Chapter One

Nanotechnology, Nanoparticles and Nanotoxicology

1.1 The PARTICLE_RISK project

The evaluation of nanoparticle (NP) toxicity to the liver was conducted as part of the European Union funded project PARTICLE_RISK, which was concerned with revealing the potential human health implications of NP exposure. This was achieved using a variety of different particles (termed the PARTICLE_RISK particle panel) namely; ultrafine carbon black (ufCB), carbon black (CB), single walled carbon nanotubes (SWCNTs), carbon fullerenes (C_{60}), and quantum dots (QDs), all of which have current or foreseeable applications within diverse products, and were fully characterised as part of the project (University of Venice). The distribution of the PARTICLE_RISK particle panel after exposure via injection, inhalation or ingestion was investigated to identify potential targets of toxicity, and conducted by partners within the project (GSF, Munich, and National Research Centre for the Working Environment, Copenhagen). The toxicity of NPs to particular areas of interest, specifically the cardiovascular (University of Edinburgh), pulmonary (National Research Centre for the Working Environment, Copenhagen) and hepatic (Napier University, Edinburgh) systems, was evaluated in more detail *in vivo* and *in vitro*, the findings of which enabled risk assessments to be carried out to evaluate the consequences of human exposure. It is important to highlight, that prior to the receipt of the PARTICLE_RISK particle panel, fluorescent polystyrene particles (of two sizes; specifically 200nm or 20nm diameter) were used as experimental tools to establish protocols for testing the PARTICLE_RISK panel. As a consequence of this work, the data generated provides an insight into the potential for NP uptake and intracellular fate in hepatocytes. These studies provided interesting pilot data and therefore provided the opportunity for discussion regarding the future development of this work. This work could not be completed within the budget or time-frame of this particular project.

It is therefore necessary to consider what NPs are, and why their production and exploitation is anticipated to increase, particularly concentrating on the PARTICLE_RISK particle panel. In addition, evidence that outlines the concern regarding NP mediated toxicity to the liver, and how liver function could be detrimentally affected by NP exposure will be discussed.

1.2.1 What is Nanotechnology?

Nanotechnology is concerned with the controlled design, characterisation, production and application of nano-sized materials (The Royal Society *et al.*, 2004, McNeil 2005). Nanotechnology is therefore involved with the creation and/or manipulation of materials within a nanometre (nm) scale, and has arisen as a consequence of the novel, exploitable properties that materials exhibit within the 'nano' size range (McNeil, 2005, Service, 2004, Hoet, 2004). Specifically, all three dimensions of NPs have a diameter of less than 100nm, and nanomaterials have one or more structural dimension that has a diameter of less than 100nm (BSi report, 2007); consequently the consideration of size is fundamental to the field of nanotechnology, as size is the defining characteristic of all NPs. It is important to highlight that throughout the thesis, the term NP has been used to encompass all nanomaterials and NPs contained within the PARTICLE_RISK panel, although it is acknowledged that particles such as CNTs are not strictly definable as NPs, according to the BSi definitions. An indication of the size dimensions that are relevant within the field of nanotechnology is suggested by the name. Firstly, the prefix 'nano' is derived from the Greek word 'nanos' which is translated as dwarf; so it can be concluded that the formation and utilisation of 'small' things is pertinent (Whatmore, 2006). Furthermore, 'nano' can also be derived from the word nanometre, which is defined as being 10^{-9} m (one billionth of a metre), so that the field is concerned with the generation of products within a nanometre scale (Whatmore, 2006). To put the size of NPs into perspective, a human hair has a width of about 80000nm and a red blood cell has a diameter of 7-8000nm (Whitesides, 2003). The size of NPs is therefore comparable to that of sub-cellular structures, including cell organelles or biological macromolecules such as proteins (McNeil, 2005).

The attraction of producing, and exploiting NPs is a consequence of the fact that the properties of nano-sized materials are expected to, and have been demonstrated to be strikingly different from bulk forms of the same material (Service, 2004). This is beneficial, as properties not previously encountered within substances can be exploited, so that characteristics such as colour, strength, and conductivity (heat and electrical) are drastically altered with modifications in size (Dreher, 2004, Tsuji *et al.*, 2006, Powell and Kanarek,

2006). As a result, the field of nanotechnology has generated substantial interest from many areas that aim to harness the properties exhibited by NPs, so that they can be incorporated into products to enhance or improve upon existing properties, or can be used as novel 'stand–alone' products (Oberdorster *et al.*, 2005b). However, paradoxically it is acknowledged that substances which are ordinarily innocuous can elicit toxicity due to the altered chemical and physical properties that become evident within nano dimensions (Lee, Qi and Copeland 2005).

Nanoscience is involved with unravelling how and why the behaviour of NPs is different to that of their larger counterparts (The Royal Society *et al.*, 2004). Therefore it is important, from a toxicological perspective, to highlight that knowledge of how NPs behave cannot simply be extrapolated from bulk forms composed from the same material, and as a result NPs therefore need to be thought of as distinct entities from their larger equivalents. Consequently, another new field of research which has developed as a consequence of nanotechnology is nanotoxicology. Nanotoxicology is concerned with evaluating the toxicity of NPs, so that the adverse implications associated with NP generation and utilisation are assessed so that their beneficial properties can be exploited safely (Donaldson *et al.*, 2004). Therefore the appeal of exploiting NPs derives from the knowledge that NPs have properties that are distinct from those exhibited by their larger equivalents; however this may also translate to unpredictable, potentially toxic effects becoming apparent within the nano size range that have not been previously encountered in their bulk counterparts, so that NP exploitation can be regarded as a double-edged sword.

1.3 The benefits and dangers associated with NP exploitation

A number of products are being developed, or are currently available that exploit the novel properties that materials exhibit within the 'nano' size range (McNeil, 2005, Service, 2004, Hoet, 2004). Through such applications it is anticipated that nanotechnology will provide great benefits for society in general, as well as financial gains [which is exemplified by the fact that the nanotechnology sector is expected to be worth 1trillion US$ by 2015 (Aitken *et al.*, 2006)]. However it would appear that the technology driving the exploitation

of NPs is ahead of the toxicology assessments that may ultimately limit their use (see later).

1.3.1 Sources of NPs

The existence and exploitation of NPs is not a novel concept, for example gold NPs were used to colour the glass in stain glass windows for centuries (Service, 2004). A number of other NPs are currently produced in large quantities for exploitation within a number of diverse products, for example carbon black (diameter <100nm) has been used as a pigment in printers and photocopiers, and titanium dioxide ($Ti0_2$, micrometre sized) has been used in sunscreens to reflect ultraviolet (UV) light, and in toothpaste and paint to provide a white colour (Powell and Kanarek, 2006). Recently, nano-sized TiO_2 has been used to make sunscreens more aesthetically pleasing, so that these sunscreens retain the ability to reflect UV light to offer the same extent of UV protection as micrometer sized TiO_2, but unlike their larger counterparts appear transparent, and not white in colour (Hood, 2004, Borm *et al.*, 2006), highlighting how the incorporation of NPs into currently available products can improve an existing property. However, although NP containing products have been in existence for a number of years, the consequences of the increased exposure of humans and the environment are largely unknown and recently concerns about their utilisation have materialised.

The deliberate production of NPs (termed engineered or manufactured NPs) either involves the precise configuration of single atoms, a process known as ‘bottom up’ production, or requires the reduction and refinement of bulk forms of materials to nanometre dimensions, termed ‘top down’ procedures (The Royal Society *et al.*, 2004, Silva, 2004) to generate the desired product. NPs can thereby be constructed to predefined requirements due to the ability to precisely configure and/or manipulate atoms and molecules within a structure. The exposure of humans to NPs can be described as intentional, whereby NPs are manufactured for a specific purpose, and are termed engineered NPs which are generated to exploit the size related properties evident at a nano-scale. Exposure to engineered NPs can occur in occupational, consumer and environmental (released during their manufacture or use) settings. In addition exposure of humans to NPs can be accidental or unintentional, originating from

natural, (such as NP release from forest fires, and volcanoes) or anthropogenic (arise as a consequence of human activities for example NP release from vehicles) sources (Borm *et al.*, 2006).

1.3.2 Risk assessment of the impact of NP exposure on human health

1.3.2a The evaluation of risk

NPs are currently being generated by universities, emerging NP companies and existing chemical and pharmaceutical companies (Aitken *et al.*, 2004). As a result, it is necessary to consider any detrimental health consequences associated with this increased production or utilisation of NPs. This is achieved by conducting risk assessments, where risk=hazard x exposure. Consequently, it is required that hazards associated with NP exposure are identified, and that this assessment is based on evidence of NP toxicity within *in vitro* or *in vivo* systems or on the knowledge of the repercussions of NP exposure on human health.

1.3.2b The consideration of exposure levels when assessing risk

It is necessary to consider NP exposure levels, as according to Paracelsus all materials are toxic if exposure occurs in sufficient quantities (Timbrell, 1999). Consequently before interpreting toxicological evidence that has identified hazards, the extent of human exposure should be assessed, which is likely to vary within consumer, occupational and environmental settings. The generation of NPs is expected to potentially expose workers to higher concentrations than the consumer. However workers are generally healthy, and it is generally within susceptible individuals that adverse health effects are induced by particulates (Oberdorster, 2000), and NPs are generally produced within enclosed systems, so the likelihood for exposure is low. In addition, it is likely that health and safety measures are enforced within workplaces to protect workers from NP exposure, including, for example, the use of face masks and gloves.

Despite the fact that NP containing products are currently on the market (see section 1.4), there is a lack of human exposure data, which represents a significant research need, and can fuel scepticism surrounding their exploitation due to the uncertainty of the implications of NP exposure on human health. NPs are often bound within materials, such in sports equipment, so that it is

unlikely that NPs are available in a ‘free form’, however the potential for their release, for example during their disposal, must be considered (Stern and McNeil, 2008). Consequently focussing risk assessments on materials that allow NPs to be biologically available is of greatest relevance. Benn and Westerhoff, (2008) investigated the quantity of silver NPs released from a range of commercially available socks (that contained silver NPs as an anti-microbial agent). It was revealed that the socks contained silver particles that ranged from 10-500nm in diameter, and that the silver NPs leached from the socks when they were washed. This has several consequences, for example silver NPs are transferred to the environment during washing, and that silver NPs were contained within the socks in reduced quantities, so that it is anticipated and the anti-microbial benefit afforded by the incorporation of silver NPs into the socks will be lost.

1.3.2c NP production volume influencing risk assessments

It is of relevance that the assessment of NP risk needs to take into consideration if NP production and exploitation will occur to the relatively high levels that have been anticipated. Some NPs are difficult to produce in large quantities, so that in fact the levels of manufactured NPs are sometimes only apparent within a small scale (Baughman Zakhidov and de Heer, 2002) limiting their potential exploitation, and hence likelihood for exposure. However this was the case in 2002 but due to the widespread interest in NP exploitation and financial investment in technologies that allow production to be scaled up, an increase in NP generation is now being realised in order to meet the demand (Aitken, 2006). For example, the Mitsubushi Corporation in Japan opened a plant (Frontier Carbon Corporation) to generate carbon fullerenes on a large scale, with production quantities increasing from 4 to 40 tons/year; however these production levels have not been verified by the company (Chaudhry *et al.*, 2005, Aitken, 2006). This is important, as it is likely that the NPs that can be most easily produced in large quantities will be exploited to the greatest extent within commercial applications potentially leading to greatest exposures and therefore risks (Aitken, 2006). However, risk assessments should not be totally driven by volume of production, as those NPs that have the potential to be very hazardous, even if made in relatively small amounts, should also be considered a priority.

1.3.2d Risk/Benefit analysis

The successful exploitation of NPs will require risk/benefit evaluations which allow a greater understanding of the implications of NP exposure, because if human health is endangered, the use of NPs will flounder (Maynard, 2007). Consequently, the risks associated with NP exposure need to be identified, and the probability of exposure occurring determined, so that the full potential of nanotechnology can be realised. Although there are uncertainties regarding the risks associated with newly engineered NPs (which is driving their toxicological assessment), analogies have been made between the potential health effects related to NP exposure and to those associated with ultrafine particle exposure (realised within epidemiological studies; see section 1.6), which leads to the concern regarding increased NP generation and exposure. Currently, the risk assessment of engineered NPs is severely lacking, but this largely derives from the large number of available NPs requiring toxicological assessment, lack of information regarding exposure levels of humans, and the uncertainty regarding the relevancy of extrapolating the toxicity of engineered NPs from existing NP toxicological databases, or from bulk forms of NPs (Dreher, 2004). Furthermore it is important to highlight that the growing concern surrounding NP exploitation derives from the uncertainty surrounding their potential toxicity, as they may in fact be safe. Consequently nanotechnology directs research towards the potential negative impacts of NPs as well as determining the benefits their exploitation offers, to provide an element of responsible research. Consequently, the unknown risks of engineered NPs need to be identified to determine if they outweigh their established benefits for society, with the uncertainty of the outcomes on human health causing the greatest scepticism (Colvin, 2003).

1.4 Current and expected examples of NP exploitation

A number of products have been introduced, or are being currently developed, as a consequence of NP exploitation within a number of diverse areas. The pharmaceutical industry has interest in NPs, as they can improve the diagnosis and treatment of disease, for example silica NPs have been used as a drug carrier for salicyclic acid, which specifically accumulated within inflamed colonic tissues to allow for the specific delivery of medication to inflamed sites (Moulari *et al.*, 2008). Furthermore NPs may also have a role in the treatment of cancer,

for example zinc oxide NPs have been demonstrated to discriminate between cancer and 'normal' cells, to enable to the preferential killing of cancer cells (Hanley *et al.*, 2008). The ability of NPs to impact on cancer treatment arises as a consequence of their preferential delivery to tumour sites, due to the increased blood flow associated with tumour development, and 'leakiness' of the associated vasculature. NP utilisation is also relevant within a clinical setting as the diagnosis of disease may be improved, for example NPs (such as quantum dots) can allow the visualisation of cancer cells within the body (Sharma *et al.*, 2006). The textiles industry contributes to increased NP generation, as NPs have been incorporated into a number of commercially available products such as socks (Benn and Westerhoff, 2008), and 'sleepsafe' pyjamas (Marks and Spencer's) which were designed to protect hospital patients from MRSA infection (http://www.telegraph.co.uk/news/uknews/1570889/MandampS-pyjamas'-silver-lining-helps-stop-MRSA, accessed on 6th October, 2008) which take advantage of the antibacterial properties of silver NPs. The cosmetics industry is already exploiting NPs within sunscreens, toothpastes, and moisturisers, to name a few; for example the cosmetics company Lancome has incorporated silica/protein NPs into face creams which are believed to exhibit anti-aging properties (http://www.nanotechproject.org/inventories/consumer/browse/categories/health_fitness/cosmetics/page6/ accessed on 6th October 2008). Sports equipment can be improved through the use of NPs, for example the Japanese company ABS used C_{60} NPs to make bowling balls scratch resistant http://www.rsc.org/chemistryworld/Issues/2005/December/nano.asp accessed on 6th October 2008). The electronics industry also has an interest in the utilisation of NPs as they have been used as LCD displays on laptops and televisions(http://www.nanotechproject.org/inventories/consumer/browse/categories/electronics_computers/display/ accessed on 6th October 2008). A number of products that contain NPs have been discussed, and demonstrated the diverse fields that exploit NPs; the Woodrow Wilson online database lists products that have been identified as containing NPs (http://www.nanotechproject.org/index.php?id=44 accessed on 6th October 2008).

It is known that the incorporation of NPs into products can enhance their function, this is exemplified by the introduction of the Samsung washing machine that has a 'silver wash' setting which is proposed to coat clothes with silver NPs to keep them bacteria and fungus free, in attempt to improve the odour of the clothing. There are a number of additional benefits, such as reduced energy consumption, and the fabric will have a lesser tendency to shrink due to the lower temperatures that can be used (http://ww2.samsung.co.za/silvernano/silvernano/ accessed on 6th October 2008). However, there is debate surrounding the constituents of the silver wash, as the product may not release silver NPs but silver ions. It would therefore be useful, from a risk assessment perspective, to define what exactly constitutes a NP is defined.

When the exact composition of a product is unknown, NPs may be wrongly accused of being responsible for toxic outcomes in humans. This is exemplified by the German product 'Magic Nano' (Kleinmann GmbH) which was generated as a bathroom cleaner and aimed to keep surfaces dirt repellent. After its use a number of consumers suffered from respiratory problems, and six hospitalisations were reported (http://www.washingtonpost.com/wp-dyn/content/article/2006/04/05/AR2006040502149_pf.html accessed on 6th October 2006). As a consequence of the adverse health effects associated with the use of this product, Magic Nano was recalled, and the constituents responsible for the observed toxicity were investigated. Initially it was unknown if the product contained NPs, as the company would not disclose details of its composition due to patent reasons, but the product's name suggested that it did. It was subsequently revealed that the product does not contain NPs. However the uncertainty of the product's composition corresponded to controversy surrounding the future exploitation of NPs, due to the fact that public acceptance is able to influence the success of new technologies. This also provides the impetus for ensuring that the composition of products is specified, and that a full safety evaluation of NP containing products is carried out before their widespread use commences, so that society has confidence that NP containing products are safe and ensures that consumers can make informed decisions about the products they are buying. NPs being falsely accused of toxicity and increased media interest surrounding such problems

could lead to increased social awareness and scepticism (based on real or perceived risks) surrounding NP exploitation which may impact on their integration into products and their popularity (Nel *et al.*, 2006).

As demonstrated by the above examples, nanotechnology is anticipated to have a broad impact in society, due to the variety of 'fields' that will utilise NPs and the number of products that will contain them. This emphasises the need for the evaluation of toxicological outcomes associated with NP exposure, which is information that is currently lacking. This is of importance as concern regarding NP toxicity may ultimately restrict their utilisation despite the widespread and beneficial applications that have been promised (Seaton, 2006).

1.5 The importance of NP physicochemical characteristics

Although size is a commonality between different NPs, there are a number of variables that could impact on NP toxicity, as NPs vary with respect to shape, composition, structure, surface area, charge, surface coating, and aggregation state (Tsuji *et al.*, 2006). It is therefore necessary to reveal if the diversity of NPs available will confer to a varied extent and mechanisms of toxicity to determine if generalisations can be made about NP behaviour, as it may transpire that certain NP properties drive NP toxicity.

1.5.1 The impact of particle size on NP behaviour

The size of NPs is specifically relevant within the field of nanotechnology, due to the fact that it is believed that it is within nano dimensions (1-100nm) that materials are most likely to have altered properties (The Royal Society *et al.*, 2004). One of the main reasons believed to be accountable for changes in NP behaviour is the increase in surface area that arises as a consequence of their small size, when compared to their larger counterparts. Therefore as particle size decreases, particle surface area per unit mass increases, which equates to a greater proportion of its atoms/molecules being displayed on the surface of the material (Nel *et al.*, 2006). This is confirmed by the demonstration that the toxicity of NPs is best correlated to the surface area of particles, and not when exposure is expressed as a mass dose (Oberdorster, 2000). The importance of increased surface reactivity in contributing to the greater reactivity of nano-materials, is demonstrated when NPs are exploited as catalysts, as substances such as gold which are ordinarily inert can acquire the property of catalysis

within nano dimensions (Roduner, 2006). 'Quantum effects' become apparent, due to the size range within which this field is constrained (The Royal Society *et al.*, 2004) and can be described as properties that are not previously encountered in larger forms of the same material, so that materials exhibit size dependent phenomena. Therefore the small size of NPs results in electron confinement and restricted bond angles, which explains the emergence of unique physicochemical properties, which not only make NPs useful for exploitation in new applications and products, but also potentially determine their biological reactivity and toxicity (Stone and Kinloch, 2007). The size dependent toxicity has been repeatedly demonstrated *in vitro* and *in vivo*, which had confirmed that toxicity increases as particle size decreases (see for example Li et al., 1999).

