solutes from the alveolar to interstitial surfaces of the epithelial cells. Recovery of the lung from oedema requires the removal of protein across the alveolar epithelium, and this is believed to occur via endocytic transcytosis routes as well, as via paracellular routes (Hastings et al. 2004). Endocytosis is the more important mechanism at low protein concentrations, but the paracellular route becomes increasingly important on exposure to high protein concentrations. During oedema or acute lung injury, it is suggested that transcellular routes become more active in order to clear protein from the lung surface (Hastings et al. 2004). Therefore, it is feasible that nanoparticles could cross this barrier by both transcellular and paracellular routes, especially in a diseased lung.

It is possible to introduce surface attachments that encourage the uptake of NPs so that they can be targeted for uptake into any cell type, including lung epithelial cells, via clathrin-mediated endocytosis. For example, a lectin coating on particles facilitates interaction with the intestinal Caco-2 epithelial cell line and thereby promotes uptake *in vitro* (Russell-Jones et al. 1999). It has also been discovered that several lectins were actively taken up into primary lung epithelial cells when applied to the apical surface *in vitro* (Yi et al. 2001).

After internalisation, the endocytic vesicle delivers its contents to an early endosome (also known as a sorting endosome) (van der Goot et al. 2006). The endocytic vesicles fuse with an early endosome, which requires the presence of EEA-1 (involved in vesicle fusion) and Rab5 (involved in movement of endosomes along the cytoskeleton) on the early endosome membrane. These proteins allow the early endosomes to

redistribute their contents to other intracellular locations (Liu & Shapiro 2003). For this reason early endosomes are often called sorting endosomes (Gruenberg 2001). The early endosome dictates the sub-cellular distribution of the internalised cargo (Patel et al. 2007), so that the vesicle is targeted to its appropriate sub-cellular destination. Such destinations include (i) delivery to late endosomes and lysosomes which subsequently target the substance for degradation, (ii) if the cargo is required by the cell it may be delivered to the endoplasmic reticulum (ER) or Golgi (van der Goot et al. 2006), or (iii) the substance may be redirected out of the cell by exocytosis (Medina-Kauwe 2007).

There are a number of studies, although mainly unpublished, which are currently investigating the localisation of nanoparticles within cells such as macrophages. Early results from Napier University suggest that carboxylated quantum dots and carboxylated polystyrene beads enter macrophages rapidly, entering endosomal, lysosomal and mitochondrial compartments (Clift et al., manuscript in preparation).

A number of studies have demonstrated that nanoparticles can be exocytosed from cells. In a study with human vascular smooth muscle cells, nanoparticles containing bovine serum albumin and 6-coumarin as a fluorescent marker were taken up by a concentration, time and energy dependent process (Panyam et al. 2003). The same study also identified that inclusion of protein in the medium (often added to aid particle dispersion) results in protein uptake along with the nanoparticles, which then interacts with the exocytosis pathways stimulating greater exocytosis of the nanoparticles. Exocytosis of nanoparticles from lung epithelial cells or macrophages was not reported in any identified publications.

Phagocytotic uptake is thought to be restricted to specialised cells such as macrophages and neutrophils (Conner & Schmid 2003), and is generally responsible for the uptake of large materials (greater than 0.5 µm) such as bacteria or cell debris (Khalil et al. 2006). In the lung this is especially important as inhaled air provides a route of delivery of foreign particles and microorganisms into the body, therefore macrophages are essential for maintaining a clear, sterile and functioning respiratory surface. During phagocytosis, the cell recognises ligands via cell surface receptors. Receptor binding then triggers the polymerisation and rearrangement of the actin cytoskeleton to form membrane extensions, so that the plasma membrane surrounds the material to be internalised (Liu & Shapiro 2003; Perret et al. 2005; Khalil et al. 2006). The phagosome that is formed then fuses with lysosomes, so that the cargo can be degraded (Perret et al. 2005).

The movement of the vesicles within any cell type requires the contribution of intact microtubules (Medina-Kauwe 2007). Treatment of macrophages *in vitro* with nanoparticle carbon black (and diesel particles), resulted in increased cytoskeletal stiffness and decreased trafficking of endocytic vesicles (Moller et al. 2005). Addition of an antioxidant provided some protection suggesting that these carbon nanoparticles can directly or indirectly interact with this pathway via a mechanism involving oxidative stress.

It has been demonstrated that a number of different entry mechanisms have been proposed to explain the uptake of nanoparticles by a variety of cell types. However it is also worth acknowledging that different types of endocytosis can operate simultaneously (Rejman et al. 2004), so that more than one type of internalisation pathway could contribute to their uptake and that if one uptake pathway does not function, another can take over.

The uptake of biodegradable nano and microparticles of polylactic polyclycolic acid co-polymer containing bovine serum albumin and 6-courmin as a fluorescent marker has

been investigated (Desai et al. 1997). This study focused on uptake by Caco-2 (intestinal) epithelial cells, and the particles used were 0.1 μm, 1 μm and 10 μm in diameter. Uptake was found to be size, concentration and temperature dependent. For example, treatment of the cells for 2 hours (100 μg/ml) resulted in uptake of the 0.1 μm particles which was 2.5-fold greater than the uptake of the 1 μm particles on a weight basis. In addition, the 1 μm particle uptake was 6-fold greater than the 10 μm particle uptake. The uptake rate was greater at 37°C than at 4°C, suggesting an active mode of uptake.

Lung Clara and type II epithelial cells treated with carbon black nanoparticles and diesel exhaust particles *in vitro* demonstrated internalisation of the particles, suggesting that the epithelial cells can take up carbon nanoparticles (Murphy et al. 1999).

Internalisation of 50nm TiO_2 particles by A549 alveolar type II epithelial cells into membrane bound vesicles after 6 and 24 hour exposures has been demonstrated (Stearns et al. 2001). It was observed that the uptake of particle by these cells was limited to their aggregated form and was mediated via phagocytosis, due to the fact that plasma membrane projections surrounded and engulfed the particles prior to internalisation. Aggregates of NPs were apparent in this study, possibly due to the use of serum-free medium and limited sonication time (30 seconds).

Fluorescent polystyrene-based latex microspheres (Fluoresbrite®) of 50, 100, 200, 500 and 1000 nm diameter have been used to investigate the size dependency of particle uptake by B16-F10 cells (a murine melanoma cell line) (Rejman et al (2004). Uptake was evaluated using confocal microscopy and FACS (flow cytometry) and was evident for all. The 50, 100, 200 and 1000 nm beads were distributed throughout the cells but 500 nm beads remained at the cell periphery. The uptake of 50 nm beads was greatest, with internalisation reducing as particle size increased, so that in comparison to 100 nm beads there was a 3 to 4 fold greater uptake of 50 nm beads, and in comparison to 500 nm beads 8 to 10 times greater, with no uptake of 1000 nm beads evident after a 1 hour exposure. No internalisation of beads (of all sizes) was observed at 4°C. After disruption of microtubules with nocodazole, 50, 100 and 200 nm beads appeared to accumulate at the plasma membrane, and the authors suggested that internalised beads could not be trafficked from early to late endosomes. Treatment with nocodazole reduced the uptake of 50 and 100 nm beads by 50%, 200 nm by 25% and the uptake of 500 nm beads was unchanged; suggesting a size dependent element to particle uptake. Intracellular potassium depletion and pre-treatment of cells with chlorpromazine was used to inhibit clathrin mediated endocytosis, to determine if this mechanism contributed to particle uptake. In this experiment the uptake of 50 nm beads was inhibited to the greatest extent, suggesting that clathrin mediated endocytosis enabled their uptake. When caveolin-mediated endocytosis was inhibited (using filipin) the uptake of 500 nm beads was affected to the greatest extent, and entry of these beads was confirmed using a fluorescent caveolin marker, that was co-localised with internalised beads. The observation of a size-dependent method of uptake suggested that smaller particles (particles up to 200 nm diameter) were preferentially internalised via clathrin-mediated endocytosis, and larger particles (>200 nm, but smaller than 1 μm) via caveolin-mediated endocytosis. Smaller particles were also observed in lysosomes.