1.5.2 The importance of aggregation and agglomeration to NP properties

The terms agglomeration and aggregation are often used interchangeably to describe the attractions that hold together a collection of particles (Oberdorster *et al.*, 2005b). However it is more appropriate to consider NP aggregation and agglomeration as distinct phenomena; as agglomerates are formed by clusters of NPs that are held together by electrostatic interactions, whereas aggregates are formed from fused NPs (by covalent bonds or sintering) that are not easily separated, and it is also possible for aggregates of NPs to agglomerate (Oberdorster, Stone and Donaldson, 2008). Therefore the tendency for NPs to interact and form larger structures must be considered, as materials may be 'made up' from particles <100nm, but that human exposure does not occur to individual particles within the nano size range. When aggregated, NPs can loose properties which are associated with their small size (ECETOC report, 2006), therefore the existence of 'free' monodispersed NPs is important when undertaking experiments that evaluate NP toxicity, as it affects how NP suspensions (administered to animals, humans and cells) are formed for use in toxicity tests. Therefore considering how NPs are encountered within humans is important when assessing their toxicity *in vitro* as it can impact on how they are dispersed, to more accurately reflect the exposure conditions experienced *in vivo,* and is important in retaining their exploitable properties. Therefore it is known that a number of physiologically relevant substances can be used to aid in the dispersal of NPs within toxicity tests and can be relevant to how NPs are

encountered by humans; for example, NPs will be exposed to surfactants present within the lung lining fluid on exposure when inhaled, and serum proteins when NPs are contained within blood. However, although determining the aggregation and agglomeration of NPs is important, Ferin *et al.,* (1992) demonstrated that aggregates of ultrafine TiO_2 were more toxic than their larger counterparts which formed similarly sized aggregates. Consequently, the size of the particles that contribute to aggregate formation appears to be critical.

1.5.3 NP behaviour is dependent on their shape and structure

NPs are available in different morphological 'types', for example they can be spherical, tubular, rod or irregularly shaped, and has been a basis for classifying NPs (Aitken *et al.*, 2006). Carbon is an example of a substance that is present in a number of naturally occurring forms, including graphite and diamond. Within the nano–size range, carbon is available in a number of different forms, for example ufCB has an approximately spherical structure (diameter of <100nm,) carbon nanotubes have a cylindrical structure and carbon fullerenes (also known as buckyballs) contain 60 carbon atoms arranged in a football-like cage structure (figure 1.1). The availability of a number of carbon based NPs demonstrates that despite having the same composition, the arrangement of the atoms within the structure dictates its shape, classification and properties. In addition, although carbon nanotubes are known to have a similar structure to graphite, it is not accurate to extrapolate nanotube behaviour from that of graphite (Dreher, 2004).

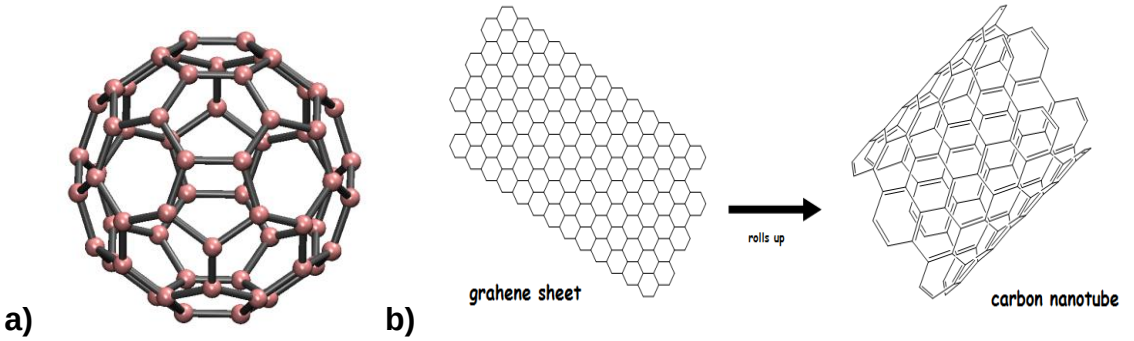


Figure 1.1 The different forms of nano-carbon. Carbon fullerenes, **(a)** have a cage–like structure and is formed from 60 carbon atoms, Carbon nanotubes **(b)** resemble a rolled up graphite sheet (imaged adaped from http://www.nanotech-now.com/nanotube-buckyball-sites.htm (accessed on 6th October 2006)), and may be 'capped' by a fullerene molecule. The pictures were generated by Nadia Vahdati (University of Southampton).

1.5.4 The chemical composition of NPs is diverse

NP composition has been the basis for NP categorisation; namely carbon based materials (including CNTs and C_{60}), organic polymer NPs, and inorganic NPs such as those based on metal oxides (for example cerium oxide), and metal derived NPs [such as gold, and quantum dots (Ju-Nam and Lead, 2008)]. NPs can therefore be composed from a variety of substances including carbon, iron, titanium, gold, silver, aluminium and cadmium, to name a few. A number of different NP 'types' can be developed from one main constituent, for example, as described above carbon is the basis for carbon fullerenes, carbon nanotubes, and ultrafine carbon black (ufCB), which vary with respect to how the carbon atoms are arranged within the structure of the particle. Conversely, one NP may contain a number of different constituents (for example, quantum dots), making its structure more complex. It is also relevant to consider the purity of NPs, as impurities may arise from their production or contaminants may become attached to the NP when incorporated into a product or when released into the environment, and these contaminants have the potential to contribute to NP toxicity (Dreher, 2004).

1.5.5 The importance of surface attachments to NP properties

The surfaces of NPs contain the components that interact with the biological system and thus it is necessary to consider the NP 'surface' with which the body interacts, as it may be a basis for their toxicity (Donaldson, Li and MacNee, 1998). The surface of NPs can be modified through the surface conjugation of moieties which allow NPs to be tailored to the desired specification, so that the manipulation of the NP surface confers advantageous properties such as increasing its solubility or specific targeting of NPs to particular sites within the body (McNeil, 2005). The influence of surface alterations to NP toxicity requires evaluation, as it has become apparent that the attachment of moieties to the NP surface may alter NP activity (including the introduction of toxicity) or the behaviour of the surface moiety, so that the NP or surface moiety may not fulfil its desired purpose (Karajanagi *et al.*, 2004).

1.5.6 Influence of charge on NP behaviour

The surface charge of NPs can be neutral, positive or negative, with the charge of NPs being dictated by its composition and surface attachments. Charge has been shown to influence the toxicity of NPs, for example Lockman *et al.,* (2004)

demonstrated that positively charged NPs were most toxic to the blood brain barrier, leading to the conclusion that NP toxicity may be charge dependent.

1.6 Why is there concern regarding NP utilisation?

Interest in evaluating the toxicity of NPs stems from two main findings which independently recognised that as particle size decreases, toxicity generally increases. Firstly it was demonstrated that following inhalation the inflammogenic potential of titanium dioxide (TiO_2) was size dependent, specifically that ultrafine TiO_2 (21nm diameter) invoked a greater inflammatory response in rats when compared to that of fine particles (250nm diameter) of identical composition, and equivalent mass (Ferin *et al.,* 1992). This finding had a number of implications, specifically that the use of TiO_2 as a 'control particulate' (as it was previously assumed to be a particulate that did not induce an inflammatory response on exposure) in experiments that examined the pathogenicity of particles such as quartz (Donaldson and Tran, 2002), had to be re-evaluated.

At the same time, epidemiological studies found a positive correlation between the level of particulate air pollution and increased morbidity and mortality rates in both adults and children, with adverse health effects being manifested predominantly in susceptible individuals who had pre-existing pulmonary or cardiovascular disease (Schwartz 1994a, Dockery *et al.*, 1993, Pope and Dockery, 1999). PM_{10} (defined as particulate matter collected through a size selective inlet that has a 10µm cut off, and 50% efficiency) is thought to be responsible for mediating these adverse health effects, and is known to contain a complex mixture of components with ultrafine particles, transition metals and endotoxin, all thought to contribute to the observed toxicity (Donaldson and Stone, 2003, Stone, 2000). The ultrafine particle component has been held principally accountable for eliciting much of the toxicity associated with PM_{10} exposure (Seaton *et al.*, 1995), which was complemented by epidemiological studies conducted by Peters *et al.,* (1997) where respiratory ill health was associated with the number of ambient ultrafine particles. The 'ultrafine hypothesis' was also supported by findings that demonstrated the size dependence of $Ti0_2$ toxicity conducted by Ferin *et al.,* (1992), as described above. Therefore particles within this size range (<100nm diameter) were

thought to be particularly hazardous to health. The size dependency of particle toxicity was subsequently extensively investigated but only using a limited number of materials, including carbon black (Li *et al.*, 1999, Brown *et al.*, 2000), and polystyrene beads (Brown *et al.*, 2001) to confirm the generalisation that ultrafine particles were more toxic than fine particles. It is now relevant to determine the applicability of findings to the toxicity of engineered NPs, to determine if the same generalisation exists.

Knowledge on how ultrafine particles behave therefore provides the basis for concern regarding engineered NP production and utilisation, whilst also providing the foundations for the research when investigating potential mechanisms of NP toxicity, by directing the most appropriate experimental approaches that should be considered with highest priority (Kipen and Laskin, 2005, see sections 5.1.2, 5,1.3, 5,1.4 and 5.1.6). The perceived risks associated with NP utilisation have therefore emanated from their small size, due to knowledge on how ultrafine particles behave, in comparison to their larger equivalents. However, nanomaterials are defined as having one dimension that is <100nm, but ultrafine particles and NPs are defined as having all dimensions <100nm (BSi Report, 2007). Therefore, although all ultrafine particles can be described as NPs not all nanomaterials can be described as ultrafine particles; consequently the extrapolation of findings should be approached with caution. The toxicity of a limited range of compositions and structures were previously tested within ultrafine particulate studies, as surrogate particles that aimed to represent particulate air pollution exposure were focused on. These studies provided a useful basis for assessing the toxicity of NPs (in terms of directing the most appropriate toxicological endpoints that should be considered with highest priority), but limits the extrapolation of the ultrafine toxicity study findings to the field of nanotoxicology where a wider variety of NP compositions (of more complex structures) are evident within engineered NPs. This is important as there is already evidence that ultrafine particles, of a variety of compositions and forms, do not behave similarly, so that their toxicity is not comparable. Dick *et al.,* (2003) used four different ultrafine particle types (carbon, cobalt, nickel and titanium dioxide) to determine the attributes of particles that influenced their toxicity *in vitro* and *in vivo*. It was found that the inflammatory response elicited by the different

particle types was not comparable, so that their toxicity can be ranked in the following order; carbon=cobalt>nickel>titanium dioxide. In addition, Xia *et al.,* (2006) compared the toxicity of ultrafine ambient particles, polystyrene NPs (positive and negative) and engineered NPs (carbon black, TiO_2, C_{60}) to macrophages, and found that ambient ultrafine particles and positively charged polystyrene NPs were most capable of inducing toxicity, suggesting that composition and charge was able to influence toxicity.

The documented toxicity of ultrafine particulates therefore serves as an early warning for the potential hazards associated with NP exposure (Donaldson *et al.*, 2006). It is also foreseeable, and therefore of concern, that the release of NPs into the environment could increase the concentration of atmospheric particles (ECETOC report, 2006), which has obvious health implications, when considering the epidemiological findings of PM_{10} toxicity.

1.7 Susceptibility to the effects of NP exposure

Increased exacerbations of inflammatory based respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) have been reported as a consequence of PM_{10} exposure (Pope and Dockery, 1999), and have been hypothesised to arise due to an increase in the 'background level' of inflammation induced by particulate exposure (MacNee and Donaldson, 2000). A predisposition to the adverse health effects elicited by PM_{10} may also derive from the fact that clearance mechanisms in these individuals are compromised due to pre-existing disease, so that particles remain in the lung for longer periods, increasing the likelihood of a detrimental response manifesting (Adamson and Prieditis, 1995). Clarke *et al.,* (1999) characterised the effect of particulate matter exposure on animals with pre-existing disease (to mimic the pathology observed in susceptible human populations) and normal rats, and provided evidence that animals with chronic bronchitis were more susceptible to the detrimental effects of particle exposure than normal rats, thereby supporting epidemiological findings.

1.8 Routes of NP exposure

A number of exposure routes are associated with NP utilisation and production that arise as a consequence of their potentially diverse applications; with

inhalation, dermal, ingestion and injection exposures are all relevant and may occur as a consequence of NP manufacture, utilisation, or release into the environment (figure 1.2). It is acknowledged that human skin, lungs and the gastrointestinal tract (GIT) are in contact with the external environment, and as a consequence have barrier functions to prevent the entry of substances into the body (Hoet, Bruske-Hohlfeld and Salata, 2004). However it also possible that NPs are able to translocate from their site of exposure, thus providing the potential for NP toxicity at multiple targets located at a distance from their portal of entry (figure 1.2).

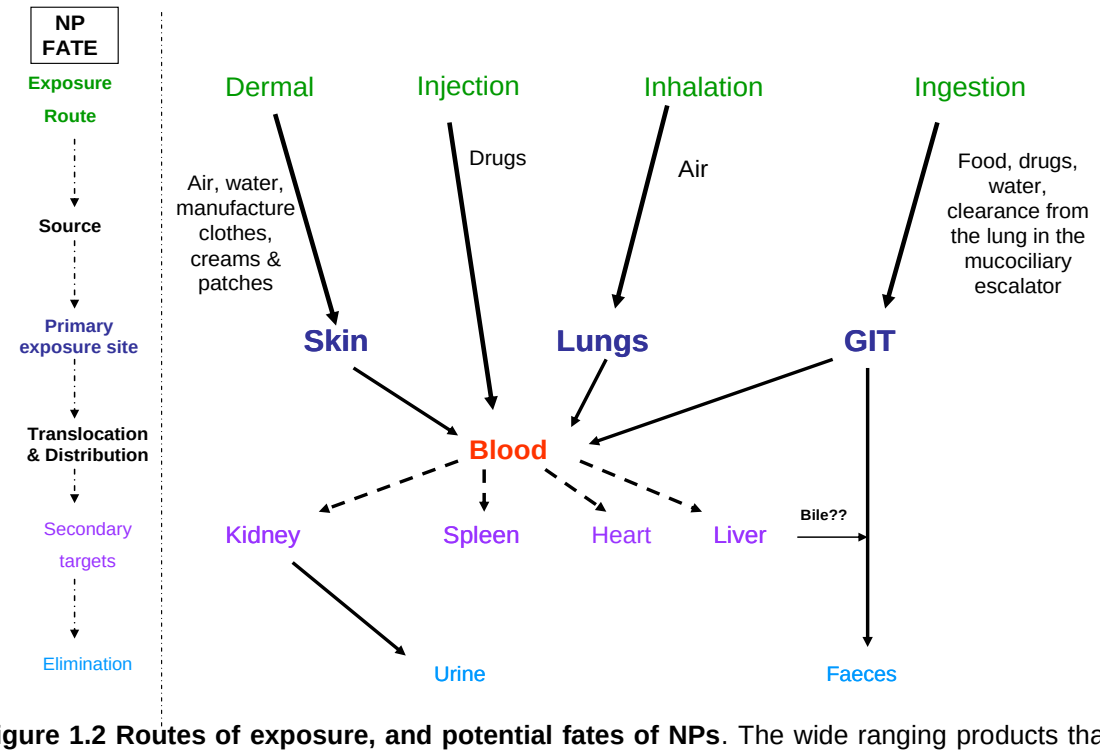


Figure 1.2 Routes of exposure, and potential fates of NPs. The wide ranging products that are expected to contain NPs allow exposure via a variety of routes. A primary target of NP toxicity is considered if toxicity has the potential to occur at the site of exposure. If toxicity occurs as a consequence of the translocation of NPs to sites distal to that of the portal of entry then the organ is considered a secondary target (with some, but not all examples outlined). The ability of NPs to translocate to distal sites is dependent on the barrier function and clearance mechanisms evident at the exposure site. Adapted from Oberdorster et al., (2005b)

1.8.1 Toxicity of NPs to the skin

The direct application of NP containing products to the skin is already existent, as NPs are currently present within a number of cosmetic products including sunscreens [which constitutes one of the largest applications of NPs (mainly TiO_2 and ZnO) where the global production for this purpose was estimated at

1000tonnes in 2003-2004 (Stern and McNeil, 2008, Nohynek, Dufour and Roberts, 2008)]. In addition, there is also the potential for airborne NPs to come into contact with the skin, as NPs contained within clothes. Furthermore it is also necessary to consider the possibility that the exposure of skin to NPs may allow for NP ingestion as a consequence of hand to mouth contact (Aitken *et al.*, 2006).

It has been demonstrated that NP characteristics are able to influence the penetration of NPs within skin; for example quantum dots (QDs) that have a neutral or positive charge were observed to penetrate into the dermis and epidermis of intact porcine skin, and negatively charged QDs were only able to do so to a more limited extent (Ryman-Rasmussen, Riviere and Monteiro-Riviere, 2006). However, it is of relevance that NP containing products may be applied to damaged skin, which may allow for a greater toxicity of NPs (Mortensen *et al.*, 2008). Mortensen *et al.,* (2008) evaluated the effect of skin condition to the penetration of QDs, and utilised ultraviolet radiation (UVR) to induce a mild level of sunburn. It was found that higher levels of QD penetration, past the stratum corneum (protective barrier of the skin), within the epidermis and dermis, were apparent in mice that had compromised skin. However there was also evidence of QD penetration (to the epidermis) within intact skin, but this was apparent to a more limited extent. Zhang *et al.,* (2008) also observed that penetration of neutral CdSe QDs within the skin was limited (where they reached the outer stratum corneum). Further studies demonstrated that badly damaged skin (by dermal abrasion) allowed the deeper penetration of QDs (Zhang and Monteiro-Riviere, 2008) that may initiate inflammation within the skin *in vivo*, due to the potential of QDs to elicit an inflammatory response by keratinocytes *in vitro* (Zhang *et al.,* 2008). The observations made using QDs parallel the findings of other NPs, such as TiO_2, that has been observed to remain within the stratum corneum, with limited further penetration in intact skin (reviewed by Nohynek, Dufour and Roberts, 2008). The findings therefore suggest that compromised skin (for example that is sunburnt or contains cuts or sores) permits NPs to access deeper layers of the skin, where their potential to cause damage is greater, but intact skin (whose integrity is not compromised) appears to protect against the penetration of NPs thereby fulfilling its barrier function, highlighting the importance of skin condition to NP penetration.

Consequently, most studies support the contention that healthy, intact skin acts as a good barrier to NPs. Therefore determining the ability of NPs to penetrate healthy or compromised skin is of relevance and may have toxicological consequences for the underlying skin cells, or allow their translocation to secondary sites (Mortensen *et al.*, 2008).

1.8.2 Pulmonary toxicity of NPs

Inhalation of NPs is likely to occur as a consequence of NP release into the environment; which could arise during their manufacture or utilisation. The consequences of lung exposure to ultrafine particles have been discussed previously (see section 1.6). Therefore if engineered NPs behave similarly, or contribute to the NP burden within air, then their propensity to elicit pulmonary damage, subsequent to inhalation, is of concern, and has been realised within recent studies which demonstrated that SWCNTs were able to elicit pulmonary toxicity subsequent to inhalation (Shvedova *et al.*, 2008). The translocation of NPs from the lung into the blood, to allow their distribution to other organs is also of concern as it provides the opportunity for multi-organ exposure and toxicity.

1.8.3 NP toxicity to the GIT

The ingestion of NPs is expected, as it is likely that NPs will be contained within drugs, water and food products that are consumed daily and so the toxicity of NPs to the GIT is of concern. Furthermore, if NPs are cleared via the mucociliary escalator from the lungs they may eventually become evident within the GIT, where NPs have the potential to pass into the systemic circulation, and become distributed within the body; as has been observed for copper NPs (25nm diameter) which translocate from the gut to the blood after oral administration, and subsequently distribute to other organs (Chen *et al.*, 2006). Furthermore Jani *et al.* (1990) demonstrated that 50nm (to a greater extent) and 100nm polystyrene microspheres were absorbed across the gut wall and redistributed to the liver (and other target organs) after oral exposure. NP presence within the GIT may therefore enable NPs to elicit toxicity in a number of secondary targets due to their transfer into blood.

1.8.4 NP presence within blood

In addition to the translocation of NPs into blood, subsequent to pulmonary, dermal and GIT exposure, direct injection of NPs is also expected due to their exploitation within drugs and diagnostics. The injection of NPs into the blood will allow for their distribution to multiple targets. In addition the presence of NPs within blood is of concern as NPs have been illustrated to have pro-thrombotic effects within blood vessels in mice (Khandoga *et al.,* 2004) and hamsters (Nemmar *et al.*, 2003), to compromise blood flow and contribute to the development of cardiovascular disease due to the occlusion of a critically situated vessel. Furthermore NPs have been demonstrated to encourage the development of inflammatory processes by endothelial cells *in vitro*, which could contribute to the progression of inflammatory events within blood vessels, such as atherosclerosis (Oesterling *et al.,* 2008). Furthermore, it is also necessary to encompass the possibility that NPs may exert toxicity at their exposure site that results in the release of systemically acting factors into the circulation that mediates toxicity at secondary sites, at a distance to their site of release.

1.8.5 Elimination of NPs

It is necessary to consider the excretion of NPs from the body, as NP elimination has the potential to limit their toxicity, and is necessary for terminating the activity of NP based drugs or diagnostics. The excretion of NPs within urine necessitates that they are removed by the kidneys, which is likely to be a size dependent phenomenon due to the limitations of renal filtration (which allow molecules <8nm to undergo renal clearance (Longmire *et al*., 2008)), and also requires that substances are hydrophillic. The accumulation of NPs within faeces would require that NPs become present within the GIT which could occur subsequent to ingestion or may be mediated by the liver through the elimination of NPs within bile. Singh *et al.,* (2006) demonstrated that SWCNTs and multi walled carbon nanotubes (MWCNTs) were excreted as intact nanotubes within urine, subsequent to i.v. exposure, indicating the potential for NP elimination from the body, but this was promoted by their functionalisation and short length. Chen *et al.,* (2008) observed that silica coated CdSe QDs distributed to the liver and kidneys, with evidence of elimination within faeces and urine subsequent to tail vein injection, with the excretion of QDs within faeces occurring more quickly and to a greater extent than that of urine, with the QDs thought to remain intact due to stability tests that were carried out.

However, the CNTs and QDs used within the studies described were functionalised to make them more water soluble, which would promote their elimination in urine. In addition, evidence that NPs are eliminated within faeces, subsequent to i.v. exposure is suggestive that the liver contributes to their elimination, due to their secretion into bile. The elimination of NPs is likely to be related to their biopersistence, so that NP composition can dictate the likelihood of breakdown and therefore longevity within the body.

1.8.6 Cell targets of NPs

A number of targets, often composed from multiple cell types, are at risk from NP toxicity. Therefore when conducting toxicity tests it is relevant to determine the cells that would be most detrimentally affected by NP exposure or to investigate what cells would contribute to particle clearance as part of host defence. Consequently, *in vitro* studies that investigate the toxicity of NPs have isolated primary cells from the tissue of interest or utilised cell lines. A vast array of cell lines have been utilised that concentrate on the toxicity of NPs at their portal of entry (skin, lung and GIT derived cells) or known sites of NP accumulation (such as those derived from the liver, heart, endothelial cells, and neuronal system) and will be frequently referred to when discussing evidence of NP toxicity, and so have been outlined in table 1.1.

Table 1.1 The species and tissue origin of cell lines. Cell lines have been used to evaluate the toxicity of NPs to the various cellular targets of NPs, and those discussed with the thesis have been outlined, with information regarding the species of origin, organ or tissue and cell type listed.

Tissue of origin	Cell line	Species of origin	Cell type
Lung	A549	Human	Epithelial type 2 cells
	L2	Rat	Epithelial type 2 cells
	SV40T2	Rat	Epithelial type 2 cells
	C10	Mouse	Epithelial type 2 cells
	NR8383	Rat	Alveolar macrophage
	BEAS-2B	Human	Bronchial epithelial cells
	16-HBE	Human	Bronchial epithelial cells
	Calu-3	Human	Bronchial epithelial cells
Skin	A431	Human	Epidermoidal
	HaCaT	Human	Keratinocyte
	HDF	Human	Fibroblast
	B16-F10	Mouse	Melanoma cells
	HT-1080	Human	Fibrosarcoma cells
Liver	HepG2	Human	Hepatocyte
	C3A	Human	Hepatocyte
	BRL3A	Human	Hepatocyte
Neuronal	NHA	Human	Astrocytes
	SBN19	Mouse	Glioblast
	T98G	Human	Fibroblasts
	N9	Mouse	Microglia
	PC12	Rat	Pheochromocytoma cells
Heart	H9C2	Rat	Cardiomyocytes
	HUVEC	Human	Vascular endothelial cells
	HAEC	Human	Aortic endothelial cells
Kidney	Vero	Money	Epithelial cell line
Blood	RAW 264.7	Mouse	Macrophage-like
	J774	Mouse	Macrophage-like
	MM6	Human	Monocyte
	THP-1	Human	Monocyte
	WTK1	Human	Lymphoblast
GIT	Caco-2	Human	Epithelial cells
Breast	MCF-7	Human	Adenocarcinoma cells
Cervix	HeLa	Human	Epithelial cells
Embryo	STO	Mouse	Fibroblasts

1.9 What particles will be used to investigate NP toxicity to the liver?

Evaluating the toxicity of NPs is still in its infancy due to the large variety of NPs available or currently under development, and number of potential targets within the body, so that presently the human health implications of NP exposure are not fully understood. However there are an increasing number of studies being conducted to determine what attributes of NPs contribute to their toxicity. Furthermore, although studies that examined the toxicity of ultrafine particles may provide insight into how NPs exert toxicity, only limited compositions and structures were tested, as studies were concerned with particles that represented the composition of PM_{10}. In contrast, NPs are a diverse group of substances, and as such a number of different types need to be evaluated in toxicity tests, to reveal if some NPs are more toxic than others, and if NPs exert toxicity through different mechanisms. The toxicity of the PARTICLE_RISK particle panel to the liver will be evaluated, therefore it is necessary to outline what NPs are included within the panel and why there is concern surrounding their exploitation.

1.9.1 Quantum Dots (QDs)

QDs are spherical in shape and generally are between 2–100nm in diameter (Hardman, 2006). Structurally, QDs consist of a core which is often surrounded by a shell that has attached surface moieties that can improve, for example, solubility or tissue targeting (Hardman 2006, Ozkan 2004). The QD core can be composed from a variety of metal complexes including cadmium selenide (CdSe) or cadmium telluride (CdTe). The shell, composed from, for example zinc sulphide is necessary to stabilise the structure of the QD, so that the core is not exposed (Sharma *et al.*, 2006). If the shell of the QD is degraded, QD stability is compromised, and the core components are released, which is of importance when evaluating the potential toxicity of QDs, as toxicity may only arise under conditions that render the QD unstable (Hardman, 2006). Furthermore, it may be difficult to come to general conclusions about QD toxicity due to the diversity of QDs available, as QDs are not a uniform population, which is related to the variety of cores, shells, and surface coating composition possibilities that are available (Hardman, 2006). The most valuable property of QDs is their fluorescence, and so they are expected to be exploited as imaging agents *in vivo* and *in vitro* (Sharma *et al.*, 2006).

There is evidence that QDs are able to localise within the liver after exposure, to potentially exert toxicity. Akerman *et al.,* (2002) investigated the possibility of targeting QDs to specific tissues, through the attachment of different surface moieties. It was observed that regardless of the surface coating, QDs

accumulated within the liver, and that Kupffer cells and liver sinusoid endothelial cells primarily participated in the clearance of the QDs, which was reduced by the presence of PEG on their surface. The main concern regarding the potential toxicity of QDs to the liver derives from the fact that they contain cadmium, which is a known liver toxicant (Patrick, 2003). The stability of QDs will therefore be of critical importance in dictating the outcome of liver exposure, as it may be dependent on cadmium release from the QD core. Derfus, Chan and Bhatia, (2004) demonstrated that cadmium release from QDs was responsible for the toxicity of QDs.

1.9.2 Carbon Nanotubes (CNTs)

CNTs are composed from carbon atoms, and have a structural arrangement that resembles a graphite sheet that is rolled up into a cylindrical structure (Donaldson *et al.*, 2006, figure 1.1). CNTs have a diameter that is in the region of a few nanometres, however their length can be up to several microns and are often 'capped' by a fullerene molecule (Aitken *et al.*, 2006). There are two classes of CNTs available; single-walled (SWCNTs) formed from one CNT, and multi-walled (MWCNTs) composed from concentric layers of CNTs. CNTs tend to aggregate by 'clumping' together to form 'bundles' or 'rope-like' structures, which are longer and wider than the CNTs that they are composed from, which may impact on their toxicity (Thess *et al.*, 1996, Donaldson *et al.*, 2006). CNTs can be exploited within a number of applications, and one of their most exploitable properties is their strength (Baughman, Zakhidov and Heer, 2002).

The cytotoxicity of CNTs was demonstrated by Shvedova *et al.,* (2003) who observed that SWCNTs were cytotoxic to keratinocytes. Lam *et al.,* (2004) observed that SWCNTs produced interstitial granulomas and inflammation in the lungs after intratracheal administration. These findings were confirmed by Warheit *et al.*, 2004, with granuloma formation thought to arise due to the presence of SWCNT aggregates, and due to high dose used (5mg/kg). A more comprehensive study was carried out by Shvedova *et al.,* (2005) that characterised the pulmonary response mediated by SWCNTs and ufCB in mice. ufCB was used so that a comparison could be made between carbon based NPs. It was found that granuloma formation was associated with SWCNT aggregates and that their formation was not apparent with ufCB administration.

The inflammatory response was most prominent with SWCNT exposure and characterised by a rise in pro-inflammatory cytokines (TNFα and IL-1ß), inflammatory cell infiltration and oxidative stress. The varied extent of toxicity of these different carbon based nanoparticles highlights that it is not necessarily the chemical composition of a NP that dictates its toxicity but a number of different parameters including shape and structure.

Concern regarding CNT toxicity is derived from their structural resemblance to pathogenic fibres such as asbestos, as they have a long, fibre like structure (Seaton, 2006). Asbestos is a naturally occurring fibre that was found in wide ranging applications, and exposure (in sufficient quantities) caused pathogenicity within the lung, exhibited as mesothelioma, lung fibrosis and cancer (Seaton, 2006). It is known that the pulmonary toxicity of the fibres, such as asbestos is dependent on their diameter, as this will dictate its pulmonary deposition, and length, as this will influence the ability of macrophages to clear them (Donaldson and Tran, 2002, Donaldson and Tran 2004, Seaton, 2006). Longer fibres, for example would present difficulties for alveolar macrophages, and a failure of macrophage mediated clearance, known as frustrated phagocytosis ensues which stimulates inflammatory mediator release from these cells, with potent inflammatory, fibrotic, and carcinogenic consequences (Donaldson and Tran, 2002, Donaldson and Tran, 2004, Seaton, 2006). The importance of CNT length has been demonstrated to be central to their toxicity, as Sato *et al.,* (2005) found that longer MWCNTs (825nm) were able to elicit a greater inflammatory response than their shorter (220nm) counterparts. Furthermore Poland *et al.,* (2008) investigated the toxicity of a variety of CNT morphologies (long vs short) and demonstrated that asbestos-like CNTs (long fibres) caused a similar pathology to asbestos. In this study, exposure of the mesothelial lining of abdominal cavity (a surrogate for the mesothelial lining within the lungs) to CNTs resulted in an asbestos-like, length dependent pathology (such as inflammation and granuloma formation). Therefore the similarities in pathologies related to CNT and asbestos exposure provides compelling evidence that NP characteristics such as shape are important to their toxicity, and therefore represent a potential threat to human health if exposed in sufficient quantities, and so harnessing the attractive properties of CNTs should be approached with caution. Consequently,

structure activity relationships (SARs) may be utilised to predict the toxicity of NPs, as it has transpired that some structural characteristics, such as length have been identified as being capable of contributing to the observed toxicity.

In addition to the length of CNTs contributing to their toxicity it is also necessary to consider the contribution of surface contaminants as metal impurities, which are introduced during the manufacture process. This is important as it is known that surface contaminants (such as transition metals) are able to contribute to the toxicity of ambient particles, through a synergistic interaction (Wilson *et al.*, 2002). There is potential for CNT manufacture to introduce substantial amounts of metal impurities onto the CNT surface (due to the use of metal catalysts such as nickel or iron) that may contribute to their toxicity (Baughman, Zakhidov, and de Heer, 2002). The importance of considering CNT impurities is illustrated by the findings of Pulskamp, Diabate and Krug, (2007) whereby 'impure' CNTs were able to increase ROS levels and decrease mitochondrial membrane potential, but purified CNTs were not.

1.9.3 Carbon Fullerenes C_{60}

Carbon fullerenes, also termed carbon buckyballs, are composed exclusively from carbon atoms (60 in total), which are arranged in a spherical, football-like, cage structure, and have a diameter of about 1nm (Whatmore, 2006). Fullerene production can occur naturally, as they can be released from combustion processes such as forest fires (Powell and Kanarek, 2006) or intentionally produced for a specific purpose.

Sayes *et al.,* (2005) found that C_{60} exerted cytotoxicity through lipid peroxidation (and resultant membrane damage), and that the administration of the antioxidant ascorbic acid could prevent the development of oxidative damage and therefore the appearance of C_{60} mediated toxicity; it can therefore be concluded that oxidative stress is central to C_{60} toxicity in HDF, HepG2 and NHA human cell lines. Kamat *et al.,* (2000) have also demonstrated that C_{60} can exhibit toxicity through the generation of ROS and lipid peroxidation within rat liver microsomes. Furthermore, it has been suggested that the surface modification of C_{60} is able to impact on its toxicity, specifically that 'pure' C_{60} is more toxic than $C_{60}(OH)_{24}$ *in vitro* (Sayes *et al.*, 2004), and that this could aid in

the design of NPs to render then less toxic. However the studies were replicated *in vivo*, and found that both types of C_{60} were non-toxic (when considering cytotoxicity, inflammation, and histopathological end points) so that it is important that surface attachments are able to influence toxicity (Sayes *et al.*, 2007).

However, contrary to findings that have demonstrated that C_{60} induces oxidative stress to mediate its toxicity, there is evidence that C_{60} can act as a free radical scavenger, and thus have antioxidant properties (Gharbi *et al.*, 2005). This was illustrated by the fact that C_{60} administration can protect the liver from carbon tetrafluoride mediated liver damage due to its free radical scavenging activity. The antioxidant potential of C_{60} is thought to be dependent on its dispersion, so that aggregates of C_{60} will not exhibit this antioxidant property (Gharbi *et al.*, 2005). Gharbi *et al.,* (2005) also provided evidence that C_{60} accumulates within the liver after injection, so that the liver may be a target for any toxicity.

1.9.4 Ultrafine Carbon Black (ufCB)

ufCB was used as a surrogate ultrafine particle to model the composition of PM_{10} in studies that aimed to characterise the implications of PM_{10} exposure, and was deemed a particularly relevant particle 'model' to use as carbon based ultrafine particles comprise about 50% of the mass of PM_{10} (Donaldson and Stone, 2003). Therefore a number of studies were conducted using ufCB (see for example Li *et al.*, 1999) to assess the toxic potential of ultrafine particles within lung models, and so there is a vast array of background information available that indicates ufCB is more potent at inducing inflammation in the lung than fine (260nm) CB (Li *et al.*, 1999, Wilson *et al.*, 2002, Brown *et al.*, 2000). This inflammation has been associated with the surface area of carbon black (Stoeger *et al.*, 2006). *In vitro* ufCB has also be shown to elicit a pro-inflammatory response, characterised by TNFα expression in macrophages (Brown et al, 2004) and IL-8 production by epithelial cells (Monteillier et al., 2007). In macrophage models, the upregulation of TNFα has been shown to be controlled by a complex series of events that ultimately results in transcription mediator activation due to rises in intracellular calcium and oxidative stress development (Brown *et al.*, 2004). Consequently, due to the known toxicity of ufCB, it is now considered to be a benchmark NP to compare the toxicity of

other engineered NPs against. Therefore it is relevant to use ufCB, when assessing the toxicity of NPs to the liver as comparisons can be made to its ability to initiate toxicity to a similar extent as that exhibited within the lung. It is also necessary to evaluate ufCB toxicity as it has a number of commercial applications, for example it can be used as a toner in photocopiers and printers and is also used by the rubber industry in the manufacture of tyres (Reijnders, 2005), so that exposure could result in toxic outcomes in those exposed in an occupational or consumer setting.

Chapter Two

Why is there concern regarding NP toxicity to the liver?

2.1 Why is it important to study the toxicity of NPs to the liver?

Studies that have evaluated the toxicity of ultrafine particles (when investigating the components responsible for driving PM_{10} toxicity) concentrated on the impact of particulate exposure within particular target organs. The respiratory system was considered to be the most likely target of particle toxicity due to the fact that exposure to PM_{10} occurred via inhalation. The impact of ultrafine particles on the cardiovascular system was also investigated as mortality associated with exposure to PM_{10} was also associated with cardiovascular events (Schwartz, 1994b, Schwartz, 1997). However the realisation that NP localisation is not restricted to their portal of entry, illustrating that NPs can become distributed to organs distal to their site of exposure, means that NP toxicity can be potentially exerted at a number of secondary targets (Oberdorster *et al.*, 2005a). Consequently there is a research need to be more far reaching in studies that investigate NP toxicity, to encompass the possibility of multi-organ involvement. In addition, systemic effects may transpire due to the release of factors that act at a distance to their site of production as a consequence of NP mediated toxicity to the liver.

2.2.1 Blood supply to the liver

It is imperative to consider the blood supply to the liver, as this will 'deliver' NPs to liver cells. The liver receives a dual blood supply; the two main vessels that supply the liver are the hepatic artery, which delivers oxygenated blood from the heart, and constitutes approximately 30% of the liver's blood supply, and the hepatic portal vein, which delivers blood rich in nutrients and xenobiotics from the intestine, and accounts for approximately 70% of the liver's blood (Malarkey *et al.*, 2005, figure 2.1). The hepatic vein removes blood from the liver (figure 2.1), and the incoming and outgoing blood vessels are connected by sinusoid vessels.