Investigations of the interaction between 1 μm polystyrene particles and a 3-D lung epithelial barrier model (Rothen-Rutishauser et al. 2005) included epithelial cells (A549 cell line), as well as macrophages and dendritic cells. Exposure of the cells via the apical surface, led to uptake by all three cell types, despite the fact that the dendritic cells were on the basolateral surface of the culture membrane. This could suggest that

the particles were passed from one cell type to another, or that the dendritic cells, via extensions of the cell body, were able to sample particles from the apical surface. Interactions between nanoparticles and this model have not yet been published.

Positive (89.8±4 nm) and negatively (96.4±6 nm) charged polyactide PEGylated particles were used to determine if the same internalisation pathway applied when entering HeLa cells (cervical cancer cell line) (Harush-Frenkel et al. 2007). It was found that neither particle type caused cytotoxicity. It was apparent that both particle types were internalised by cells in a time dependent manner (using confocal microscopy), however positively-charged nanoparticles were internalised at an earlier time, and accumulated to a greater extent when compared to their negatively charged counterparts. The uptake of the positively-charged nanoparticles plateaued at 45 minutes, which was suggestive that the uptake mechanism was saturable. It was determined that negatively-charged particles were not internalised by clathrin or caveolin dependent pathways, in contrast to the positively-charged nanoparticles that were internalised by these pathways, in addition to a smaller contribution of macropinocytosis. It was reported that positively-charged nanoparticle uptake was evident despite the inhibition of the clathrin and caveolin endocytic processes, suggesting that there were compensatory mechanisms that mediate uptake, and despite having a preferential pathway of uptake, other pathways can contribute to their uptake when necessary. Therefore nanoparticle surface charge affects the amount internalised, and the uptake pathway that is utilised in HeLa cells. It would be assumed that particles with a positive surface charge would interact with the negatively charged plasma membrane to facilitate uptake. With respect to the lung and inhaled particles, charge may change in the atmosphere, in the humid environment of the airways and within the lung lining fluid. The ultimate charge of the particle interacting with lung cells needs to be determined for such particles, and how this influences the potential for particle uptake.

Cell models are a potentially fertile area for development of screening systems for translocation, but these are not well-developed. Presently optimal conditions for measuring translocation across a cell monolayer have been described using Trans-Epithelial Electrical Resistance (TEER) to determine tightness of monolayer, in at least one system (Geys et al. 2007). When fluorescent nanoparticles were studied, 6% transfer was reported for polystyrene NP (Geys et al. 2006). This work focused primarily on the potential uptake and trafficking of NPs through cells and provides an indication of ways in which to address exposure and dose within a biological system. Such approaches can then be used to study specific toxicity indicators such as measures of oxidative stress, inflammation and cytotoxicity.

In summary, determining the mechanism of uptake is complicated by the fact that there are a large number of cells that are potentially exposed to a variety of nanoparticle types. Following inhalation, a number of different lung cells are exposed to nanoparticles, therefore attributing translocation of nanoparticle into the systemic circulation to one cell type is difficult.

4.3 BLOOD

The issue of whether inhaled nanoparticles reach the blood in humans is inconsistent. Although it has been reported that nanoparticles inhaled by human subjects reached the blood in a study using a ^{99m}Technetium-labelled carbon nanoparticle *Technegas* (Nemmar et al. 2002), other groups have failed to replicate the Nemmar results (Mills et al. 2006; Wiebert et al. 2006). *Technegas* is produced by a sealed commercial apparatus and is used for assessing lung ventilation, a function that requires that the

particles do not release soluble radioactivity, otherwise radioactivity in the blood would cloud the assessment of ventilation. In the more recent studies (Mills et al. 2006; Wiebert et al. 2006), translocation of soluble Technetium to the blood was recorded but was very low and there was essentially full retention of the Technegas particles in the lungs. Contamination of the nanoparticles in the Nemmar studies with soluble *pertechnate* ($^{99m}TcO_4^-$) may have explained the rise in radioactivity in the blood seen in the Nemmar study (Mills et al 2006). *Pertechnegas* is soluble and is produced accidentally when the usual pure argon environment of the *Technegas* generator contains oxygen above 100 ppm by volume.

Although there is a lack of any substantial epidemiology on the effects of exposure to engineered nanoparticles, the best guide as to likely effects on the cardiovascular system comes from studies such as the American Cancer Society studies (Pope et al. 2003) and the implication of combustion-derived nanoparticles (CDNP) in such adverse cardiovascular effects of $PM_{10}/PM_{2.5}$. As regards mechanisms, the evidence is accumulating for an impact on the endothelium and on atherothrombosis (Brook et al. 2003; Mills et al. 2007a). As a result, there is considerable interest in investigating how or if engineered nanoparticles might similarly impact cardiovascular disease.

There is very little known of the role of structure on translocation to the blood in so far as only low toxicity low solubility nanoparticles have been studied. However, analogously to the situation with interstitialisation, the increased permeability caused by inflammation could aid penetration to the blood. Changing the size and surface reactivity, factors that would influence inflammogenicity, may be considered likely modifiers of the potential of any nanoparticle to translocate to the blood.

From the few studies of the toxicokinetics of a range of different nanoparticles in rats following inhalation (Kreyling et al. 2002a; Kreyling et al. 2004; Kreyling et al. 2007; Oberdorster et al. 2002), there is some evidence that inhaled nanoparticles of various types of materials (e.g. iridium) enter the blood at a rate of about 1% of the deposited dose (Kreyling et al. 2007). Similar values (1-2% of the deposited dose entering the blood) have been reported for TiO_2 nanoparticles following instillation (Chen et al. 2006). Lower concentrations (0.05%) of inhaled gold nanoparticles were reported to enter the blood following instillation (Takenaka et al 2006). Results from electron microscope morphometry (Muhlfeld et al. 2007) have suggested that there is rapid transfer of 22 nm TiO_2 nanoparticles (count median diameter) into the blood via the interstitium, within a very short space of time following deposition. Although the mass fraction entering the blood may be small, the particle number that reaches these other targets can be very great (Kreyling et al. 2007) and these organs are not equipped, as the lungs are, to deal with large numbers of particles and so the effects may be greater.

Caution must be observed over the relevance of translocation studies following instillation delivery, since the bolus of saline must lead to an imbalance in the normal fluid flow across the airspace barrier.

Red blood cells have been used to investigate the uptake of fluorescent polystyrene particles and gold nanoparticles (Rothen-Rutishauser et al. 2006). Erythrocytes were chosen due to the fact that they are not phagocytic so that other mechanisms of nanoparticle uptake could be studied. However, due to their unusual membrane structure and lack of a nucleus, the generalisability of these results must be questionable. Fluorescent particles varying in size (1 μm, 0.2 μm and 78 nm) and surface properties (positively, negatively and uncharged) were investigated by confocal microscopy, and gold nanoparticles were studied by electron microscopy. The 1 μm polystyrene particles were observed attached to the cell surface, and were not internalised by the erythrocytes during any of the incubation times studied (4-24 hours).