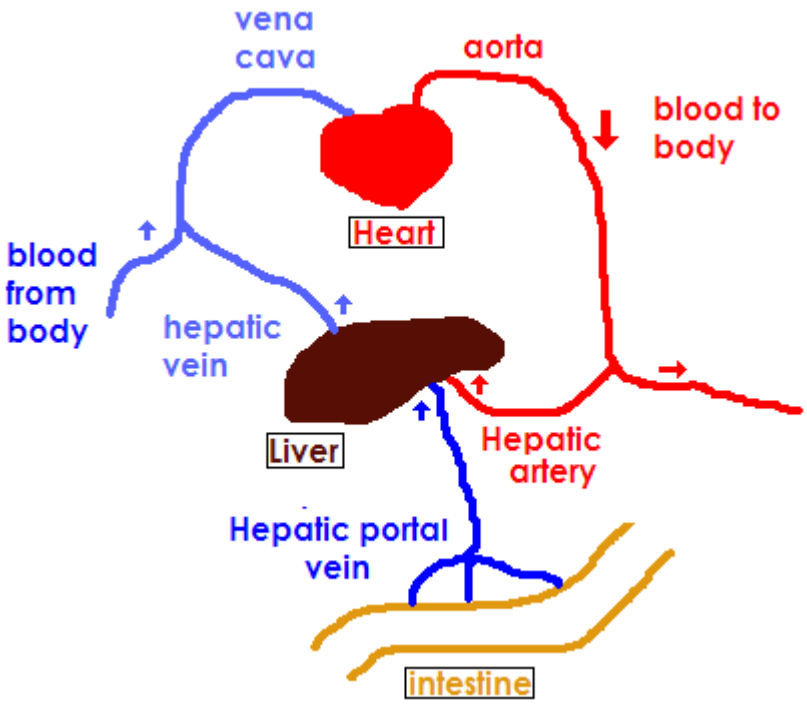


Figure 2.1 The blood supply to the liver. Oxygenated blood from the heart is delivered to the liver from the hepatic artery, and blood from the GIT is brought to the liver via the hepatic portal vein. Blood is removed from the liver by the hepatic vein.

2.2.2 Redistribution of NPs from their site of deposition and their accumulation within the liver

There are a number of observations that have confirmed the ability of NPs to accumulate within the liver after exposure via a variety of routes. Nemmar *et al.,* (2001) demonstrated that denatured albumin NPs (<80nm) were able to pass into the systemic circulation, and preferentially accumulate within the liver of hamster after intratracheal instillation. In addition, Oberdorster *et al.*, (2002) demonstrated that a 6 hour whole body exposure of rats to radio-labelled carbon NPs (20-29nm) resulted in their accumulation within the liver. Furthermore Takenaka *et al.,* (2001) demonstrated that silver NPs (4-10nm) were detected in the blood after inhalation by rats, from where they distributed to other organs, with predominant localisation within the liver. The finding that NPs were able to accumulate within the liver was then verified in humans, when ^{99}Tc labelled, 5-10nm, carbon NPs were inhaled (Nemmar *et al.*, 2002).

However, on the contrary, NP accumulation within the liver after pulmonary exposure is not a universal finding, and as such may be dependent on the exposure conditions and type of NP used. Kreyling *et al.,* (2002a, 2000b) assessed the ability of radiolabelled iridium NPs (15 and 80nm) to translocate

from the lungs after inhalation by rats and demonstrated that a very small fraction of NPs accumulated within the liver, and that there was a size dependency to the findings of particle translocation; so that larger (80nm) NPs were distributed to 'secondary' target organs to a lesser extent than the 15nm NPs.

Mills *et al.,* (2005) aimed to replicate the findings of Nemmar *et al.,* (2002) but found that the (same) NPs were unable to pass into the systemic circulation from the lungs in exposed humans. This may arise as a consequence of differences in particle size, aggregation state, or the methods used for analysis (including the imaging of particles). Alternatively, it is believed that the radio-isotope (which is attached to the carbon NPs used within the Mills and Nemmar studies) may detach from the NP and enter the circulation (Wiebert *et al.*, 2006) and could thereby account for the apparent systemic presence of NPs in the Nemmar studies. To overcome these limitations, Wiebert *et al.,* (2006) studied the systemic translocation of NPs in humans (after inhalation), with the radiolabel more tightly bound to the carbon NPs. It was found that the particles largely remained within the lungs after exposure, and were not apparent in the systemic circulation. The findings from these studies therefore highlight the difficulty in reliably predicting NP behaviour, as the findings could not be replicated by different lab groups despite the use of the same NP. The discrepancies found, regarding the ability of NPs to access the liver from the lungs, also highlights why it is important to evaluate different NP compositions and different routes of exposure, as they have the potential to impact on NP distribution, and therefore toxicity, within the body.

NP accumulation within the liver necessitates that NPs are present within the blood so that they can access the liver via a supplying vessel (either the hepatic portal vein or the hepatic artery). As a result, a greater localisation of NPs within the liver would be expected when NPs were exposure was intravenous (i.v), and not by inhalation or instillation where there are more barriers to prevent against liver accumulation. Ogawara *et al.,* (1999a, 1999b) and Furumoto *et al*., (2001) demonstrated that 50nm polystyrene beads predominantly accumulated within the liver after i.v. administration, and Chen *et*

al., (2008) demonstrated that QDs were capable of being distributed to the liver after i.v. exposure.

There are a number of different exposure scenarios that are therefore able to result in the accumulation of NPs within the liver. There is potential for the delivery of NPs to the liver from the blood, skin, lung, and GIT (see section 1.8), which allow NPs to access the liver via a supplying vessel after transfer into the systemic circulation from the site of exposure (figure 2.2).

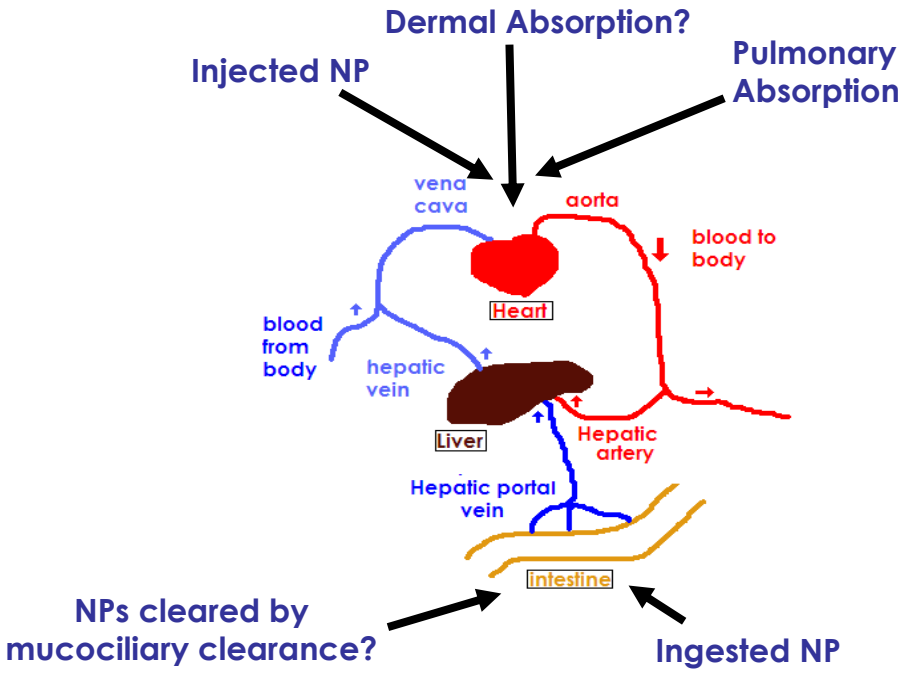


Figure 2.2 Routes of exposure that lead to the exposure of the liver to NPs. NPs have been reported to distribute to organs distal to their site of entry, so that their toxicity can be potentially exerted at a number of targets within the body. Therefore, regardless of the route of exposure, it is apparent that NPs are able to enter the circulation and gain access to the liver via the hepatic portal vein from the gastrointestinal tract, or via the hepatic artery from the general circulation following pulmonary, ingestion, dermal or intravenous exposure.

2.3 LIVER STRUCTURE

2.3.1 Cells required for normal liver function

The functions undertaken by the liver require the activity of a collection of cells; parenchymal cells, termed hepatocytes, and non-parenchymal cells; namely endothelial cells, Kupffer cells and stellate cells (Selden, Khalil and Hodgson, 1999). The importance of each distinct cell sub-population in maintaining the

vast array of liver functions can be appreciated when considering the numbers of each cell type present. Hepatocytes are the main liver cell population in terms of number, constituting 60% of total liver cells; their predominance thereby reflects their importance to normal liver function (Malarkey *et al.*, 2005). Kupffer cells are resident liver macrophages, and comprise about 15% of total liver cells (Malarkey *et al.*, 2005). Endothelial cells line the blood vessels that supply hepatocytes and represent up to 20% of total liver cells. Finally stellate cells comprise about 5-8% of total liver cells (Malarkey *et al.*, 2005, Reynaert *et al.*, 2002). It is recognised that hepatocytes carry out the majority of liver activities and non-parenchymal cells modulate and regulate the activity of hepatocytes (Seldon, Khalil and Hodgson, 1999).

2.3.2 The Liver Lobule

Macroscopically the liver is a wedge-shaped dark red/brown coloured organ that can be divided into 4 lobes, and microscopically, the liver can be organised into structural units known as liver lobules (figure 2.3, Malarkey *et al.,* 2005). Liver lobules have a hexagonal structure and consist of hepatocytes arranged in 'plates' (rows of cells that 1-2 cells thick), which radiate out from the central vein (located in the centre of the lobule) towards the periphery of the lobule, where 4-6 portal triads (composed from a bile duct, branch of the hepatic artery and branch of the hepatic portal vein) are situated. At the periphery of the lobule the venous and arterial blood supplies mix in vessels called sinusoids, with blood flowing through the sinusoids between the 'plates' of hepatocytes towards the centre of the lobule from where it exits the lobule via the central vein (figure 2.3)., and can therefore be regarded as the capillaries of the liver vasculature,. The central veins coalesce into the hepatic vein to exit the liver. The presence of sinusoids allows the exchange of substances between the blood and hepatocytes, and they are therefore essential to the maintenance of normal liver function (Enomoto *et al.*, 2004). Bile is formed by hepatocytes and is excreted into bile ducts, which are responsible for the delivery of bile to the gall bladder. The direction of bile flow is opposite to that of blood flow, within the lobule.

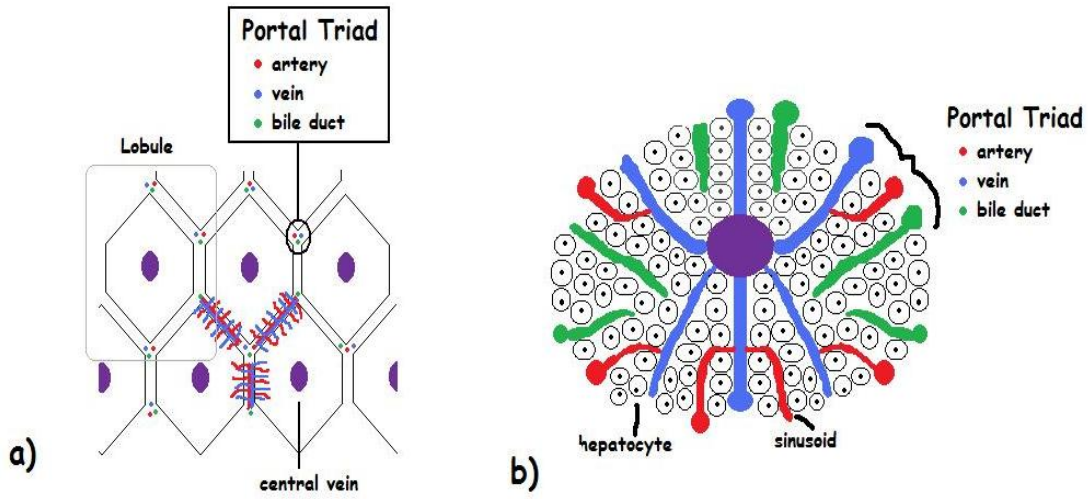


Figure 2.3 The liver lobule. The liver can be macroscopically organised into liver lobules that have a hexagonal shape. Within the liver lobule, 4-6 portal triads supply hepatocytes within the lobule with blood (**a**), with blood from a branch of the hepatic portal vein and a branch of the hepatic artery mixing in the sinusoids, passing between hepatocytes and then exiting the liver via the hepatic central vein (**b**). The images are adapted from Davies *et al*., 2001.

2.3.3 The Liver acinus

Another way of structurally defining the liver is into acini, which is based on functional considerations. A liver acinus contains a diamond shaped region of hepatocytes that extends from one central vein to another in an adjacent lobule, and is supplied by a terminal branch of a terminal hepatic portal vein and hepatic artery (figure 2.4).

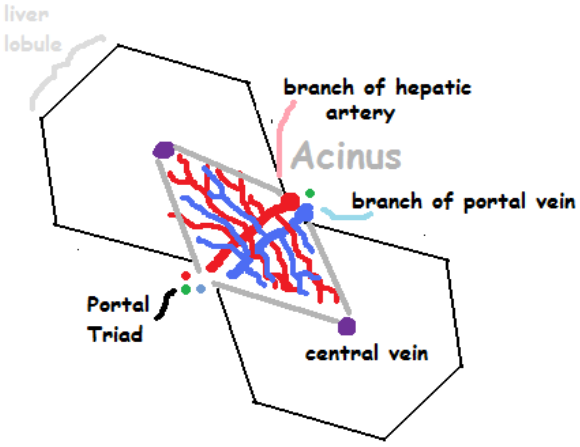


Figure 2.4 The Liver Acinus. The acinus refers to the diamond shaped region of hepatocytes supplied by a terminal branch of the hepatic portal vein and hepatic artery. The liver acinus includes hepatocytes located within different lobules. Adapted from: http://medical-dictionary.thefreedictionary.com/_/viewer.aspx?path=dorland&name+acinus_liver_liver.jpg (accessed on 6th October 2006).

A liver acinus is described as a 'functional unit' of the liver, due to the fact that it is orientated around the hepatic vasculature, with the composition of the blood dictating the function of cells. The structure of the liver acinus was first described by Rappaport, (1954), who observed that as blood flows through the

sinusoids between the plates of hepatocytes its composition changes as substances are taken up and excreted by cells, and as a consequence the function of the liver cells is altered. As a consequence the cells contained within the acinus can be divided into 3 zones, depending on their proximity to the portal triad (figure 2.5). Cells located within Zone 1 (periportal) are situated closest to the blood supply and receive the highest concentration of oxygen and nutrients, zone 3 (perivenous) hepatocytes are located furthest from the blood supply, closest to the central vein and therefore receive lower concentrations of oxygen and nutrients from the sinusoidal blood, zone 2 (midzonal) is located between these zones (Rappaport, 1958).

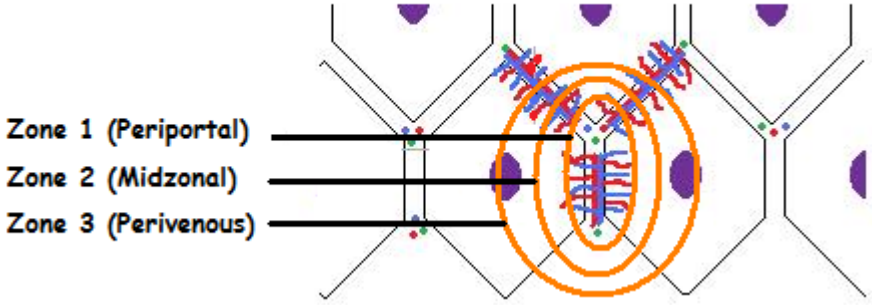


Figure 2.5 Zones of the liver acinus. The hepatocytes located within the liver acinus can be divided into three zones, based on their distance from the blood supply, as this has functional consequences for the cells. The figure is adapted from: http://biology.about.com/library/organs/bldigestliver.htm (accessed on 6th October 2008).

Rappaport, (1958) stated that cells located in zone 1 were “privileged by the circulation [due to the higher oxygen and nutrient content] and cells farther from the portal triads get blood of a poorer quality” and that “the more distant the hepatic cells are from the site where the terminal branches [of portal vessels] empty into the sinusoids, the poorer the quality of blood which bathes them and the less their resistance to damage”. Therefore the progressive removal of oxygen and nutrients by hepatic cells nearest to the portal triad reduces the supply for hepatocytes that are located further from the blood supply, so that cells in different zones, adopting heterogeneous functions, and morphologies (Bykov *et al.*, 2004), and arises due to differences in the composition of blood that the cells are exposed to.

2.4.1 What cells are important to normal liver function?

The liver has a unique structure and is formed from a collection of cells that have varied morphologies, functions and locations. The arrangement of different cell populations within the liver can be observed in figure 2.6.

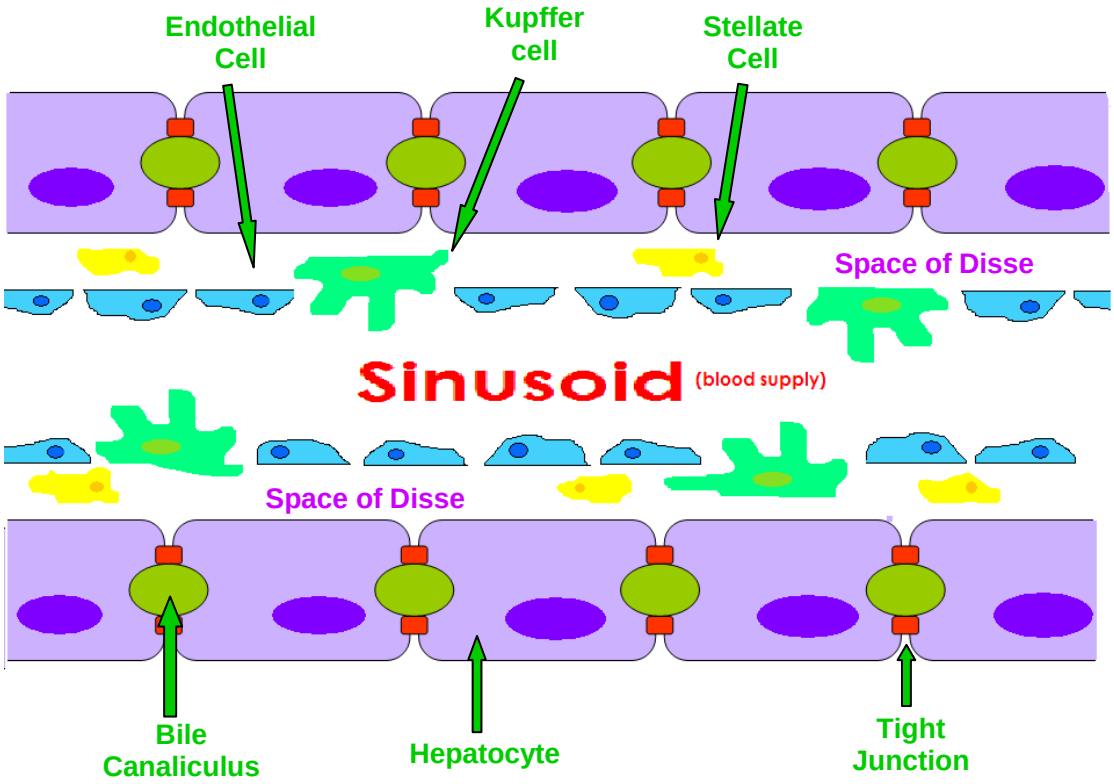


Figure 2.6 The arrangement of cells within the liver. Hepatocytes are the main liver cell population and are supplied with blood contained within sinusoids. Adjacent hepatocytes are joined by tight junctions that restrict the passage of substances from the extracellular fluid into the bile canaliculus. The sinusoids are lined by endothelial cells and Kupffer cells. The Space of Disse (also known as the perisinusoidal space) is found in the space between endothelial cells and hepatocytes and is particularly important for the rapid passage of nutrients to hepatocytes from the blood, to allow the efficient transport of substances to the hepatocyte surface, and is also the location of stellate cells. Bile is formed in the intercellular channels (bile canaliculi) between adjacent hepatocytes. Adapted from Weiler-Normann and Rehermann, 2004.