Negatively and uncharged 200 nm particles were found within cells, as were the positively charged particles, however these were also apparent at the cell surface. The 78 nm particles (uncharged and negatively charged) were also found within cells. Positive polystyrene fluorescent particles were not available in this size range, so that positively charged gold particles were used to investigate the impact of NP charge on uptake by erythrocytes. The uptake of positive gold NPs (25 nm) did not occur to a greater extent, when comparing the uptake of NPs of a variety of charges. It was highlighted that imaging particles within the nanometre size range is challenging, and that even using analysis like TEM the identification of NPs is difficult due to their similarity in size (and appearance) to cellular structures e.g. ribosomes. Therefore confocal visualisation allowed the uptake of NPs to be compared with that of larger sized particles, and further analysis using TEM could be utilised to further investigate the intracellular fate. It was observed that the gold NPs were not membrane bound within the erythrocytes. It was therefore concluded that NPs were taken up by erythrocytes, but that endocytosis was unlikely to contribute to their internalisation (due to the lack of NP localisation within vesicles), or actin based mechanisms (as erythrocytes do not contain actin). Again, although this study does not use specific lung cells, the results are important to understand how particles interact with cells in general.

Studies suggest that surface charge can affect ability of polybeads to modify thrombus size in a hamster model of experimental thrombosis (Nemmar et al. 2003) where aminated beads were more active than carboxylated nanobeads. This could be a consequence of differential translocation to the blood for these two particle types but this was not studied. Other studies suggest that the aminated polybeads used in this case are cytotoxic and inflammogenic (Donaldson et al. unpublished data) and this could explain the differences. Likewise surface derivatisation with hydroxyl and carboxylate groups, which are known to modify C_{60} and nanotube toxicity (Sayes et al. 2004; Sayes et al. 2006) might affect transfer to the blood.

The endothelium is the key cell that particles must cross in order to gain access to the blood. Studies with medical nanoparticles derived from maltodextrins (e.g. dipalmitoyl phosphatidyl glycerol) with and without cationic ligands show charge-dependent binding to endothelial cells, that may be related to passage across the cell layer (Jallouli et al. 2007). Many studies have made the assumption that particles definitely cross the endothelium and have reported effects on the endothelium and cellular elements of the blood. Carbon black nanoparticles and PM_{10}/$PM_{2.5}$ affect endothelial cells in ways that enhance coagulation (Gilmour et al. 2005) and similarly pro-thrombotic effects in liver endothelium following treatment *in vivo* have been reported (Khandoga et al. 2004). Several studies have consistently described pro-coagulant effects of a range of nanoparticles on platelet aggregation and activation (Nemmar et al. 2003; Nemmar et al. 2006; Radomski et al. 2005). In one study where a large range of nanoparticles were tested for their pro-coagulant effects on platelets, there were dramatic difference between types, some being highly potent and others having no activity (Radomski et al. 2005), suggesting that surface structures dictate the activity.

4.4 BLOOD VESSEL WALL

The endothelium, atherosclerotic lesions and the clotting system are key targets for nanoparticles in circulatory system. It is important to point out that the literature reviewed here concerns the role of the endothelium in atherothrombosis, not in translocation, the latter being discussed in the section above.

The key pathogenic process underlying atherosclerosis in the blood vessel wall is well understood and centres on inflammation. This arises from endothelial damage and accumulation of foamy macrophages and myofibroblasts in the vascular wall with an overlying fibrous cap. The inflammatory activity of this plaque lesion is central to its stability. Increased inflammatory activity is associated with potential for catastrophic plaque rupture which leads to thrombosis and to acute coronary syndrome with angina and acute myocardial infarction. Inflammation at sites remote from the plaque are well known to enhance inflammatory activity in the plaque and the potential for rupture. NP could contribute to plaque rupture by i) causing inflammation in the lungs which could influence inflammatory activity in the plaque; ii) entering the blood, gaining access directly to the plaque and increasing oxidative stress and inflammation in the plaque; iii) enhancing thrombosis so that any thrombosis associated with rupture is worsened.

The potential cardiovascular impact of engineered nanoparticles is a major concern given the data showing adverse cardiovascular as lead effects of $PM_{10}/PM_{2.5}$ (Schwartz & Morris 1995) and recent work on diesel inhalation showing effects on the endothelium that are very likely occurring via a mechanism involving oxidative stress (Mills et al. 2005; Mills et al. 2007b). There is experimental evidence that deposition of $PM_{10}/PM_{2.5}$ in the lungs accelerates and worsens atherosclerotic plaque development in animal models (Lippmann et al. 2005; Sun et al. 2005; Suwa et al. 2002). The only data that indicates that engineered nanoparticles might have similar effects is the finding that intratracheally administered CNT causes oxidative stress and mitochondrial dysfunction in the aortae of mice (Li et al. 2007). This does suggest that effects on the vascular wall might be a generic effect of nanoparticles. These effects could be driven by the oxidative stress from the particle or the inflammation resulting from the particles being deposited in the lungs. However, an alternative explanation is that the nanoparticles enter the blood and cause a direct effect on the plaques by affecting the overlying endothelium of entering the atherosclerotic lesion and affecting the stability of the plaque. Paradoxically, given this concern, nanoparticulate iron has been used to image plaques where the particles have been found to enter the macrophages in the plaque with, perhaps surprisingly, no adverse effects reported (Trivedi et al. 2006).

4.5 PLACENTA / FOETUS

Little data beyond an overview of the issues and research priorities for investigating the interaction between nanoparticles and the placental barrier (Saunders 2007) is available. However, an on-going study has shown uptake of blood-borne gold nanoparticles into the placenta and foetus (Kreyling, personal communication).

4.6 BRAIN

The present interest amongst the toxicology community in engineered nanoparticles translocating to non-pulmonary organs, in particular the CNS, was stimulated in-part by a 2002 editorial (Oberdorster & Utell 2002) and subsequent acknowledgement of the need for research in a number of reviews (see for example Borm & Kreyling 2004; Gwinn & Vallyathan 2006; Hoet et al. 2004; Oberdorster et al. 2005; Peters et al. 2006).

The translocation of inhaled nanoparticles to the CNS has been postulated to occur via three pathways: i) across the BBB after their translocation into the blood circulation from deposits anywhere in the respiratory tract, ii) via the olfactory nerve from deposits on the olfactory mucosa and uptake into the olfactory bulb, and iii) via paracellular or perineural pathways across the olfactory mucosa and ethmoid bone into cerebrospinal

fluid (Oberdorster et al. 2004). Translocation to the brain via the olfactory nerve has been a focus of recent research, given the estimated 80% probability of inhaled NPs of ~1 nm in size depositing in the nasopharyngeal region (ICRP 1994).

Research on translocation of nanoparticles to the CNS is widely cited to originate from studies in the 1940s using 30 nm polio virus particles (Bodian & Howe 1941) and 1970s using 50 nm colloidal gold particles (De Lorenzo 1970) instilled intranasally. These early studies revealed the olfactory nerve and olfactory bulbs as portals of entry to the CNS. An interesting finding in this study was that the gold nanoparticles in the olfactory bulb were no longer freely distributed in the cytoplasm but were preferentially located in mitochondria. More recent studies have indicated that this translocation pathway is also operational for inhaled nanoparticles. Inhalation of elemental 35 nm ^{13}C nanoparticles resulted in a significant increase of ^{13}C in the olfactory bulb on day 1, which increased further throughout day 7 post-exposure (Oberdorster et al. 2004).

It is often acknowledged that conflicting studies have been reported regarding particle translocation after inhalation or instillation of nanoparticles in the lung. For example, rapid translocation toward the liver of more than 50% of ^{13}C nanoparticles has been observed within 24 hours in a rat model (Oberdorster et al. 2004), whereas <1% translocation of iridium nanoparticles (15–20 nm in size) was observed into the blood of rats, albeit reaching not only the liver, but also the spleen, kidneys, brain and heart (Kreyling et al. 2002b). Many factors are anticipated to influence the behaviour of nanoparticles and their interaction with biological membranes, many of which will be associated with physicochemical parameters that remain to be fully understood.