2.4.1a Hepatocytes

Hepatocytes, constitute the main liver cell population (Malarkey *et al.*, 2005, Seldon, Khalil and Hodgson, 1999, Weller-Normann and Rehermann, 2004). A vast array of functions are undertaken by the liver in general, and by

hepatocytes in particular, so that they are principally accountable for sustaining the majority of liver functions (Wilton and Mathews, 1996, Seldon, Khalil and Hodgson, 1999). Hepatocytes can be described as polarised cells, due to the fact that their cell surface can be divided into specialised regions that are; responsible for distinct functions, exposed to different environments and have a characteristic membrane composition (Zegers and Hoekstra, 1998). Hepatocytes have a sinusoidal (basolateral) domain which faces and interacts with the blood. A canalicular (apical) domain is also evident where bile is elaborated into the bile canaliculus (Coleman and Roma, 2000). The polarity of hepatocytes is dependent upon the presence of tight junctions (which seal the canaliculus) so that it is separate from the other membrane domains, and the differential distribution of the cytoskeleton which allows distinct proteins and lipids to be specifically targeted to particular domains so that they acquire distinct compositions and functions (Roma *et al.*, 2000, Coleman and Roma, 2000, Chandra and Brouwer, 2004). Bile canaliculi are intercellular channels that form between adjacent hepatocytes and are responsible for the formation, and accumulation of bile (Coleman *et al.*, 1995). The contraction of the bile canaliculus is responsible for the periodic expulsion of its contents into bile ducts (Oshio and Phillips, 1981).

There is evidence that hepatocytes are able to internalise 20nm polystyrene NPs *in vivo*, that accounted for 28% of the total hepatic uptake observed (Ogawara *et al.*, 1999a) and it is therefore important to determine if NP accumulation initiates functional disturbances within hepatocytes. In addition particulates, such as the contrast agent AMI-HS have been demonstrated to accumulate within bile canaliculi after i.v. exposure, and be eliminated unchanged, within bile (Dupas *et al.*, 2001), so that the localisation of NPs within canaliculi is of relevance and warrants investigation. Therefore it is relevant that hepatocytes may internalise NPs to facilitate their breakdown or removal from the body, within bile.

2.4.1b Endothelial cells

Endothelial cells line sinusoidal vessels, and regulate the access of blood borne substances from the sinusoidal lumen to the hepatocyte surface (Seldon, Khalil and Hodgson, 1999). As a consequence, endothelial cells are thought to have

a protective role and act as a barrier to the access of substances to hepatocytes from the blood (de Leeuw *et al.*, 1990).

The liver sinusoids can be regarded as unique capillaries that differ from other capillaries within the body, owing to differences in their endothelial morphology. Sinusoidal endothelial cells have fenestrae, and lack a basal lamina, which ordinarily acts as a barrier to restrict the passage of substances to underlying cells (Braet and Wisse, 2002), and as a result, the liver endothelium can permit the free, unrestricted access of substances from sinusoids into the space of Disse, so that they interact with the underlying hepatocytes. Endothelial cells, are perforated with small fenestrations that typically have a diameter of 150-175nm, (Braet and Wisse, 2002, Gatmaitan *et al.,* 1996) and allow the direct, unrestricted passage of substances from blood to the hepatocyte surface (Arii and Imamura, 2000), this morphology of sinusoidal endothelial cells thereby allows the sinusoidal endothelial lining to act as a sieve and is necessary for the efficient, rapid transport of substances such as nutrients to hepatocytes from the blood (de Leeuw *et al.*, 1990). However, paradoxically, the fenestrated structure of endothelial cells may increase the likelihood of hepatocyte exposure to NPs, by facilitating NP passage from the blood to hepatocytes, due to the fact that NPs are smaller than the average fenestration size (Ogawara *et al.*, 1999b). Contact of NPs within endothelial cells is also of concern as there is evidence that endothelial cell function may be compromised by NP exposure. Khandoga *et al.,* (2004) investigated the effects of carbon NPs (14nm diameter) on sinusoidal vessels within the liver. It was found that NPs promote platelet accumulation on endothelial cells within the sinusoids, so that they have a pro-thrombotic action that may impact on normal liver function, as it will compromise blood flow within the liver which would inevitably impact on hepatocyte function.

2.4.1c Kupffer cells

Kupffer cells are found in the sinusoidal lining, and are the resident macrophage population in the liver (Arii and Imamura, 2000, Malarkey *et al.*, 2005). Kupffer cells are derived from circulating monocytes that migrate to the liver and are transformed into macrophages, and constitute 80-90% of tissue macrophages within the body (Bilzer*,* Roggel and Gerbes, 2006). The position of Kupffer cells

within the sinusoidal lining is paramount to their role in defence against circulating toxicants, whose contact with hepatocytes should be minimised.

The behaviour of Kupffer cells is particularly relevant when considering NP-mediated toxicity, as their counterparts within the lung, termed alveolar macrophages, have been shown to be integral to the toxicity of inhaled NPs (Oberdorster *et al.*, 1992, Donaldson, Li and MacNee, 1998). Therefore the ability of ultrafine particles to stimulate alveolar macrophages, and initiate an inflammatory response within the lung, is of relevance (Oberdorster *et al.*, 1992). Kupffer cells could therefore potentially play a prominent role in the clearance and toxicity of NPs present within the hepatic circulation. This has been observed by Ogawara *et al.,* (1999a) who demonstrated that Kupffer cells were the liver cell population that were predominantly responsible for the uptake of NPs (after i.v. administration), thereby accounting for the preferential localisation of NPs within the liver. In addition, evaluating the consequences of NP exposure on Kupffer cell function is essential due to evidence that has demonstrated the ability of ultrafine particles to impair macrophage function within the lung (Renwick *et al.,* 2001). An impairment of Kupffer cell function would have implications for normal liver function as the removal of potentially toxic substances that are ordinarily removed from the liver would be compromised. However, Khandoga *et al.,* (2004) have shown that the accumulation of carbon NPs within the liver does not affect Kupffer cell function which could explain why NPs were not observed to exert an inflammatory reaction was associated with NP exposure in the liver in this study.

It is of interest to determine if Kupffer cell mediated NP uptake can be avoided, as under some circumstances it will be necessary to evade NP clearance from the blood. Avoidance of cell uptake by NPs may be achieved through the attachment of surface moieties, such as polyethylene glycol (PEG). PEG coating is thought to prevent the opsonisation of blood components (that are integral to phagocytosis) onto drug surfaces to prolong blood circulation time, by decreasing their recognition by cells such as Kupffer cells within the liver (Garnett and Kallinteri, 2006).

2.4.1d Stellate Cells

Stellate cells are star shaped cells that lie in the space of Disse, (Malarkey *et al.*, 2005). Stellate cells are responsible for the storage of vitamin A, fat and deposition and expansion of the extracellular matrix through the release of growth factors (Senoo, 2004, Seldon, Khalil and Hodgson, 1999, Reynaert *et al.*, 2002). Stellate cells are thought to contribute to the regulation of sinusoid diameter, and therefore sinusoidal blood flow (Reynaert *et al.*, 2002).

2.5.1 The liver and the production of bile

The liver is solely responsible for the formation and secretion of bile. The secretion of bile serves a number of important purposes. It functions not simply to enable the removal of waste products from the liver (the function with which it is most commonly associated) but is also involved in lipid digestion and absorption after its secretion into the intestine (Coleman, 1987, Trauner and Boyer, 2002, Aresse and Trauner, 2003).

The formation of bile is dependent on a number of processes; specifically the delivery of bile constituents to hepatocytes from the sinusoidal blood, their internalisation, potential metabolic modification, transport to the canalicular membrane (within vesicles or by diffusion), and subsequent secretion across the canalicular membrane into the bile canaliculus (Trauner and Boyer, 2002, Milkiewicz *et al.*, 2002, Meier and Stieger, 2000). The elimination of substances within bile is facilitated by the polar nature of hepatocytes, and requires the presence of ATP-dependent export pumps that transport substrates into bile and are exclusively found on canalicular membrane such as the bile salt export pump (Bsep) (Chandra and Brouwer, 2004). If NPs are eliminated within bile they must therefore be recognised by these transporters, or it is also possible for bile components to be emptied into bile canaliculi via vesicles.

Bile formed within the canaliculus is expelled into bile ductules and then bile ducts where bile is subject to modification by biliary epithelial cells (known as cholangiocytes), and is stored within the gall bladder in humans (where it is concentrated) and secreted into the intestine when stimulated (Milkiewicz *et al.*, 2002, Arrrese and Trauner, 2003, Trauner and Boyer, 2002). Substances eliminated within bile are secreted into the intestine from where they undergo elimination from the body within the faeces (Chandra and Brouwer, 2004). A

number of bile constituents (such as bile salts, and cholesterol) are reabsorbed from the small intestine, so that they are re-circulated via the hepatic portal vein to the liver for reuse (Trauner and Boyer, 2002). If NPs are eliminated within bile they may be recycled into the systemic circulation as there is evidence of NP translocation across the gut wall (Jani *et al.*, 1990). Ogawara *et al.* (1999b) have demonstrated that 50nm polystyrene microspheres were found in bile after i.v. administration.

2.5.2 Study of Hepatobiliary function

Cholestasis is defined as a reduction of bile formation (Milkiewicz *et al.*, 2002), and as the impact of NP exposure on cholestasis development has not been previously investigated, cell models that will be considered, when evaluating the impact of the PARTICLE_RISK particle panel on bile formation and secretion will be discussed.

2.5.2a Isolated cells

Cells can be isolated from the liver (termed primary cells) and cultured, from a number of different species, including humans (Grondeberg, Grosse-Siestrup and Fischer, 2002). Isolated cells have the advantage that a number of substances, at a range of concentrations, can be studied from cells isolated from one liver. However isolated primary cells can only be used in the short term as they cannot be grown indefinitely in culture due to the onset of replicative senescence. The polarised nature of hepatocyte structure and function is also lost on isolation, so that primary cells cannot be used to study bile secretion, and are predominantly used to study the uptake and metabolism of substances by hepatocytes (Oude *et al.*, 1995). The maintenance of primary hepatocyte function and polarity, can be improved through the use of sandwich culture, which culture hepatocytes between two layers of collagen (to represent the extracellular matrix) to promote the polarisation of hepatocytes (Talamini *et al.*, 1997). Furthermore the liver is ordinarily reliant on the co-ordinated activity of a variety of cell types, which is not taken into consideration when using isolated primary cells. However, this may be overcome through the use of co-cultures (Chandra and Brouwer, 2004).

2.5.2b Hepatocyte cell lines

Hepatocyte cell lines are available in human derived forms. However there are concerns about their relevancy, as they have been immortalised and therefore have the ability to proliferate indefinitely, which is not a property ordinarily exhibited by cells *in situ*. However, there is evidence that canalicular structures can form between adjacent hepatocytes of the WIF-B and HepG2 cell line, so that they may potentially be used to study the uptake and accumulation of substances within bile (Shanks *et al.*, 1994, Sormunen, Eskelinen and Lehto, 1993).

2.5.2c Isolated Rat Hepatocyte couplet

Oshio and Phillips, (1981) were the first to observe that hepatocytes can remain attached during isolation from the liver using collagenase, so that a functional canalicular structure is apparent between adjacent cells. Collagenase is an enzyme that breaks down the connective tissue that holds adjacent cells together, and allows cells to become separated from their neighbours. When the concentration of collagenase and exposure time is reduced (from that used to isolate single cells), two hepatocytes fail to separate from one another so that they remain connected via their tight junctions and are termed hepatocyte couplets (Coleman *et al.*, 1995). Once isolated, hepatocyte couplets are incubated for 4-6 hours to allow the re-establishment of morphological, structural and functional polarity (Coleman *et al.*, 1995, Wilton *et al.*, 1993). The canaliculus that forms in hepatocyte couplets is larger than observed *in situ*, and is sealed rather than being open ended (as ordinarily it is an inter-cellular channel that forms between adjacent cells) however despite this feature it is a useful *in vitro* cell preparation when the maintenance of cell polarity is required, and therefore relevant when investigating the effect of substances on bile secretion (Coleman and Roma, 2000, Gallin, 1997).

The ability of substances to affect canalicular function, and therefore bile formation can be investigated by determining the ability of hepatocyte couplets to take up, transport, secrete and accumulate a fluorescent bile acid analogue within bile canaliculi (Stone *et al.*, 1994). This is useful when investigating the potential cholestatic effects of substances. Cholyl lysyl fluorescein (CLF) is an example of such a substance and can be used to evaluate the effect of potential toxicants on bile formation, as it is taken up by the hepatocyte, secreted across

the canalicular membrane to accumulate within the canaliculus (Fentem *et al.*, 1990, figure 2.7). Therefore if the accumulation of CLF is inhibited by exposure to a toxicant it may have a cholestatic action.

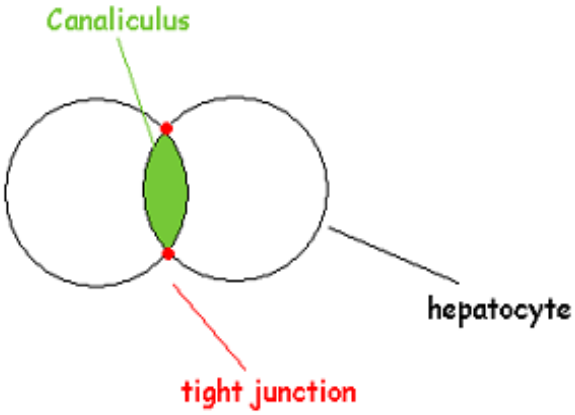


Figure 2.7 The hepatocyte couplet. Two hepatocytes remain attached via tight junctions, that maintain functional bile canaliculi,. Hepatocytes excrete bile components across the canalicular membrane into the canaliculus, which is sealed by tight junctions. CLF (green) can accumulate within the canaliculus after exposure.

2.6 Aims & Objectives

2.6.1Aim:

To evaluate the toxicity of NPs to the liver, as part of the EU funded project PARTICLE_RISK.

2.6.2 Objectives:

2.6.2a Determine the size and time dependent uptake, and the subsequent sub-cellular localisation of NPs in hepatocytes.

The uptake of 20nm and 200nm fluorescent polystyrene particles by hepatocyte cell lines (C3A and HepG2), and isolated primary rat hepatocyte couplets was investigated using confocal microscopy. The mechanism of uptake, and intracellular fate of internalised NPs was revealed using fluorescent probes and immunofluorescent staining, in order to identify the accumulation of NPs within sub-cellular compartments, specifically early endosomes, lysosomes, mitochondria and bile canaliculi. Accumulation of particles within these structures was anticipated to give an indication of the processes that contribute to NP internalisation or elimination from cells. The uptake and fate of the PARTICLE_RISK particle panel can be determined through TEM (Transmission Electron Microscopy) analysis

2.6.2b Determine the ability of NPs to induce oxidative stress in hepatocytes.

Oxidative stress development was detected by determining the ability of NPs to deplete the reduced form of the cellular antioxidant glutathione, and to accumulate the oxidised form (namely GSSG).

2.6.2c Investigate the ability of NPs to initiate an inflammatory response in the liver.

An insight into the ability of NPs to induce inflammation within the liver was gained by detecting increases in the release of pro-inflammatory cytokines from hepatocytes. Multiplex analysis (that allowed the measurement of multiple cytokines at one time) was conducted initially so that any suggestion of NP mediated increases in cytokine production were further investigated using conventional enzyme linked immunosorbant assay (ELISA) kits.

2.6.2d Investigate the potential of NPs to induce cytotoxicity

The ability of NP exposure to compromise cell viability was investigated using the lactate dehydrogenase (measures membrane integrity as an indictor of cell death) and MTT (measures mitochondrial activity to assess metabolic competence and therefore cell viability) assays. Assessment of gross and sub-cellular cell morphology, using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis, subsequent to particle exposure was utilised to gain insight into the ability of particles to induce cell death.

2.6.2e Evaluate the impact of NP exposure on bile secretion.

Determining the impact of NP exposure on the canalicular accumulation of a fluorescent bile acid analogue in isolated rat hepatocyte couplets, was used as an *in vitro* model of bile secretion.

2.6.2f Determine the contribution of inter-cellular interactions to NP toxicity within the liver.

The liver is an example of an organ where multiple cell types are evident and must co-operate to sustain normal liver function, so that cell-cell interactions are an important consideration when evaluating the impact of NP exposure on normal liver function. Accordingly, macrophages were exposed to particles to obtain a conditioned medium (CM) that was exposed to hepatocytes to gain an insight into the ability of macrophages to influence NP mediated toxicity within

the liver. The impact of CM exposure to hepatocytes on the following toxicity endpoints of oxidative stress, cytokine production, cytototoxicity and bile secretion was then assessed.

2.6.3 Hypothesis

NPs will be able to induce toxicity within the liver, which requires that they are taken up by hepatocytes to induce oxidative stress to initiate an inflammatory response and to disrupt normal cellular processes such as bile secretion. Resident liver macrophages will phagocytose NPs, and become activated to initiate an inflammatory response to compromise normal hepatocyte function. It is also likely that as the NPs contained within the PARTICLE_RISK panel have different physicochemical characteristics they will not be equally toxic.

Chapter Three

General Materials and Methods

3.1 Materials

3.1.1 Nanoparticles

The PARTICLE_RISK particles were purchased, as stated; SWCNTs (Thomas Swan and Co, Consett, UK), C_{60} (Sigma-Aldrich, Poole, UK,) ufCB (Printex90) and CB (Degussa, Frankfurt Germany,) and QD621 and QD620 (American Dye source Inc, Quebec, Canada,). Fluorescent carboxylate-modified microsphere polystyrene beads (200nm and 20nm diameter) were purchased from Molecular Probes (Eugene, Oregon, USA).

3.1.2 Equipment

The following equipment was employed: Sartorius BP211D balance, Grant XB6 ultrasonic sonicator, ESCO Class II BSC Airstream biohazard safety cabinet, SLS Galaxy R incubator, Grant SUB Aqua water bath, 101U peristaltic pump (Watson, Marlow, UK), and silicone tubing (MacKay and Lynn, Edinburgh, UK).

3.1.3 Chemicals

The chemicals used included, minimum essential medium (MEM) Eagle growth medium, sodium pyruvate, MEM non essential amino acids, calcium chloride, heparin sodium salt, Trypan blue, Hanks balanced salt solution (HBSS), potassium hydroxide, and were purchased from Sigma-Aldrich (Poole, UK).

Fetal calf serum (FCS), phosphate buffered saline (PBS) Trypsin-EDTA, L-glutamine, penicillin streptomycin (pen/strep), Leibovitz (L-15) medium and collagenase from clostridium histolyticum were purchased from Gibco Invitrogen (Paisley UK). Saline (0.9% NaCl) was obtained from Baxter (Cumbernauld, UK) and halothane was purchased from Merial Animal Health Ltd (Harlow, UK).

3.2 Particle Characteristics

The PARTICLE_RISK particle panel was characterised by Venice University as part of the PARTICLE_RISK project, so that the size, surface area, porosity, chemical composition, purity, and concentration (if supplied within solution) of each particle was determined, with the findings summarised in table 3.1. I was not involved within the characterisation process, and so only information supplied by partners (Venice University) within the project is supplied.

For the characterisation process, particles were dispersed in MilliQ water, and sonicated for 20 minutes at 100W. Particle size was determined by TEM, atomic force microscopy (AFM) and dynamic light scattering (DLS); analysis provided similar results despite the use of different techniques, and the sizes for ufCB, CB, SWCNTs and C_{60} are derived from TEM, and for QDs DLS was used due to technique size limitations. The surface area and pore size of particles was evaluated using Brunauer Emmett and Teller (BET) analysis. The chemical composition investigated using carbon nitrogen hydrogen (CHN) analysis, inductively coupled plasma mass spectroscopy (ICP-MS), high performance liquid chromatography (HPLC-MS) and purity using by ICP-MS, HPLC-MS, field flow fractionation (FFF). A number of QD characterisations could not be completed due to the fact that they were supplied in solution. The QDs were cadmium telluride (CdTe) based and had a cadmium sulphide coating that allowed the attachment of carboxylic acid (QD620) or amine (QD621) groups to modify the charge of the QD, and were dispersed in water. It was observed that cadmium and telluride release from the QDs occurred over time, so that if opened, the QDs were deemed to be unstable within a month. The length of the CNTs was not provided as its measurement was impeded by their tendency to aggregate.

Table 3.1 PARTICLE_RISK particle characterisation. The size, surface area, pore size, chemical composition and purity are outlined, and were determined using the techniques previously stated.

NP	Average Size (nm)	Surface area (m^2/g)	Average Pore size (nm)	Chemical composition	Purity	Impurities of concern
Carbon Black	30 (ufCB), 410 (CB)	338	60	89% C, 0.8% N	> 98%	Polycyclic aromatic hydrocarbons (PAHs)
C_{60}	0.7	< 20	< 20	> 99.9% C	> 99.9%	-
Carbon Nano Tubes	15	730	15	SWCNTs high purity	> 96%	3-4 ring PAHs and substitued PAHs
Quantum Dots (5.5mg/ml)	6.4	-	-	-	-	Cd, Te

Particles were weighed into bijoux tubes (3-6mg) and a stock concentration of 1 mg/ml (in the appropriate vehicle) was made on the day of the experiment. The particle suspensions were then sonicated for 20 minutes, with subsequent serial dilution of the stock solutions to the required concentrations.

3.3 Hepatocyte cell line cell culture

The human hepatocyte carcinoma HepG2 cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK) and maintained in MEM growth medium (supplemented with 10% FCS, 2mM L-glutamine, 100U/mL pen/strep, 1% MEM non essential amino acids (termed complete HepG2 cell medium) in plastic culture flasks and incubated at 37^{o}C, 5% CO_2.

The human hepatoblastoma C3A cell line was obtained from the American Type Culture Collection (Manassas, USA). The cells were maintained in MEM growth medium (supplemented with 10% FCS, 2mM L-glutamine, 100U/mL pen/strep, 1% MEM non essential amino acids, and 1mM sodium pyruvate (termed

complete C3A cell medium)), in plastic culture flasks and maintained at 37^{o}C, 5% CO_2.

HepG2 and C3A cells were sub-cultured using 0.5% trypsin (in saline).

3.4 Determining cell viability and number

Cell counts were performed using a Neubauer haemocytometer, with cell viability assessed using 0.5% Trypan Blue (in PBS).

3.5 Statement of animal welfare

The experiments described were performed under a Project License and Personal Licence issued by the United Kingdom Home Office.

Sprague Dawley rats (male, >3 months old) were obtained from Biomedical Research Resources, Royal Infirmary Edinburgh (Edinburgh, UK) and housed in conditions that were approved by the UK Home Office. Food and water were available *ad libitum*.

3.6 Isolation and culture of rat hepatocyte couplets

Hepatocyte couplets were isolated according to a two step collagenase perfusion method adapted from Gautam, Ng and Boyer, (1987). The day prior to the couplet isolation procedure, HBSS (without calcium), was brought to room temperature (from 4^{o}C) and oxygenated for 20 minutes (95% O_2, 5% CO_2) and the pH adjusted to 7.10 using 0.1M NaOH. The pH of the solution was observed to increase after passage through the perfusion apparatus, so that the pH of the HBSS was adjusted to that lower than required, pH 7.4, to compensate for the observed pH change. The buffer was then left at room temperature overnight until required. Heparin (5000units/ml) was made up in 0.9% saline, and the collagenase was weighed and kept at 4^{o}C until required. On the day of the couplet isolation the perfusion apparatus (figure 3.1) was filled with calcium free HBSS medium, and the water bath pre-warmed to 38^{o}C (so that, on perfusion of liver, the temperature of the perfusate was equivalent to that of blood) prior to the start of surgery.

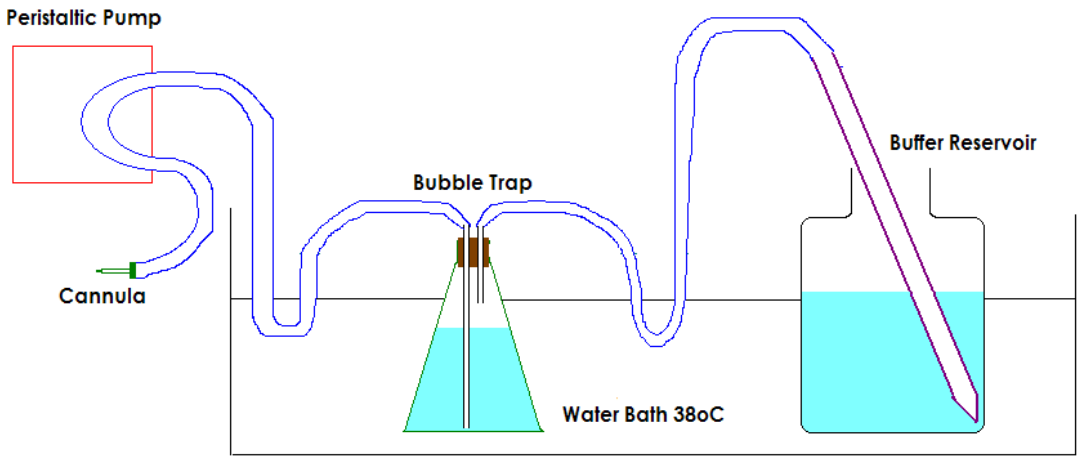


Figure 3.1 Couplet perfusion apparatus. The buffer was introduced into the buffer reservoir, and allowed to circulate within the apparatus, to allow the contents to equilibrate to the water bath temperature. The bubble trap prevents bubbles entering and blocking the liver vasculature during the perfusion. The peristaltic pump controls the circulation of the buffer through the system, and the cannula connects the system to the hepatic portal vein of the rat. It is important to ensure that transit through the perfusion apparatus does not affect the temperature or pH of the perfusate, and if it does that this is compensated for.

Sprague Dawley rats were anaesthetised with halothane, the abdomen opened using a midline incision, and the liver exposed. Heparin (0.1ml) was injected into the posterior vena cava to prevent coagulation of the blood which would otherwise block the liver vasculature and compromise the perfusion, and a cannula (pre-filled with heparin) was inserted into the hepatic portal vein, secured with the tie, and connected to the perfusion apparatus. Calcium free HBSS (250ml) was then allowed to enter the liver (20ml/min) and the posterior vena cava cut to allow the escape of blood and buffer from the animal, at which point the liver turned a mustard yellow colour. The liver was then perfused with 0.05% collagenase (80-90ml), in 4mM calcium chloride (1.2ml 400mM in 120ml HBSS) containing HBSS. After the perfusion was complete the liver was removed, coarsely minced in ice cold HBSS (30-40ml), with scissors, and the hepatocytes released by gentle agitation on ice. Hepatocytes were collected by filtering through a sterilised 150µm nylon mesh. The remaining tissue was re-incubated in collagenase solution and agitated at 38^{o}C for 7.5 minutes, and the hepatocytes collected as described. Both preparations were maintained separately, so that the cells collected from each preparation were not combined. After settling, the hepatocytes were re-suspended in Leibovitz, (L-15) medium (containing 100U/mL Pen/Strep), and the cells washed with L-15 medium (x2)

before the cell suspension contents (i.e. single cells, couplets etc) and viability was assessed using 0.5% Trypan Blue. The second preparation of hepatocytes was routinely used (due to their higher viability), diluted in L-15 medium, and plated at the appropriate concentration and incubated for a minimum of 4 hours (to allow the bile canaliculus to re-seal) at 37^{o}C in atmospheric air prior to exposure to particles.

Chapter Four

Investigating the uptake and intracellular fate of fluorescent polystyrene particles in hepatocytes *in vitro*

4.1 Importance of studying the internalisation of NPs by liver cells

NPs have been repeatedly observed to localise within the liver after administration via a variety of routes (see for example Takenaka *et al*., 2001, Nemmar *et al*., 2002). It is therefore of interest to determine if the localisation of NPs within the liver can be replicated *in vitro* within the individual liver cell populations. Consequently, the ability of NPs to cross the plasma membrane (PM) of cells to access the cell interior may impact on their ability to alter cell physiology and function, thereby justifying the investigation of particle uptake by liver cells.

The importance of evaluating particle uptake to the toxicity of NPs is of great relevance due to the ability of particle internalisation to contribute to the extent, or mechanisms of NP mediated toxicity. This is exemplified by the findings of Lovric *et al.*, (2005b) who postulated that a greater extent of QD uptake enhanced their toxicity. Furthermore, diesel exhaust particles have been observed to be internalised by 16-HBE bronchial epithelial cells, which was suggested to initiate an inflammatory response through the release of pro-inflammatory mediators (Boland *et al.*, 1999). An insight into NP fate subsequent to uptake is also important to cover, to give an indication of the longevity of NPs within a cell. Further impetus for investigating NP internalisation by cells derives from the knowledge that to allow some NPs to execute their desired function, NPs will have to reach particular target sites within cells, so that the ability of NPs to traverse the PM may be the limiting factor to their activity.

4.1.2 Cellular barriers to NP uptake

In order for NPs to enter the body and access the liver, they must cross cellular barriers, for example the lung and gastrointestinal tract are lined with epithelial cells, which must be crossed to allow NPs to enter the circulation, and be delivered to the liver by the hepatic portal vein or hepatic artery (see section 2.2.1). Adjacent epithelial cells are joined by tight junctions which are strictly regulated, to provide a element of control for the passage of substances between cells (termed paracellular transport), and act to limit the interaction of substances with other body compartments, thus allowing them to fulfil their barrier role (Patel, Zaro and Shen, 2007). The ability of toxicant exposure or

disease to inflict damage to the epithelium can lead to an increase in tight junction permeability, and increase the transport of substances between adjacent epithelial cells. It is therefore relevant that MWCNTs have been demonstrated to impair the paracellular permeability of Calu-3 epithelial cells, however, it was observed that this effect was not demonstrated with SWCNT or ufCB exposure (Rotoli *et al.*, 2008). Despite the fact that the effect was not apparent with all NPs tested, the potential of NPs to alter the paracellular permeability of airway epithelial cells is an important consideration as it would allow for greater NP translocation into the circulation to increase the likelihood of liver exposure. Furthermore, the permeability of airway epithelial cells is known to increase during inflammatory based pulmonary diseases, which could allow for a greater translocation of NPs into the bloodstream, and thereby make individuals more susceptible to NP toxicity. It is possible to encourage NP transport across cellular barriers, for example the penetration of radiolabelled (^{125}I) latex 100nm NPs across the nasal mucosa of rats into the blood was enhanced by coating the NPs with chitosan (Brooking, Davis and Illum, 2001). Furthermore, it was also found that smaller particles (20 and 100nm) were able to pass into the circulation to a greater extent than their larger counterparts (1000nm) suggesting that the small size of NPs may allow them to bypass the protective barriers that are in place, so that they pass in or between cells unnoticed.

NPs may be taken up directly by epithelial cells, and then pass through them to gain access to underlying cells or to the circulation, which is termed transcellular transport. However the transcellular transport of chemicals is normally limited by the presence of plasma membrane transport proteins, including efflux transporters such as P-glycoprotein (P-gp) so that this transport route is tightly regulated (Patel, Zaro and Shen, 2007).

Once NPs have crossed epithelial barriers and are contained within the systemic circulation they are able to enter liver sinusoids, where NPs must cross the endothelial barrier that line these vessels, to reach the underlying hepatocytes. Therefore the ability of endothelial cells to restrict the access of NPs to the underlying hepatocytes is of relevance to the ability of NPs to inflict toxicity within hepatocytes. However, the endothelial cells that line the

sinusoidal blood vessels are fenestrated (150-175nm), which has been suggested to increase the likelihood of hepatocyte NP exposure, by facilitating NP passage from the blood (Braet and Wisse, 2002, Ogawara *et al.*, 1999b). In addition, the ability of NPs to reach underlying hepatocytes may also occur via transcellular or paracellular transport, as described for epithelial cells.

4.1.3 Internalisation pathways

4.1.3.1 Passive transport mechanisms

The plasma membrane (PM) allows the segregation of the internal and external environments of a cell, and is responsible for regulated the entry and exit of substances (Conner and Schmid, 2003). Substances that require entry into the cell interior must therefore pass through the PM, with the uptake of substances accomplished via a variety of processes that can be described as active (energy requiring) or passive (non-energy requiring). It is therefore important to outline the different uptake pathways available for enabling the uptake of substances by cells, and the intracellular events that occur as part of them.

Some molecules are able to penetrate cells via diffusion, the principle method of is passive transport, and is dictated by the physicochemical characteristics of the substance in question. Diffusion allows substances to 'pass' through the PM into the interior of the cell from an area of high to low concentration, consequently a concentration gradient is required. As the PM is lipophyllic, like natured substances will most easily pass through the PM, and the entry of large, hydrophyllic, or charged molecules is restricted (Khalil *et al.,* 2006). Alternatively an electrical gradient is able to contribute to the diffusion of charged substances across the PM. The charge of the PM could therefore influence the way in which charged particles interact with cells., as it has been illustrated to influence the uptake of substances, which is thought to derive from the strong attraction of cationic molecules to the cell surface due to the non-specific electrostatic interaction that occurs with the negative charge of the PM interior (Patel, Zaro and Shen, 2007). However, it is generally accepted that charged molecules cannot pass through the PM directly via diffusion, but can access the cell interior via transport through transmembrane channels. Whether NPs can move into cells via diffusion remains unclear, but there is

some evidence supporting this (see for example Geiser *et al.*, 2005), and will inevitably be dictated by NP physicochemical characteristics.

Another form of passive transport is facilitated diffusion, which allows substances to pass from an area of high to low concentration through selective transmembrane protein channels. The carrier proteins and channels responsible for facilitated diffusion are open or closed depending on the requirements of the cell; for example ligand and voltage gated ion channels are evident. Furthermore as the entry of substances will be limited by the number of carriers present within the PM, the process can be described as saturable (King, 1996). Movement of NPs into the cell via such means would require the particles to be small enough to move through naturally occurring channels (which are 100-300 Å (where $1Å=1x10^{-10}m$, and therefore the channel size is 10-30nm)), as it is likely that their passage would not occur due to a case of mistaken identity, unless they were specifically designed to do so through the attachment of an appropriate surface moiety.

4.1.3.2 Transporter mediated active transport

In active transport, substances move across the PM against a concentration gradient, and thus energy, in the form of adenosine trisphosphate (ATP), is required. Active transport is conducted by specific transport proteins located within the membrane, which allow substances to be 'actively' pumped into and out of the cell, and occurs against a concentration gradient. The structure of such transporters are very specific for the molecules that they transport, and so it seems unlikely that NPs could use this pathway as a route of entry into the cell, unless their structure was physically and chemically designed to mimic that of a transported molecule. Furthermore, the ion selectivity of ion channels is thought to be maintained by the size of the pore in question so that a 'wide' channel pore is considered to be 10Å in diameter, and 'narrow' pores are generally 3-4Å in diameter (Gu *et al*., 2000).

4.1.3.3 Endocytosis

Endocytosis is a collective term that describes the active, energy dependent internalisation of substances, and is characterised by incorporation of substances into membrane bound cytoplasmic vesicles that derive from the

pinching off of a portion of the PM, which permits the carriage of the cargo into the cell interior (Johannes and Lamaze, 2002, Conner and Schmid, 2003, Patel, Zaro and Shen, 2007).

A number of distinct endocytic pathways have been identified, and include phagocytosis, clathrin-mediated endocytosis, caveolae mediated endocytosis and clathrin and caveolae independent endocytosis (Conner and Schmid, 2003). All processes share the commonality that they require the formation of endocytic vesicles within the cell interior that are encapsulated by a region of the PM (Watts and Marsh, 1992). However, it is known that the size of the vesicles formed is different for each of the pathways, for example clathrin coated pits are approximately 120nm in diameter, caveolae generally 50-80nm and phagosomes 1 to 5µm (Patel, Zaro and Shen, 2007). Although the size of the vesicles is not definitive and there are exceptions, it is thought that the size limitations of each pathway exist to restrict the size of the cargo that is internalised, thus introducing a form of selectivity to the process (Patel, Zaro and Shen, 2007). Such pathways therefore form a likely route of uptake of NPs, and NP aggregates into cells.

Although it is acknowledged that some substances preferentially enter cells by a particular pathway, it is possible that more than one pathway contributes to the internalisation of a substance (Patel, Zaro and Shen, 2007). For example, albumin is known to be taken up by caveolae mediated endocytosis, however when this pathway is disrupted, albumin is still internalised, thus suggesting that there are compensatory mechanisms that enable albumin uptake (Johnannes and Lamaze, 2002), so that when one pathway cannot function perhaps another can take over its role. Furthermore within the lung, it is known when there is an excessive burden of NPs, macrophages are overwhelmed by NP presence and therefore remaining NPs are not cleared effectively (known as particle overload), increasing their ability to interact with other cell types within the lung, thereby increasing their propensity for damage (Donaldson, Li and MacNee, 1998). This may be important to the uptake of NPs by other cell types, whereby uptake pathways may be overwhelmed if NPs are present in sufficient numbers.