A historical perspective and a summary of findings from recent translocation studies are presented in a 2005 review of nanotoxicology (Oberdorster et al. 2005). Within the area of neuronal uptake and translocation, the review highlights the size-dependent findings from inhalation studies with manganese oxide particles in rats that showed a predominance for uptake in the olfactory bulb, compared to the lung. When one nostril was occluded, manganese accumulation in the olfactory bulb was restricted to the side of the open nostril only. Although additional neuronal translocation pathways for solid nanoparticles were suggested by Oberdorster et al. (2005) via the trigeminus nerve and tracheobronchial sensory nerves, as yet this is not an area that have received any significant investigation. The trigeminus nerves sense chemicals and irritants in a number of areas including the nasal cavity, but their potential to sense or even transport NPs is not yet known.

Studies of manganese nanoparticles their accumulation, and effects in the brain feature in the literature largely from interest in an accelerated onset of parkinsonism in welders and the high levels of the element in welding fumes. Recent research sought to address the hypothesis that a major translocation route for inhaled poorly soluble manganese oxide nanoparticles from deposits in the nose is to the olfactory bulb in the CNS (Elder et al. 2006). The study characterised the size, oxidation state, and *in vitro* solubility of manganese oxide particles and also compared the translocation kinetics to the olfactory bulb of manganese oxide and manganese chloride that were applied to the nasal epithelium of rats via instillation. The accumulation of manganese in lung, liver, and olfactory bulb were measured after repeated inhalation exposures with both nares patent or with one naris occluded. Their findings show that 30 nm manganese oxide nanoparticles are translocated to and retained in the olfactory bulb (ipsilateral to the patent naris only) and present evidence of exposure-induced effects in that region of the brain. The authors mention that the earlier studies of olfactory translocation of nanoparticles of different types (viruses, gold, carbon), with results from their study, together imply that all nanoparticles deposited on the olfactory mucosa will translocate to the brain. However, uptake into sensory nerve endings and subsequent

translocation is likely to depend not only on size, but also on many other particle characteristics, such as shape, chemistry, surface properties (area, porosity, charge and surface modifications), agglomeration state, solubility, and dose. They recognise their findings may not be directly applied to nanoparticles in general until more data are available on mechanisms controlling neuronal uptake and translocation. The authors conclude that the olfactory neuronal pathway represents a significant exposure route of CNS tissue to inhaled solid manganese oxide nanoparticles. In rats, which are obligatory nose breathers, translocation of inhaled nanoparticles along neurons seems to be a more efficient pathway to the CNS than via the blood circulation across the BBB. Given that this neuronal translocation pathway was also demonstrated in non-human primates, they suggest it is likely to be operative in humans as well.

In a recent study examining the body distribution of inhaled nanoparticles (Kwon et al. 2008), BBB penetration by 50 nm fluorescent magnetic nanoparticles was observed in mice using T2-weighted spin-echo magnetic resonance imaging. The authors speculate that their fluorescent magnetic nanoparticles may have entered the brain at discontinuities in the BBB but also suggest an alternative pathway for the observed brain distribution by referencing the evidence of translocation along the olfactory nerve into the olfactory bulb.

Studies using gold nanoparticles are also appearing more frequently in the literature; the rationale being that i) gold nanoparticles can be synthesized at a large size (1-100 nm diameter) and shape range (1:1 to 1:5 aspect ratio), ii) they are easy to characterise by UV-visible spectrophotometry, inductively coupled plasma atomic emission spectroscopy (ICP-AES), and transmission electron microscopy (TEM),and iii) they have recently been demonstrated in cell imaging, targeted drug delivery, and cancer diagnostics and therapeutic applications.

In a study of the effect of nanoparticle size, shape, concentration, and incubation time on cellular uptake kinetics (Chithrani et al. 2006), it is speculated that non-specific adsorption of serum proteins mediates the uptake of the nanoparticles and that the presence of these proteins on the surface of the nanoparticles dictates uptake half-life, rates, and amount. A quantitative comparison of citrate-stabilised gold nanoparticles versus transferrin-coated nanoparticles showed greater uptake of citrate-stabilised gold nanoparticles. As serum proteins contain a diverse set of proteins, the surface of the citrate-stabilised gold nanoparticles was thought to contain a variety of serum proteins on its surface. Many of the serum proteins (e.g. α- and β-globulin proteins) are known to be taken up by cells. Therefore, the diversity of the proteins may allow entrance into the cells via multiple receptors. The authors also reported a large difference in the uptake of the different size and shaped gold nanoparticles. For example, the uptake rates for 74 x 14 nm rod-shaped nanoparticles were lower than those for 74 or 14 nm spherical nanoparticles. Postulated reasons for the difference were the i) curvature of the different-shaped nanoparticles, ii) the retention of a surfactant molecule used in their synthesis onto the rod-shaped nanoparticle surface precluding the binding of serum protein, and iii) the protein coating on the surface of the rod-shaped gold nanoparticles being heterogeneous such that ligands on the surface couldn't bind to receptors on the cell surface as strongly.

Most recently reported are the findings from a study undertaken to test the hypothesis that gold nanoparticles can accumulate in the olfactory bulb, and translocate from the lung to other organs after inhalation exposure (Yu et al. 2007). The authors report little accumulation of gold nanoparticles in most parts of the brain regardless of exposure duration and believe this to be due to the blood brain barrier. The exceptions were the olfactory bulb, septum, entorhinal cortex and cerebellum. The authors suggest that the findings of increased levels of gold in the olfactory bulb after 5 days exposure, and in

the olfactory bulb, septum and entorhinal cortex after 15 days exposure, support the view that gold nanoparticles could, with time, be transported from the olfactory bulb to other parts of the brain. They conclude that inhaled gold nanoparticles accumulate in the olfactory bulb, and, somewhat ambiguously, are able to translocate from the lung to other organs with time. No gross abnormalities were observed in the exposed rats, even though changes in muscle-related genes and phosphatidylserine species in the lung suggest that there could be health effects after exposure to the gold nanoparticles.

In a recent study to examine the possible neurotoxicity of titanium dioxide (Long et al. 2007), nerve cells critical to the pathophysiology of neurodegeneration (i.e. microglia, neurons) were exposed to the commercially available nanomaterial Degussa P25. The authors suggest their data indicate that the titanium dioxide nanoparticles stimulate BV2 microglia to release reactive oxygen species (ROS) and affects genomic pathways associated with cell cycling, inflammation, apoptosis and mitochondrial bioenergetics. P25 appeared to be non-toxic to isolated DA neurons (N27) even after 72 hours. However, when examined in primary cultures of brain striatum which contain microglia, neuronal loss occurred by 6 hours in response to only 5 ppm. The authors report that the shift in dose-response, coupled with cellular and genomic evidence of P25's effect on inflammatory and apoptotic pathways and disruption of energy pathways in BV2 microglia, suggests that the potent neurotoxicity of P25 seen in complex cultures was mediated though microglia-generated ROS. Their study emphasises that for valid interpretation of nanotoxicity data, physicochemical properties must be determined under conditions that parallel the biological exposures. Their data indicated that the exposure conditions (i.e. vehicle, temperature) significantly modified P25's particle size and zeta potential which could affect its interaction with biological systems and its ultimate toxicity. Particle (or aggregate) size determines if a particle enters the cellular environment though ROS-producing phagocytosis, through endocytosis, or some undefined mechanisms. The surface charge or zeta potential of a particle affects its aggregation in solution and its behaviour in an electric or ionic field. The surface charge of a particle also determines its interactions with specific biological receptors. Polymodal receptors located in the cellular membrane of microglia and macrophages (e.g. TRPV1, Mac-1) are sensitive to protons (i.e. charge) or repeating patterns of charge like those found on crystalline metal oxide nanoparticles. In an earlier publication of their study (Long et al. 2006), the negative zeta potential of P25 particles and the ordered arrangement of charged O^- sites on their surface could be activating either type of receptor and subsequent oxidative stress pathways in the microglia.