4.1.3.3.1 The intracellular events that dictate the fate of cargo internalised by endocytosis

For all forms of endocytosis the same basic principle applies; that the substance is internalised into a PM enclosed vesicle. This vesicle, regardless of the route of uptake, generally follows a sequence of events that results in the organisation of the vesicle components into specific cellular compartments, so that the cargo is sorted for delivery to specific destinations within the cell (figure 4.1). The common element evident within the majority of endocytic pathways, is the delivery of internalised substances to early endosomes [also known as a sorting endosome (Cavalli, Corti and Gruenberg., 2001, van der Goot and Gruenberg., 2006)], which dictates the sub-cellular distribution of internalised cargo, as it is targeted and redistributed to the most appropriate sub-cellular destination. Specifically, delivery to late endosomes and lysosomes targets the substance for degradation, however if the cargo is required by the cell it may be delivered to the endoplasmic reticulum (ER) or golgi apparatus, or alternatively may be redirected out of the cell (Mellman, 1996, Medina-Kauwe, 2007). In this way early endosomes therefore fulfil their role in 'sorting' internalised cargo. Therefore despite several routes of endocytosis being available for the internalisation of substances, they are connected due to the cellular events that occur subsequent to the incorporation of the contents of the endocytic vesicle into early endosomes (see figure 4.1). However, there are exceptions whereby substances internalised by endocytic pathways do not follow the same intracellular route (see section 4.1.4.4).

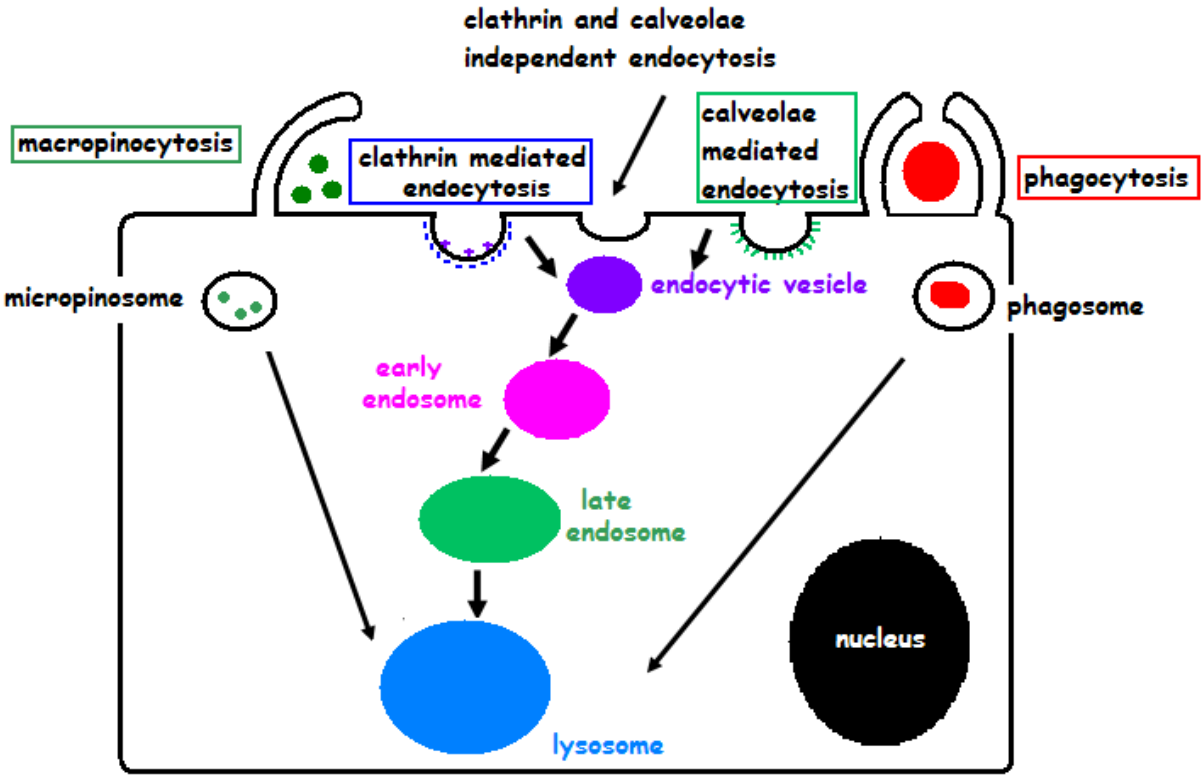


Figure 4.1 Organisation of endocytic pathways leading to degradation. Despite the endocytic pathway of entry, endocytic vesicles generally deliver their cargo to early endosomes. After internalisation the cargo passes through a series of distinct compartments. Within the cell, the internalised cargo is directed to the most appropriate cellular location, such as lysosomes to enable their degradation due to the sorting role that early endosomes adopt. Adapted from Khalil *et al.* (2006) and van der Goot and Gruenberg, (2002).

4.1.3.3.2 The importance of the early endosomes in dictating the fate of internalised substances

As the early endosome is a point of convergence for many endocytic pathways, it is important to consider their formation and function in more detail. There are two models surrounding the incorporation of internalised substances from early to late endosomes. The alternative model states that early and late endosomes are pre-existing compartments within the cell, and the maturation model suggests that early endosomes gradually mature into late endosomes and lysosomes due to their acidification (Dunn and Maxfield, 1992, Mu, 1995, Gruenberg and Maxfield, 1995, Medina-Kauwe, 2007, Johnannes and Lamaze, 2002). In the alternative model, substances are transferred from early to late endosomes via carrier vesicles, and in the maturation model, substances are evident within late endosomes due to the transformation of early endosomes (Dunn and Maxfield, 1992).

Therefore despite the endocytic route of entry, the pathway that internalised vesicles follow appears to have common elements. This is exemplified by the knowledge that the Cholera toxin can enter cells via numerous routes that all lead to its incorporation into the same intracellular compartment, illustrating the convergence of the pathways, despite differences in the endocytic mechanism of uptake (Perret *et al.*, 2005).

4.1.3.3.3 The contribution of lysosomes to the degradation of internalised substances

There are several fates for internalised substances; some are destined for degradation (which is fulfilled by lysosomes), whereas others are retained for utilisation by the cell (van der Goot and Gruenberg, 2006), and others are redirected out of the cell, with the persistence and fate of internalised cargo ultimately dictated by events within endosomes (Patel, Zaro and Shen, 2007).

Lysosomes are known to be the final destination for the components of endocytic vesicles that are targeted for destruction and degradation (Eskelinen *et al.,* 2003). The transfer of endocytosed material from endosomes to lysosomes has been proposed to occur by a number of mechanisms; it has been suggested that endosomes mature into lysosomes, that there is a direct fusion between endosomes and lysosomes, that vesicles carry cargo from endosomes to lysosomes, or the 'kiss-and-run' model where endosomes make direct contact with lysosomes and the contents are transferred to the lysosome, after which they dissociate (van Meel and Klumperman, 2008). To fulfil their degradative function, proteins are inserted into the lysosomal membrane to enable them to recognise endosomes and substances to be degraded, and soluble hydrolases are delivered from the ER and golgi (van Meel and Klumperman, 2008). The cell interior is protected from the unregulated activity of the hydrolytic enzymes due to the lysosomal membrane and due to fact that they act optimally at an acidic pH, and not the neutral pH that is found within the cytosol (and is maintained by the proton pump) (Eskelinen *et al.*, 2003, Luzio, Pryor and Bright, 2007). The importance of lysosome function is highlighted by the existence of lysosome storage disorders, such as Danon disease (Eskelinen *et al.,* 2003), which are related to a defect in normal lysosomal function, resulting in an accumulation of un-degraded substances within lysosomes.

4.1.4.1 Clathrin-mediated endocytosis (CME)

CME was the first endocytic mechanism to be identified and, as a result is the most extensively characterised (Gruenberg, 2001). CME was previously referred to as receptor mediated endocytosis, as it involves a receptor-ligand interaction at the PM (Conner and Schmid, 2003). The receptors, which enable CME, are concentrated in clathrin coated pits within the PM (Conner and Schmid, 2003). The clathrin pits form due to the binding of clathrin to the PM, from where it polymerises to form a cage like structure that pulls a portion of the PM inside the cell, otherwise known as membrane invagination (Liu and Shaprio, 2003). Receptors are clustered within the coated pits, and on ligand binding an endocytic vesicle pinches off from the PM (Conner and Schmid, 2003). On internalisation, the clathrin coat depolymerises (Khalil *et al.,* 2006), described as the vesicle uncoating, and facilitates their fusion with early endosomes (Mellman, 1996). Within early endosomes ligands are released from their receptors (due to the mildly acidic pH apparent), and the contents are transported to lysosomes for degradation and the receptors transported back to the PM within recycling vesicles that bud from the early endosomes (Gruenberg and Maxwell, 1995, Mellman, 1996, Gruenberg, 2001).

Although stated that receptors are concentrated within the coated pit region of the PM, there are other hypotheses that explain the internalisation of substances via CME. It has been suggested that the PM wraps around the substance to be internalised, due to the fact that receptors are uniformly distributed along the PM, and on binding of the appropriate ligand, the receptor density increases due to the diffusion of receptors in the immediate region to the contact site, so that over time the size of the 'contact area' increases, as more receptors are captured within it, thus encouraging further wrapping of the PM around the substance to be internalised (Gao, Shi and Freund, 2005). A mathematical model was used to explain this phenomenon and it is acknowledged that the size of the particle, and availability of receptors will limit the process (Gao, Shi and Freund, 2005). It is believed that the particle entry is size dependent and an optimal radius is 27-30nm for spherical particles (Gao, Shi and Freund, 2005).

4.1.4.2 Macropinocytosis

Macropinocytosis allows large volumes of extracellular fluid to be encapsulated by the PM, due to the actin-driven formation of membrane protrusions (Perret *et al.*, 2005). It is also known that the PM 'ruffles' (actin rich protrusions) are able to surround the area to be internalised, collapse, and fuse with the PM to form large PM bound vesicles (Patel, Zaro and Shen, 2007) which is a non specific process (Khalil *et al.,* 2006). Large endocytic vesicles named macropinosomes are formed that fuse with lysosomes to enable the degradation of the cargo or are recycled back to the cell surface where the contents are redirected out of the cell (Perret *et al.*, 2005).

4.1.4.3 Phagocytosis

Phagocytosis is predominantly associated with specialised cells such as macrophages and neutrophils (Conner and Schmid., 2003) and is responsible for the uptake of large materials (greater than 0.5µm) such as bacteria or cell debris (Khalil *et al.,* 2006). During phagocytosis, the cell recognises ligands via cell surface receptors, which then triggers the polymerisation and rearrangement of the actin cytoskeleton to form membrane extensions that allow the PM to surround or 'engulf' the material to be internalised (Liu and 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, as the contents are exposed to hydrolytic enzymes, and free radicals (Perret *et al.*, 2005).

4.1.4.4 Caveolae dependent endocytosis

Information regarding the alternative, clathrin independent pathways is more limited due to the fact that CME pathway has been most extensively studied. Caveolae are omega, 'flask' shaped invaginations (50-80nm) found within the PM, and are enriched with cholesterol (Johannes and Lamaze, 2002, Pelkmans and Helenius, 2002). It is known that caveolae are abundant in some cell types including endothelial cells, and muscle cells, and absent from others (Parton and Richards, 2003). The shape of the invaginations is determined by the protein caveolin which binds cholesterol at the PM (Conner and Schmid, 2003). Caveolin is required for the formation, morphology, maintenance and stability of caveolae, and in the absence of this protein or cholesterol, caveolae are absent

from cells (Pelkmans and Helenius, 2002). It is known that toxins such as the cholera toxin can trigger the formation of caveolae within the PM to facilitate and encourage its internalisation (Perret *et al.,* 2005).

In general it is acknowledged that the distinct endocytic pathways all follow the same sequence of intracellular events (figure 4.2), however there are exceptions to this, which is exemplified by the uptake of the Simian Virus 40 (SV40) by caveolae mediated endocytosis, which delivers the virus to the ER (Nichols and Lippincott-Schwartz, 2001, Pelkmans and Helenius, 2002). If the entry of SV40 is typical for all substances taken up by this pathway, substances to be internalised are thought to become trapped within the caveolae situated on the PM, perhaps due to an attraction. As a consequence of this interaction, caveolae become smaller, and an unknown receptor binds the cargo tightly to the caveolae membrane, and then caveolae activates (by an unknown mechanism) a phosphorylation cascade which triggers a vesicle to pinch from the PM (Pelkmans and Helenius, 2002). It is thought that the endocytic vesicle then transfers its contents to caveosomes, which are pre-existing organelles found within the cytoplasm that have a neutral pH, and thus are not involved in the degradation of caveosome contents, like their early endosomal counterparts. It is unlikely that caveosomes fuse with endosomes or lysosomes, but instead deliver their contents to the ER, further proving that this process does not adhere to the 'typical' intracellular events that the other endocytic pathways follow (Nichols and Lippincott-Schwartz, 2001, Pelkmans and Helenius, 2002, Patel, Zaro and Shen, 2007). Therefore, perhaps this route is adopted by substances that require delivery to compartments within the cell that cannot be easily accessed within other endocytic pathways (Pelkmans and Helenius, 2002). Knowledge that substances internalised by endocytosis are not automatically destined for degradation is useful when considering the design of NPs that need to remain intact to exert their activity within the cell.

4.1.4.5 Clathrin and caveolae independent endocytosis

Included within the definition of clathrin and caveolae independent pathways are phagocytosis, and macropinocytosis, but there are also mechanisms that allow the internalisation of substances that are more poorly understood (Conner and Schmid, 2003). Lipid rafts are composed from lipids such as cholesterol and

glycosphingolipids and are termed microdomains within the PM. Lipid rafts are mobile structures, which can move within the PM to the site where they are required (Mishra and Joshi, 2007). Caveolae are thought to derive from lipid rafts, with the caveolin protein responsible for the invagination of the PM (Liu and Shapiro, 2003). However raft invaginations, in the absence of caveolin are also evident within the PM, and can pinch off the PM to enable the uptake of substances, however the mechanisms that drive vesicle formation are unknown (Nabi and Le, 2003, Mishra and Yoshi, 2007, Hanzal-Bayer and Hancock, 2007). Lipid rafts are also implicated in cell signalling, which in addition to their role in the internalisation of substances, is thought to derive from the compartmentalisation of proteins (such as signalling molecules), termed 'sites of enrichment', within distinct regions the PM (Cavalli, Corti and Gruenberg, 2001, Nichols, 2003). It is thought that lipid rafts exhibit diverse functions, due to the fact that their composition is heterogenous, and their composition can be altered due to the recruitment and exclusion of membrane components; lipid rafts are therefore assumed to be a diverse population (Mishra and Yoshi, 2007). Within lipid rafts, cholesterol is concentrated in the PM, so that the lipid rafts have a different composition to the surrounding regions of PM, and this can also result in these regions of the PM being thicker (Nichols, 2003).

4.1.4.6. Methods of investigating NP uptake

A number of approaches can be utilised to interfere with endocytosis in general, or specific endocytic processes to elucidate their involvement in the internalisation of substances. Confocal microscopy can be used to identify the uptake of fluorescent particles by cells, with fluorescent probes able to give an indication of the specific mechanisms involved. This is possible due to the fact that the different components of the endocytic pathway have distinct compositions that can be identified with fluorescent labels, for example early endosomal antigen-1 (EEA-1) is present on the surface of early endosomes. Electron microscopy (EM) can be used to view the exterior or interior of cells in greater detail and can be used to reveal if substances are taken up by cells, and provide evidence regarding the mechanism of uptake, for example endocytosis is assumed to involve the integration of substances into membrane bound vesicles in the cell interior. There are two types of EM, that involve the same basic principle: an electron beam is directed onto the sample, under vacuum

conditions and an image is created from electrons that reflect from a sample that has been metal coated (Scanning Electron Microscopy, SEM) or that are able to pass through the sample (Transmission Electron Microscopy, TEM). Consequently SEM allows information about the cell surface to be obtained and TEM enables the imaging of the internal environment of a sample. It is acknowledged that imaging particles within the nano 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 at the high level of magnification used (Rothen-Rutishauser *et al.,* 2006). However it now possible to elucidate the chemical composition of regions imaged by EM by energy dispersive x-ray analysis.

It is known that endocytosis is an energy dependent process and can be inhibited by a low temperature due to the fact that ATP hydrolysis cannot occur (Patel, Zaro and Shen, 2007). This method can therefore be used to determine if endocytosis is involved in the internalisation of a substance (by comparing uptake at 4^{o}C to that apparent at 37^{o}C), but cannot elucidate which endocytic particular pathway is involved. The administration of pharmaceutical agents is also able to inhibit endocytosis in general, or events involved within specific endocytic pathways. For example, cytochalasin D causes the depolymerisation of the actin cytoskeleton and is able to inhibit uptake via caveolae and macropinocytosis, but not clathrin dependent endocytosis (Khalil *et al.,* 2006). Potassium depletion, hypertonicity of the cell medium (for example using a high sucrose concentration), cytosol acidification and chlorpromazine are known to inhibit clathrin dependent endocytosis, which is thought to occur due to the dissociation of the clathrin coat (Rejman *et al.*, 2004, Khalil *et al.,* 2006, Patel, Zaro and Shen, 2007). However the specificity of these treatments is disputed and their use may interfere with more than one type of endocytosis or uptake processes, so that they should be used with caution, and more than one approach should be utilised to gain insight into the mechanism of uptake. Genetic interference is also possible, whereby the expression of genes necessary for certain aspects of endocytosis are inhibited, so that components necessary for particular endocytic events are not formed, or are not functional and so endocytosis cannot occur. The depletion of essential components to

specific endocytic pathways, can therefore give insight into the contribution of specific endocytic processes to the uptake of particular substances.

4.1.5 Evidence of NP internalisation by cells

It is known that the size of NPs is similar to biological molecules (such as proteins, and DNA) and structures (including bacteria, and viruses) which are readily taken up by cells. Within the literature the investigation of NP internalisation by cells has utilised a variety of cell types, due to the fact that a number of organs are known to be targets of NP toxicity (see section 1.8). Consequently, as an array of evidence relating to NP uptake *in vitro* will be discussed, the origin cell lines, which have been extensively used can be found in table 1.1.

The shape and size of NPs has been demonstrated to impact on NP uptake. Chithrani, Ghazani and Chan, (2006) used negatively charged spherical (14, 30, 50, 74, and 100nm diameter) and rod (14x40nm, and 14x74nm) shaped gold NPs and investigated their uptake by HeLa epithelial cells after a 6 hour exposure (in cell culture medium containing serum). The uptake of spherical particles was greatest and it was observed that they were contained within vesicles in the cytoplasm of the cells, and it was suggested that NP uptake was mediated by proteins bound to the particle surface. It was also found that NP uptake increased with time until it plateaued, suggesting that the uptake process reached saturation. Consequently it was also speculated that the saturation of NP uptake may be a result of the extent of protein binding, due to the fact that protein that is not adsorbed onto the particle surface and therefore 'unbound' protein can compete for receptor sites on the site against the protein-adsorbed NPs. The uptake of 50nm particles was greatest, so that it was suggested that larger particles, which have a smaller surface area, have less protein adsorption to the particle surface, which explains their more limited uptake. It was therefore hypothesised that the uptake of gold NPs was mediated by the adsorption of serum proteins onto the particle surface, and these proteins enable NP entry via CME. However the uptake of smaller particles was less than that of their 50nm counterparts; this could be explained by the fact that there are size restrictions to CME, so that these particles are too small to be recognised. To confirm uptake by CME, particle uptake was

compared at 37^{o}C versus 4^{o}C, and it was found that uptake was reduced by the low temperature. As there are an array of serum proteins (which are ordinarily taken up by cells), NP entrance into cells may be mediated by multiple receptors. This study therefore makes a number of important observations, specifically that NP uptake occurs by CME, but that uptake is shape, size, charge, and serum dependent. The results of this study were expanded upon by Chithrani and Chan, (2007), where the spherical and rod shaped gold NPs were coated with the serum protein transferrin and uptake by HeLa, STO (fibroblasts) and SNB19 (neuronal) cell lines was investigated. Uptake of these NPs was confirmed via confocal microscopy (through the attachment of a fluorescent tag to the transferrin protein adsorbed onto the NP surface) and then low temperature, and ATP depleted environment used to confirm the involvement of endocytosis. To determine if a clathrin mediated mechanism was involved, cells were pre-treated with sucrose or potassium depletion, and uptake of the gold NPs was reduced under these circumstances. The findings were found to be similar for both gold NP shapes, and so the mechanism of uptake was assumed to be identical. These findings therefore demonstrated the ability of a variety of cell types to internalise NPs by CME and highlighted the importance of particle shape, in addition to highlighting that external factors such as protein adsorption are able to influence NP uptake.