The role of the surface groups and the charge they carry is paramount to the behaviour of nanomaterials *in vivo*. Surface groups can make the material hydrophilic, hydrophobic, lipophillic, lipophobic, or catalytically active or passive. In a study to evaluate the effect of neutral, anionic and cationic charged nanoparticles on BBB integrity and permeability using nanoparticles composed of emulsified wax (Lockman et al. 2004), neutral and low concentrations of anionic nanoparticles were found to have no effect on BBB integrity, whereas, high concentrations of anionic and cationic nanoparticles disrupted the BBB. The brain uptake rates of anionic nanoparticles at lower concentrations were superior to neutral or cationic formulations at the same concentrations. The authors concluded that i) neutral and low concentrations of anionic nanoparticles can be utilised as colloidal drug carriers to brain, ii) cationic nanoparticles have an immediate toxic effect at the BBB and iii) surface charges must be considered for toxicity and brain distribution profiles.

It is evident that the pharmacology literature offers significant insight into understanding the translocation of nanoparticles across the BBB and the influence of surface chemistry, although a major gap in communication is acknowledged (Borm & Kreyling

2004) between pharmacology developing nanoparticles for drug delivery and toxicology seeking knowledge of the mechanisms of their local and systemic adverse effects.

Nanoparticles have been used for pharmaceutical and medical applications for over 30 years and those developed for pharmacological uses are currently made from a wide array of materials such as poly(alkylcyanoacrylates); poly(methylidene malonate); polyesters such as poly (lactic acid), poly(glycolic acid), poly(ε -caprolactone) and their copolymers; polysaccharides; and proteins. The choice of nanoparticle materials is based on biodegradability, intrinsic immunogenicity, and toxicity.

A recent review (Teixido & Giralt 2008) of the role played by peptides in blood-brain barrier interactions highlighted that the binding of a peptide-coated nanoparticle to a given receptor can result in the nanoparticle being transported across a barrier, often with a mechanism other than that expected of the coating. Hydrophilic surfactants have been shown to reduce nanoparticle absorption by reticuloendothelial system organs that alters biodistribution of the nanoparticles. Coating of colloidal nanoparticles with block copolymers such as poloxamers and poloxamines induces a steric repulsion effect, minimising the adhesion of particles to the surface of macrophages, which in turn results in the decrease of phagocytic uptake and in significantly higher levels in the blood and organs including the brain, intestine, and kidneys among others. Surface PEGylation increases the blood half-life of nanoparticles; and polysorbate-80 improves BBB transport of nanoparticles.

In a comparable review of nanocarrier-based CNS delivery systems (Tiwari & Amiji 2006), a number of mechanisms proposed for the BBB transport of polymeric solid and lipid nanoparticles were summarised. The authors state that it is possible that combination of some or all of the mechanisms may act to facilitate transport. The various mechanisms proposed include:

1. Adhesion of nanoparticles to the inner endothelial cells of brain capillaries and the subsequent transport by passive diffusion, possibly by a larger concentration gradient. Nanoparticle degradation products may also act as adsorption enhancers, thus contributing to increased passive diffusion;
2. Surfactants used in coating of nanoparticles may solubilise the endothelial cell membrane lipids, thus enhancing the transport across the BBB;
3. Surfactant-coated nanoparticles, particularly polysorbate coated, administered intravenously, becomes further coated with absorbed plasma proteins especially, apolipoprotein E (Apo-E), leading to this final product being mistaken for low-density lipoprotein (LDL) particles by the cerebral endothelium and internalised by the LDL uptake system. Solid lipid nanoparticles may also transport drugs across the BBB by this mechanism;
4. Components of nanoparticle structures might open the tight junctions of the brain capillary endothelial cells, and allow the penetration of surfactant coated nanoparticles into the CNS;
5. Excipients used in the manufacture of nanoparticles (e.g. polysorbate 80) may inhibit the drug efflux system and improve the drug absorption across brain capillary endothelial cells;
6. Nanoparticles might be endocytosed or transcytosed through the brain capillary endothelial cells.

The chemical identity of the surface of a nanoparticle is key to many of these possible mechanisms and is the basis of many pharmacological approaches using the activation of natural transport routes to penetrate the blood-brain barrier.

For example, it has been shown that surface modification with thiamine enhanced the interaction of the nanoparticles with the cells due to specific association with the BBB thiamine transporter (Lockman et al. 2003). It was postulated that such an association may create an accumulation of nanoparticles at the BBB, which may ultimately increase the drug uptake over the period of time.

A methodology was developed to investigate the intracellular distribution of PEG-coated polyhexadecylcyanoacrylate (PEG-PHDCA) nanoparticles after their incubation with rat brain endothelial cells (Garcia-Garcia et al. 2005). The results showed that PEG-PHDCA nanoparticles were able to be internalised to a higher extent than PHDCA nanoparticles (after 20 min incubation). Additionally, these nanoparticles displayed different patterns of intracellular capture, depending on their specific surface composition: PEG-PHDCA nanoparticles were 48% in the plasma membrane, 24% in the cytoplasm, 20% in vesicular compartments and 8% associated with the fraction of the nucleus, the cytoskeleton and caveolae suggesting that PEG-PHDCA nanoparticle uptake by the endothelial cells is specific and presumably due to endocytosis.

From theoretical modelling of the translocation process across liquid-like membranes (Livadaru & Kovalenko 2006), it has been suggested that a combined action of the peptide insertion and the particle adhesion to the membrane can lead to translocation of the particle by opening a tight pore in the membrane, large enough to accommodate its size, and simultaneously sealed by it (against leakage of material from the other side of the membrane). The authors developed a statistical-mechanical approach to such a system and explored the possible mechanism of translocation of a functionalised nanoparticle through a membrane. They predicted a new possible mechanism of nanoparticle translocation or trapping, which does not involve total enveloping of the particle by the membrane. Initially, the nanoparticle functionalised with a peptide aggregate gets adsorbed onto the membrane. Following fluctuations in nanoparticle position and orientation, the peptide aggregate incorporates into the membrane, which becomes locally destabilized by it. A pore starts forming with the assistance of the adhesion of the membrane to the particle. Even in the absence of a driving engine against the membrane energy barrier, the fluctuations in the pore size eventually induce translocation of the particle for certain systems.

In summary, the unique bio-kinetic behaviour exhibited by nanoparticles, including cellular endocytosis, transcytosis, neuronal and circulatory translocation and distribution, makes them desirable for therapeutic and diagnostic medical applications, but may also convey potential toxicity. The routes of entry of nanoparticles to the CNS are becoming increasingly recognised, although the influence of physicochemical properties on the mechanisms remains to be fully elucidated. There is evidence of uptake in the CNS both of particles translocating from the lungs and via the olfactory bulb. It is expected that transport of nanoparticles across the BBB is possible by either passive diffusion or by carrier-mediated endocytosis (Hoet et al. 2004). Moreover, as the BBB is defective in a number of locations (e.g. pineal gland, pituitary gland, area postrema, choroid plexus), nanoparticle entry in these areas may be possible. Surface modified particles can interact with the receptors leading to uptake by endothelial cells. Also, other processes such as tight junction modulation or P-glycoprotein (Pgp) inhibition also may play a role (Kreuter 2001). Surface characteristics of nanoparticles (e.g. chemistry, charge, shape, aggregation) are clearly essential determinants, however the research interests and nanoparticles under investigation by the pharmacology and toxicology communities are quite distinct and there is limited commonality between their studies to date. Of particular relevance to the public health consequences of nanoparticle exposure are the conclusions of a critical update on the translocation and potential neurological effects of fine and ultrafine particles (Peters et

al. 2006), namely that immune responses and damage to endothelial barriers may disrupt the tight junctions and facilitate particle translocation. Through such processes, inflammatory cytokines can increase CNS levels of NF-κB activation and up-regulate the innate immune receptor Toll-like receptor 2. Such activation and the subsequent events leading to neurodegeneration have recently been described in BALB/ c mice exposed to ambient Los Angeles particulate matter. Such data indicate a neuroimmunological pathway to explain PM-CNS neurodegeneration. Together, these reports confirm that the brain is a critical target of nanoparticle exposure and implicate oxidative stress as a predisposing factor that links particulate exposure and neurotoxic susceptibility.

5 WORKSHOP

The CELL PEN workshop, discussing aspects of cellular penetration of nanoparticles in the lung epithelium, was held on Tuesday 22nd April 2008, at DEFRA's Central Science Laboratories in York. Its aim was to bring together a diverse range of stakeholders from the nanotechnology field, to disseminate the primary findings of the scoping study, and then through facilitated breakout discussions establish research priorities for further investigation of the mechanisms of nanoparticle translocation across the respiratory epithelium and the resulting possible toxic effects in and beyond the lung.

Stakeholders from across the UK and wider European nanotechnology community attended the workshop, with representation from the following institutes present:

IonBond Ltd.
Joint Research Centre (JRC)
Leeds University
Napier University
Institute of Occupational Medicine
Manchester University
Health and Safety Laboratory
Edinburgh University
Aberdeen University
Durham University
Cardiff University
University of Chester
Health Protection Agency (HPA)
Imperial College, London
European Commission (EC)
Central Science Laboratory (CSL)
University of Torino, Italy
University of Parma, Italy
Department for Environment, Farming and Rural Affairs (DEFRA)
United Bristol Healthcare NHS Trust
Nanotechnology Industries Association (NIA)
Centre for Environment, Fisheries and Aquaculture Science (CEFAS)

Workshop Format

The workshop was divided into three main sessions – scientific presentations throughout the morning, followed by facilitated breakout discussions, and a summary and discussion of findings in the afternoon.

The morning's presentations were given by both CELL PEN's authors and invited speakers from other institutions. The smaller group breakout, and final summary discussions in the afternoon aimed to build on the morning's dissemination and work toward reaching a consensus on future research.

Morning Session: Plenary presentations

The morning's scientific sessions began with an introductory presentation from Dr Steve Hankin of the IOM. Dr Hankin introduced delegates to the key concepts behind the CELL PEN study, explaining that a major concern regarding the potential toxic effects of nanoparticles is the capacity of these materials to translocate into cells, and the potential critical step this may be in the movement of particles into the bloodstream and to other tissues following deposition in the lung. He then provided a brief overview of potential pathways of exposure and translocation, and the potential interactions nanoparticles may have following translocation with an emphasis on those areas concentrated on within the study. Finally, Dr Hankin emphasised to delegates that current understanding of the mechanisms and physico-chemical driving factors is

incomplete and there is evidence of inconsistent results, hence the need for a strategy of research into the mechanisms involved in translocation, particularly across the respiratory epithelium.

The second presentation of the morning was from Professor Vicki Stone of Napier University, who summarised for delegates study findings on Translocation of NPs across the lung epithelium. Prof. Stone gave an overview of bronchial epithelial anatomy and potential mechanisms of transport across epithelial cells. During this she introduced paracellular and transcellular transport, and outlined in more detail the processes of diffusion, active transport, macrophage associated transport, and the various types of endocytosis. After summarising data from *in vivo* studies available to date, Prof. Stone concluded that the evidence suggests that inhaled and instilled NPs cross the pulmonary epithelium gaining access to the interstitium and perhaps the CV system. In addition, she provided an overview of the evidence to suggest that NP uptake may be more rapid and extensive for smaller NPs in certain cell types, and illustrated this with examples. Prof. Stone observed that disease is likely to increase permeability to the lungs and thus transport via the paracellular route (through 'leaky' tight junctions), and that transcellular transport via endocytosis and consequent exocytosis is likely but as yet unproven. Finally, the possible role of macrophages as a route for transport across the epithelium was considered, although Prof. Stone observed that there was some evidence to suggest that NPs may impair macrophage function.

Professor Ken Donaldson of Edinburgh University provided an overview of NP translocation to target systems other than the lungs, focussing on the brain (via lungs & nose), blood, foetus, athermatous plaques, and excretion from blood via the kidney. The key idea Prof. Donaldson stressed was that NPs exert both localised effects at their portal of entry, and distal target tissue specific effects after translocation, but that data available to date is weak and there a consequent urgent need for generation of high quality translocation data.

Following a summary of existing studies on each of the target areas, Prof. Donaldson focussed on the role of NPs in cardiovascular dysfunction, outlining two possible routes of action: 1. via oxidative stress and inflammation in the lung, and 2. via free NPs in the blood. A number of studies which indicated cardiovascular damage following diesel exhaust inhalation were reported to illustrate this, including evidence of endothelial dysfunction, decreased 'clot-busting' t-Pa antigen release, increased thrombus volume, increased platelet aggregation (indicative of thrombus formation), and impaired blood flow in the heart.

The last part of Prof. Donaldson's talk concentrated on translocation of NPs to the brain, and their possible resulting health effects. Evidence considered included a study by Oberdorster et. al. (in press at the time of the workshop) which showed increased numbers of NPs localising in the cerebrum and cerebellum following inhalation (although Prof. Donaldson commented that the increase was only slight), and evidence to date for a link between NP presence in the brain and neurodegeneration based on results of studies that indicated a loss of dopanimergic neurons following exposure to high concentrations of ambient particles, and Alzheimer's disease-like pathology in individuals exposed to severe air pollution. To conclude, Prof. Donaldson related the evidence presented that morning to the concept of micro-toxicokinetics and size-related compartmentalism of NPs, with reference to potential compartment-related toxicity.

The penultimate talk of the morning was from invited speaker Dr Terry Tetley of Imperial College London, who spoke on aspects of cell penetration by particulates based on her recent work on cells of the alveolar surface. The study she reported focussed on uptake of nano sized latex beads into alveolar type I (AT1) and type II (AT2) cell lines, and investigation of the mechanism of uptake via inhibition of specific pathways. The results to date indicated that using 50nm beads, AT1 cells internalised

the majority present, whereas a minimal amount was internalised in the AT2 line. In the AT1 line, particle uptake was affected by both particle charge and size: neutral beads were not internalised, whereas negatively charged and positively charged particles were internalised, with smaller beads being taken up faster than larger ones. In addition, Dr Tetley commented that following internalisation of positively charged beads, there was some cell death indicating that these beads may be toxic. Endocytosis and clathrin mediated uptake were also investigated by blocking proteins mediating these mechanisms. To date, the role of microtubules, caveolae and clathrin have been examined and all except caveolae appeared to have some role in uptake of the beads. However, Dr Tetley added that there was an indication that around 50% of the beads internalised may have done so via passive diffusion.

The final presentation of the morning was from Dr Margaret Saunders of the United Bristol Healthcare NHS Trust, who gave a summary of her current work on nanoparticles and the placenta. Dr Saunders began by introducing the placental cell barrier model used by her group: the immortalised placental choriocarcinoma BeWo cell line cultured in Transwells® on permeable inserts as a monolayer suitable for examination of translocation and uptake of nanoparticles. The group's experimental work to date had focused on a range of sizes of polystyrene based latex Fluoresbrite® particles and cobalt chrome nanoparticles (cobalt chrome being chosen due to its use in metal joint replacements). In the studies examining Fluoresbrite beads, Dr Saunders reported that results to date indicated that both translocation and uptake of the beads differ according to particle size. In the cobalt chrome nanoparticle studies, Dr Saunders reported that exposure of cells to 2.4 µm or 29nm particles had no obvious effect on the barrier function of the BeWo monolayer as determined by measurement of trans-epithelial electrical resistance (TEER) or transport of paracellular markers. However, Comet assay analysis by Dr Patrick Case's group (University of Bristol) of fibroblast cells cultured in the bottom of the well underneath the BeWo barrier did reveal increased DNA damage following direct exposure of the BeWo cells, this effect apparently independent of dose administered. Dr Saunders concluded that although research using this technique is fairly new, findings so far indicated that placental translocation of nanoparticles occurs and cell function could be altered by nanoparticles, and thus there were potential implications for functional outcomes during pregnancy.

Afternoon Breakout sessions

For the afternoon breakout sessions, delegates were split into two randomly generated groups, and presented with three themes for discussion:

1. Identification of which features of nanoparticles (NP) are important in particle-cell interactions, considering the potential role of NP chemistry, structure, mass, numbers, shape, surface area, charge and functionalisation;
2. Discussion of how NPs may be modified to enhance or reduce their capacity to enter cells;
3. Discussion of how interactions between NPs and cultured human cells might be studied.

Groups were led by Professor Ken Donaldson and Professor Vicki Stone. Rapporteurs were Dr Lang Tran and Bryony Ross.

6 CONCLUSIONS

This review has highlighted the existing knowledge and uncertainties regarding the interactions of nanoparticles and cells, the physico-chemical factors that influence translocation and the consequences of such interactions/translocation.

A summation of the principal physico-chemical factors and their influence on nanoparticle translocation as identified across the six target sites reviewed, is presented below.

Size

Particle uptake by cells has been observed to be size and temperature dependent. Examples from the literature reviewed include:

- Nanoparticles of TiO_2 (25 nm) have been observed within the lung interstitium to a greater extent than 250 nm particles, following instillation or inhalation (Ferin et al. 1990b; Ferin et al. 1992; Ferin et al. 1990a).
- Internalisation of 50 nm TiO_2 particles by A549 alveolar type II epithelial cells into membrane bound vesicles after 6 and 24 hour exposures has been demonstrated. It was observed that the uptake of particle by these cells was limited to their aggregated form and was mediated via phagocytosis, due to the fact that plasma membrane projections surrounded and engulfed the particles prior to internalisation (Stearns et al. 2001).
- In a study using nano and microparticles of polylactic polyclycolic acid co-polymer containing bovine serum albumin and 6-courmin as a fluorescent marker, treatment of the Caco-2 epithelial cells for 2 hours (100 μg/ml) resulted in uptake of the 100 nm particles which was 2.5-fold greater than the uptake of the 1000 nm particles on a weight basis. In addition, the 1000 nm particle uptake was 6-fold greater than the 10,000 nm particle uptake. The uptake rate was greater at 37°C than at 4°C, suggesting an active mode of uptake (Desai et al. 1997).
- The uptake of fluorescent polystyrene-based latex microspheres by B16-F10 cells was observed to be greatest for 50 nm beads, with internalisation reducing as particle size increased, so that in comparison to 100 nm beads there was a 3 to 4 fold greater uptake of 50 nm beads, and in comparison to 500 nm beads 8 to 10 times greater, with no uptake of 1000 nm beads evident after a 1 hour exposure. No internalisation of beads (of all sizes) was observed at 4°C (Rejman et al. 2004).
- Polystyrene particles of 1000 nm diameter attach to the surface of erythrocytes and were not internalised over a period up to 24 hours, whereas 200 nm and 78 nm polystyrene particles (negatively charged and neutral) were found within cells (Rothen-Rutishauser et al. 2006).
- The observation of a size-dependent method of uptake suggested that smaller particles (particles up to 200 nm diameter) are preferentially internalised via clathrin-mediated endocytosis, and larger particles (>200 nm, but smaller than ~1000 nm) via caveolin-mediated endocytosis.

Specific surface area

The precise influence of specific surface area on particle uptake by cells has not been extensively reported. However, high specific surface area of particles is proven to enhance inflammation (Duffin et al., 2007, Stoeger et al. 2006) which could lead to interstitialisation (e.g. Ferin et al., 1992). Since surface area is inversely related to size it could be hypothesised that increased surface area results in increased uptake into cells and penetration of cell barriers, but it is not possible at this time to assess whether such an observation is size or surface area driven.

Surface chemistry

Particle uptake by cells has been observed to be influenced by the chemical nature of the particle surface. Examples include:

- A lectin coating on particles facilitates interaction with the intestinal Caco-2 epithelial cell line and thereby promotes uptake *in vitro* (Russell-Jones et al. 1999), presumably by providing a specific interaction with a cell surface receptor. Several lectins are actively taken up into primary lung epithelial cells when applied to the apical surface *in vitro* (Yi et al. 2001).
- Citrate-stabilised gold nanoparticles have exhibited a greater uptake compared to transferrin-coated nanoparticles. As serum proteins contain a diverse set of proteins, the surface of the citrate-stabilised gold nanoparticles was thought to contain a variety of serum proteins on its surface. Many of the serum proteins (e.g. α- and β-globulin proteins) are known to be taken up by cells. Therefore, the diversity of the proteins may allow entrance into the cells via multiple receptors (Chithrani et al. 2006).
- Carboxylated quantum dots and carboxylated polystyrene beads enter macrophages rapidly, entering endosomal, lysosomal and mitochondrial compartments (Clift et al., manuscript in preparation). In comparison amino polyethylene (PEG) coated quantum dots or organic coated quantum dots are less readily taken up by such cells.
- Coating of colloidal nanoparticles with block copolymers such as poloxamers and poloxamines induces a steric repulsion effect, minimising the adhesion of particles to the surface of macrophages, which in turn results in the decrease of phagocytic uptake and in significantly higher levels in the blood and organs including the brain, intestine, and kidneys among others. As suggested above surface PEGylation increases the blood half-life of nanoparticles; and polysorbate-80 improves BBB transport of nanoparticles (Teixido & Giralt 2008).
- Poly(MePEG2000cyanoacrylate-co-hexadecylcyanoacrylate) (PEG-PHDCA) nanoparticles have however been shown to transit rat brain endothelial cells in vitro, presumably via endocytosis, to a higher extent than the non-PEGylated polyhexadecylcyanoacrylate counterpart particle (Garcia-Garcia et al. 2005).
- Surface modification of nanoparticles with thiamine has been observed to enhance their interaction with cells due to a specific association with the BBB thiamine transporter (Lockman et al. 2003).

Charge

Particle uptake by cells has been observed to be influenced by the particle’s charge.

Polymodal receptors located in the cellular membrane of microglia and macrophages (e.g. TRPV1, Mac-1) are sensitive to protons (i.e. charge) or repeating patterns of

charge like those found on crystalline metal oxide nanoparticles. According to Long et al. (2006), the negative zeta potential of TiO_2 particles and the ordered arrangement of charged O^- sites on their surface could be activating either type of receptor and subsequent oxidative stress pathways in the microglia.

Positive (89.8±4 nm) and negatively (96.4±6 nm) charged polyactide PEGylated particles have been observed to be internalised by cells in a time dependent manner (using confocal microscopy), however the positively-charged nanoparticles were internalised at an earlier time, and accumulated to a greater extent when compared to their negatively charged counterparts (Harush-Frenkel et al. 2007).

Rothen-Rutishauser et al. (2006) compared uptake by non-phagocytic red blood cells of very small particles ($\leq$200 nm) and nanoparticles of TiO_2, polystyrene and gold. Polystyrene beads were well dispersed and therefore were taken up as single particles. TiO_2 and gold tended for form aggregates, although the colloidal gold was better dispersed due to protein coating. Both particles were taken up into cells, with aggregates of up to 200 nm being seen within cells, and larger aggregates remaining at the cell surface. The authors concluded that size influenced uptake of particles into red blood cells, but charge and chemistry did not.

In conclusion, it is apparent from the literature reviewed that many findings are reported from studies which have not yet set out to investigate systematically the physico-chemical factors which control the capacity of nanoparticles to penetrate cells. It is challenging to deconvolute the influence of any one physico-chemical factor on the cell penetration capacity of nanoparticles from such studies where multiple particle types and variables are reported (e.g. size, surface coating and charge). Furthermore, properties of nanoparticles such as surface chemistry or aggregation in biological or other fluids may not be in equilibrium or even at steady state and these properties may well change as a function of time.

Modelling and the investigation of structure-activity relationships have been acknowledged as being in the infancy with further work required. These, along with more appropriately designed systematic studies, are required to elucidate the role of physico-chemical properties.

Whilst it is beyond the scope of this review to elucidate the basis of inconsistencies between studies and why some have not shown translocation from the lung to other organs where others have, the potential for translocation of nanoparticles has been established and a number of the influencing factors including physico-chemical properties (e.g. size, surface chemistry, charge), physiological mechanisms and pre-existing disease within the population have been highlighted.

On the basis of current literature, it is premature to draw definitive conclusions however, the review has highlighted those that can be expected to play an important role in determining the capacity of nanoparticles to penetrate cells. The recommendations from this project provide a framework for future studies to consider as priorities.

7 RECOMMENDATIONS

The recommendations from the project fall into two main areas: cross-cutting themes for translocation and penetration studies, and research requirements specific to aspects of penetration and translocation studies.

Programmes of research on the relationships between size, composition etc on interstitialisation are needed. The role of nanoparticle structural features and effects of modifications to the structure are likely to be important and the ability to produce inflammation is likely to be an important factor in rendering a particle able to enter the interstitium. Development of cellular models is urgently needed but this will be difficult to validate, given the lack of understanding of the events that occur *in vivo*. Research is needed on the ability of particles to cross the placenta and blood brain barrier. In particular, the role of reactive surfaces in causing inflammation means that surface reactivity might be important and should be the target for research. Studies with nerve cells in culture, measuring transit of particles along them, may illuminate the factors that dictate neural transfer.

A summary of the gaps revealed in course of the review and the contribution from the workshop discussions are outlined below.

Cross Cutting Themes for penetration & translocation studies

Analysis of group discussion summaries identified a number of clear cross-cutting themes to be considered for future Cell Penetration/Translocation Studies. These are summarised in Table 1.

Table 1: Cross-Cutting Themes for Cell Penetration & Translocation studies

Cross Cutting Themes for Cell Penetration Studies	
Theme	Recommendations
Establishment of a clear definition for Cell Penetration of Nanoparticles	This definition must recognise that NP penetration is different for different cell types; and take into account phagocytosis related vs non-phagocytosis related mechanisms of penetration
Development of well characterised NPs for use in future studies	A number of key characteristics were identified: • Aggregation kinetics • Charge • Coating (including hydrophobicity/phillicity of coating) • Composition • Size (including size distribution within NP sample) • Shape • Surface area • Surface chemistry (including further investigation of role of protein corona on surface)

Cross Cutting Themes for Cell Penetration Studies	
Theme	Recommendations
Near-term preparation of widely available, suitably characterised 'stock' NPs for penetration & localisation studies, as well as toxicity studies.	Particles identified as priority substances were: • Non-toxic polystyrene beads for imaging of penetration and localisation • Au/Ag NPs for toxicity studies and EM imaging of localisation In addition, the group recognised that guidance for this could be taken from the REFNANO Project (Aitken et al. 2008).
Development of suitable labelling systems for particle penetration/translocation studies.	Labels must allow accurate tracking in such a way that NP activity is not compromised and labels remain attached to particles throughout study duration. (The group referenced the use of Iron NPs to label tumours in MRI scans, the use of fluorescent tags, and the use of labels placed *within* NPs as being relevant examples of recent progress in this area.)
Use of a wide range of concentrations for toxicity studies to establish a dose-response relationship that can be applied to the risk assessment process.	This had a dual purpose: 1. to provide worst and best case scenarios until exposure data becomes available 2. to ensure a more accurate risk assessment when exposure information becomes available

Research Priorities for Nanoparticle Translocation Studies

Feedback from the breakout groups led to development of recommended research priorities for investigating interactions between NPs and cells which naturally divided into two main subsections:

i. research to aid understanding of mechanistic toxicology

ii. research to generate suitable hazard data for NP risk assessment

Recommendations outlined in both areas assumed parallel application of cross-cutting themes for penetration/translocation studies.

i. Mechanistic Toxicology

Priorities identified by workshop attendees were further sub-divided into i) general study priorities; ii) *in vitro* study priorities; and iii) *in vivo* study priorities. These are summarised in Table 2, below.

Table 2: Mechanistic Toxicology Research Priorities

Mechanistic Toxicology	
General Study Requirements	Investigation of nanoparticle translocation: • into cells • across cells • between intracellular organelles from cell to destination Studies must investigate each area of translocation: • under normal conditions • under inflammatory conditions • with regard to potentially compromised tight junction integrity Development of appropriate representative cell models for use in *in vivo, in vitro* and *in silico* studies.
In Vitro Study Priorities	Investigate the role of Tight Junctions in controlling transfer of NPs across barrier Investigate the effects of Inhibition of uptake and intracellular trafficking pathways on NP translocation Identify NPs within intracellular organelles using a combination of Confocal and Electron Microscopy Relate NP penetration/translocation to hazard investigate the role of absorption of biological molecules and characterisation in biological media Investigate effects of dispersion media on NP penetration Investigate whether aerosol exposure alters NP penetration Inhalation Studies to investigate NP toxicokinetics following: • short term exposure • chronic exposure • multi-dose exposure • in pregnant and non-pregnant models
	In all studies conducted, include examination of: • Cells in Bronchio-Alveolar Lavage Fluid • Non-lavageable cells • Lymph • Pleura

ii. Hazard Data Generation for Risk Assessment

Discussion of hazard data generation requirements for risk assessment of NPs led to formation of a tiered set of research priorities. These are outlined in Table 3.

Table 3: Hazard Data Generation Priorities

<table>
<tr><th colspan="3">Hazard Data Generation for Risk Assessment</th></tr>
<tr><td>1</td><td>Use appropriate in vitro barrier models with good tight junctions to investigate uptake and translocation between and through cells:
• Using healthy and compromised cells
• With easily defined controls e.g. poly beads
• Use existing well-established pharmaceutical industry models as a guideline was suggested to avoid attempting to ‘re-invent the wheel’</td><td rowspan="3">For all studies conducted, generate full dose-response data over a wide range of concentrations</td></tr>
<tr><td>2</td><td>Investigate both single bolus dose vs repeated dosing</td></tr>
<tr><td>3</td><td>Validate in vitro models with in vivo models</td></tr>
</table>

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