Stearns, Paulaukis, and Godleski, (2001) demonstrated that 50nm TiO_2 particles were internalised by A549 alveolar type 2 epithelial cells into membrane bound vesicles after 6 and 24 hour exposures. However, it was observed that the uptake of particles by these cells was limited to their aggregated form and was mediated via by phagocytosis, due to the fact that PM projections surrounded and engulfed the particles prior to internalisation. This study therefore demonstrates that lung epithelial cells are able to internalise NPs by phagocytosis, and highlights the usefulness of microscopy in determining the mechanisms of NP uptake.

Rejman *et al.,* (2004) used fluorescent microspheres (50, 100, 200, 500 and 1000nm diameter) to investigate the size dependency of particle uptake by melanoma B16-F10 cells. Uptake was evaluated using confocal microscopy and flow cytometry and was evident for all, but the 1000nm beads, with 50, 100

and 200nm beads distributed throughout the cells but 500nm beads were observed to remain at the periphery of the cell. The uptake of 50nm beads was greatest, with internalisation reducing, as particle size increased. No internalisation of beads (of all sizes) was observed at 4^{o}C, and after disruption of microtubules with nocodazole, 50, 100 and 200nm beads appeared to accumulate at the PM, which lead the authors to suggest that an endocytic mechanism of uptake was central to particle internalisation. To investigate this further intracellular potassium depletion, and pre-treatment of cells with chlorpromazine confirmed that clathrin mediated endocytosis enabled their uptake. When caveolin-mediated endocytosis was inhibited (using filipin) the uptake of 500nm beads was affected to the greatest extent. A size dependent method of uptake was therefore observed, specifically that smaller particles (particles up to 200nm diameter) were preferentially internalised via clathrin mediated endocytosis and larger particles (with particles ranging from 200nm, but smaller than 1µm) via caveolin mediated endocytosis. This study demonstrates the size dependent endocytic uptake of particles, and illustrates the importance of experimental tools to study uptake processes. The size dependent uptake of particles has also been demonstrated by Desai *et al.,* (1997) who demonstrated that the uptake of 100nm NPs (polylactic polyglycolic acid co-polymer) was 2.5 times greater, when compared to the uptake of larger sized particles (1µm diameter), with the uptake of 1µm particles being 6 fold greater than 10µm in endothelial Caco cells, although different methods of uptake are expected. Furthermore Rothen-Rutishauser *et al.,* (2006) investigated the uptake of fluorescent particles (1µm, 0.2µm and 78nm, positively, negatively and non-charged) by red blood cells (RBCs). NP uptake was identified using confocal microscopy, and it was observed that 1µm polystyrene particles were attached to the cell surface but were not internalised by cells during any of the incubation times (4-24hours). Negatively and non charged 200nm particles were found within cells, as were positively charged particles, however these particles were also apparent at the cell surface. The 78nm particles (non charged and negatively charged) were also found within cells. Positive polystyrene fluorescent particles were not available in the nano size range, so positively charged gold particles were used to investigate the impact of NP charge on uptake by RBCs (as they are non-phagocytic and allowed other mechanisms of NP uptake to be revealed). TEM analysis of the

cells was conducted using gold NPs to investigate the influence of NP charge (positive, negative and neutral) to their uptake and it was found that uptake was comparable. It was highlighted that identifying particles within the nano size range was difficult due to their similarity in size (and appearance) to cellular structures. Therefore confocal visualisation allowed the size dependence of particle uptake to be compared, with TEM analysis utilised to further investigate the intracellular fate of particles. It was observed that the gold NPs were not membrane bound within the RBCs, and it was therefore concluded that endocytosis was unlikely to contribute to their internalisation.

NP uptake has also been demonstrated to be charge dependent. Harush-Frenkel *et al.,* (2007) used positively (89.8±4nm) and negatively (96.4±6nm) charged polyactide PEGylated particles to determine if they adopted the same internalisation pathway to enter HeLa cells. It was apparent that NPs were internalised by cells in a time dependent manner, however positively charged NPs were internalised at an earlier time, and accumulated to a greater extent, when compared to their negatively charged counterparts. The uptake of positive NPs plateaued, 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, which was in contrast to the uptake of positive NPs. It was reported that positive NP uptake was evident despite the inhibition of the clathrin and caveolin endocytic processes, suggesting that there were compensatory mechanisms that mediated NP uptake, and despite having a preferential pathway of uptake, others can contribute to their uptake when necessary. Therefore NP surface charge dictated the amount of NPs internalised, and the uptake pathway that was utilised.

Geiser *et al.,* (2005) used primary porcine pulmonary macrophages and human RBCs to investigate the uptake of fluorescent polystyrene particles (1µm, 0.2µm and 78nm diameter). Rats were also exposed (inhalation) to TiO_2 particles (22nm) to identify the distribution of particles within the different lung cells (including fibroblasts, epithelial and endothelial cells). Within *in vitro* studies, all polystyrene particles were found within macrophages, however the particles within the nano size range were taken up by cells to the greatest extent.

Cytochasin D was used to inhibit phagocytosis, and this treatment was unable to inhibit the uptake of the smaller particles, but did inhibit 1µm particle uptake. These findings therefore confirm that the mechanism of particle uptake is size dependent, and that larger particles are preferentially taken up by phagocytosis. The *in vivo* experiments demonstrated that inhaled TiO_2 NPs were found within the majority of lung cell types and were predominantly localised within the cytoplasm (and were not membrane bound), and rarely within the nucleus. Regardless of an *in vitro* or *in vivo* scenario, internalised NPs were not contained within membrane bound vesicles, consequently the mechanism of uptake is thought to be non-endocytic in nature. Therefore, the studies discussed highlight that dissecting out the role of particular cell types and uptake processes to NP internalisation is made difficult by the conflicting evidence, and may be NP and cell specific.

Phagocytosis of NPs by alveolar macrophages has been suggested to be central to the uptake of NPs within the lung (Donaldson, Li and MacNee, 1998). However it is acknowledged that large numbers of particles (such as ufCB and TiO_2) are able to overwhelm macrophage defences, so that the particles are able to evade clearance to exert toxicity through prolonged interactions with other lung cell populations such as epithelial cells (Donaldson, Li and MacNee, 1998). Geiser *et al.,* (2008) argued that alveolar macrophages were not primarily responsible for the uptake and clearance of NPs within the lung *in vivo*. This derived from evidence that large TiO_2 particles (3-6µm) were more effectively cleared by alveolar macrophages, than their smaller (20nm) counterparts, which were able to bypass this important clearance mechanism within the lung due to their small size. These findings are therefore in keeping with the size limitations of uptake processes such as phagocytosis. The ability of TiO_2 NPs to evade phagocytosis was confirmed by the findings that they were not enclosed by a vesicular membrane equivalent that was apparent surrounding the larger TiO_2 particles. Therefore it was suggested that phagocytosis was not integral to the uptake mechanism, with a sporadic, non-specific mechanism of uptake hypothesised to be responsible for NP uptake into macrophages. Alternatively it was suggested that NPs were internalised unintentionally when the macrophages phagocytosed other material. The findings of Geiser *et al.,* (2008) are also able to provide an explanation for the

finding that only TiO_2 agglomerates, and not particles evident within nano dimensions, were internalised by A549 cells *in vitro* (Stearns, Paulauskis and Godleski, 2001). Consequently, these findings suggest that the removal of NPs by macrophages may be a size dependent phenomenon, and its success is also likely to be dependent on the number of particles present. However other macrophage populations have been instrumental to the size dependent uptake of particles, for example Ogawara *et al.,* (1999a) observed that Kupffer cells were largely responsible for the accumulation of NPs within the liver *in vivo*, and that smaller polystyrene particles (50nm) were taken up by Kupffer cells to a greater extent that the larger counterparts (500nm).

The studies discussed demonstrate that a number of different entry mechanisms have been proposed to explain the uptake of NPs 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 which may be necessary when one pathway is overwhelmed. In addition it is foreseeable that deciphering the properties of NPs that facilitate NP internalisation can aid in the design of NPs that facilitate or prevent uptake by cells, when required.

4.1.6 Principles of immunofluorescent staining

Immunofluorescent staining was utilised in order to visualise sub-cellular structures within hepatocytes, and therefore the principles underlying this process will be discussed. Immunofluorescence staining is a technique that utilises antibodies to recognise a particular site of interest within the cell, whereby successful binding of an antibody to specific targets can be identified due to a fluorescent signal. Indirect immunofluorescence uses two types of antibody; the primary antibody is directed against the cellular target (antigen) of interest, and the secondary antibody is fluorescently tagged, and enables the recognition of the primary antibody (that is bound to its target), so that a fluorescent conjugate is formed that can be identified by fluorescent microscopy. Prior to any staining procedure, treated cells must be fixed to immobilise the cell components, but allow the original cell structure to be retained, which is enabled through the use of the fixative paraformaldehyde.

Next, it is necessary that the antibodies used gain access to the cell interior in order to allow for the recognition of the intracellular target. As a consequence, the plasma membrane is permeabilised, through the exposure of fixed cells to Triton-X100, as Triton-X100 is a detergent that is able to create holes within the PM to allow the access of antibodies to the cell interior. To enhance the specificity of the antibody binding, bovine serum albumin (BSA) can then be used as a blocking buffer. Therefore when cells are exposed to the primary antibody, it will displace BSA from the antigen of interest due to its stronger binding affinity.

It is also necessary to carry out a number of 'controls' when conducting indirect immunofluorescent staining, to ensure that the correct cellular structure is being visualised, and that auto-fluorescence is not responsible for the image that is produced. Within the experiments discussed this was achieved by exposing cells to 1) primary antibody only, 2) secondary antibody only (to determine if a non-specific interaction occurred with the cells) 3) no antibody (cells only). The same 'scanning parameters' on the confocal microscope used to detect the fluorescence in samples is used to determine if there is autofluorescence within the sample and to confirm that any fluorescence visualised is indicative of successful binding of the primary and secondary antibody.

4.1.7 Aim

To investigate the uptake and cellular fate of fluorescent carboxylated polystyrene NPs (20nm or 200nm diameter) by HepG2 and C3A cell lines and isolated rat hepatocyte couplets (IRHC) in order to ascertain whether the cell lines are appropriate models to represent primary cell behaviour.

4.2 Materials and Methods

4.2.1.1 Equipment

The following equipment was employed: Carl Zeiss LSM 510 Meta confocal microscope, and PeCon GmbH perfusion, open and closed cultivation (POC-R) chamber. All other equipment utilised has been described previously.

4.2.1.2 Materials

The chemicals used included: Poly-D-lysine hydrobromide, bovine serum albumin (BSA) paraformaldehyde, Triton-X100 and Hoescht nuclear stain were purchased from Sigma-Aldrich (Poole, UK). Phenol red free MEM growth medium was purchased from Gibo-Invitrogen (Paisley, UK). Fluorescent carboxylate-modified microsphere polystyrene beads, (200nm and 20nm diameter) were obtained from Molecular Probes (Oregon, USA). Mouse anti-α-tubulin monoclonal antibody, Alexa Fluor 633 goat anti-mouse IgG, Goat anti-rabbit Cy5, MitoTracker Green FM, LysoTracker Red DND-99 were purchased from Molecular Probes (Cambridge, UK). EEA-1 Rabbit polyclonal antibody was purchased from Santa Cruz (Wembley, UK). Mowiol was purchased from Calbiochem, Gibbstown USA. CLF (NRL972) was kindly provided by Norgine, UK.

All other materials were purchased from sources previously stated.

4.2.2.1 Evaluating the uptake of fluorescent polystyrene particles by HepG2 and C3A cell lines and IRHCs over time, using fixed cell imaging

HepG2 and C3A cells were plated onto glass coverslips (in 24 well plates) at a concentration of $2x10^5$ cells/ml (at a volume of 1ml) and incubated overnight at 37^oC, 5% CO_2. The following day, cells were treated with 20nm or 200nm diameter fluorescent polystyrene NPs (250µl) at a concentration of 125µg/ml (suspended in cell culture medium containing 10% FCS) or vehicle control for 10, 30 or 60 minutes at 37^oC, 5% CO_2. Subsequent to exposure, cells were fixed with 3% paraformaldehyde (500µl, in PBS) at 4^oC for 30 minutes. The fixed cells were then permeabilised with 0.1% Triton-X100 (250µl, in PBS) at room temperature for 20 minutes. Next, cells were washed with PBS and exposed to anti-α-tubulin monoclonal antibody (250µl, 1:200 in PBS) for 30 minutes, the cells were then washed with PBS and then treated with the

secondary antibody Alexa Fluor 633 (250µl, 1:400 in PBS (excitation/emission: 633/650) for 30 minutes. The cell nuclei were stained with Hoescht nuclear stain (500 µl) for 30 seconds, the cells washed with PBS and the coverslips mounted onto slides using Mowiol. The cells were observed using confocal microscopy using the 510 Meta LSM programme.

Hepatocyte couplets were isolated as previously described (see section 3.6), and plated at a concentration of $3x10^5$ cells/ml onto sterilised 10mm glass coverslips (in 24 well plates) which had been coated with poly-D-lysine (0.1 mg/ml in PBS). The cells were then incubated for a minimum of 4 hours at 37^oC, 5% CO_2 and then treated, fixed, permeabilised and stained as described for the cell lines.

4.2.2.2 The live imaging of fluorescent polystyrene particle uptake by hepatocyte cell lines

C3A and HepG2 cells were plated onto 42mm glass coverslips in 55mm Petri dishes, at a concentration of $2x10^5$ cells/ml and volume of 7ml, and incubated overnight at 37^oC, 5% CO_2. The following day the coverslips were transferred to a POC-R chamber, and 4ml complete cell culture medium (phenol red free) added. The chamber was placed on the incubated stage (37^oC) of the confocal microscope and the CO_2 supply (5%) connected to maintain cell viability during the observation time. The cells were visualised for a 2 minute control period using the confocal microscope. The culture medium (phenol red free) was then removed and replaced with 4ml of 15.6µg/ml fluorescent polystyrene particle suspension (20nm and 200nm, dispersed in cell culture medium in the presence or absence of serum) and the cells visualised for 1 hour.

4.2.2.3 Investigating the incorporation of 20nm fluorescent polystyrene NPs into early endosomes within cell lines and IRHCs

C3A and HepG2 cells and hepatocyte couplets were plated, exposed to fluorescent polystyrene 20nm particles, fixed, and permeabilised, as previously described (see section 4.3.3.3). The permeabilised cells were blocked with 1% BSA (250µl, in PBS) at room temperature for 1 hour. Next, the cells were washed with PBS, exposed to EEA-1 rabbit polyclonal antibody (250µl, 1:250 dilution in PBS) for 1 hour, washed with PBS and then exposed to the secondary antibody goat anti-rabbit Cy5 (250µl, 1:200 dilution in PBS,

excitation/emission: 649/670) for 45 minutes. The cells were washed with PBS and the coverslips mounted onto slides using Mowiol and imaged using confocal microscopy.

4.2.2.4 Evaluating the incorporation of fluorescent 20nm polystyrene NPs within lysosomes or mitochondria of cell lines and IRHCs

C3A and HepG2 cells, were plated at a density of $2x10^5$ cells/ml (at a volume of 4ml) onto sterilised 42mm glass coverslips (in 55mm Petri dishes) and incubated overnight at 37^{o}C, 5% CO_2. IRHCs (isolated as described in section 3.6) were plated at a density of $2x10^5$ cells/ml (at a volume of 4ml) and incubated for 4 hours in atmospheric air, at 37^{o}C. When imaging lysosomes, cells were treated with 125µg/ml fluorescent polystyrene NPs (3ml, suspended in complete cell culture medium, excitation/emission: 505/515), for 10, 30 or 60 minutes at 37^{o}C, 5% CO_2. During the last 10 minutes of NP exposure, cells were exposed to 50nM LysoTracker (excitation/emission: 577/590). The cells were then washed with complete cell culture medium and the coverslip transferred to a POC-R chamber (with 2ml cell medium), and lysosomes imaged using confocal microscopy. When imaging mitochondria, cells were pre-treated with 500nM MitoTracker (4ml, in complete cell culture medium, excitation/emission: 490/516) for 30mins at 37^{o}C, 5% CO_2. The cells were then washed (with complete cell culture medium) and treated with 125µg/ml fluorescent polystyrene NPs (3ml, excitation/emission: 580/605). After particle exposure, cells were washed and the coverslip transferred to a POC-R chamber (with 2ml cell medium) and the mitochondria contained within the cells were observed using confocal microscopy.

4.2.2.5 Evaluating the incorporation of fluorescent 20nm polystyrene NPs into the bile canaliculi of hepatocyte cell lines and IRHCs

Hepatocyte couplets were isolated as previously described (see section 3.6) and cultured at a density of 1 x 10^5 cells/ml onto 42mm sterilised glass coverslips (at a volume of 4ml), in 55mm Petri dishes, and incubated for at least 4 hours at 37^{o}C in atmospheric air. Fluorescent polystyrene NPs (20nm) were exposed to hepatocyte couplets at a concentration of 62µg/ml (3ml) for 30 minutes (suspended in L-15 cell culture medium with 10% FCS) or appropriate control. During the last 15 minutes of the treatment 5mM cholyl lysyl fluorescein

(CLF, 3µl, in saline) was added, so that cells were exposed to a concentration of 5µM CLF. Cells were then washed with L-15 medium, and the coverslip transferred to a POC-R chamber, 2ml L-15 cell culture medium added, and the cells immediately visualised on a heated stage (37^{o}C) by confocal microscopy.

When investigating CLF uptake by hepatocyte cell lines; HepG2 and C3A cells were plated at a concentration of 2x10^{5} cells/ml onto 42mm glass coverslips, in 55mm Petri dishes (4ml). The plates were incubated overnight at 37^{o}C, and 5% CO_2. The cells were then exposed to particles and CLF as described.

4.3 Results

4.3.1 Evaluating the size and time dependent uptake of fluorescent polystyrene particles by hepatocytes

It was observed using fixed cell imaging (conducted in the presence of serum), that the uptake of 20nm polystyrene NPs by both the HepG2 and C3A cell lines, as well as the primary hepatocyte couplets, occurred earlier and to a greater extent than for 200nm polystyrene particles. It was apparent that the internalisation of 20nm polystyrene NPs increased progressively over the time points investigated, in all cell types. Specifically, after a 10 and 30 minute exposure time, 20nm particles were observed to co-localise with the tubulin cytoskeleton (figure 4.2B-C). The 20nm particles were then observed to become incorporated in compartments located within and between adjacent cells, at later time points of 30 and 60 minutes for all cell types (figure 4.2C-D). Although the size, and time dependent uptake of particles was consistent between the different hepatocyte cell types there were some variations, within the pattern of uptake described. Within the C3A cell line, there was evidence of 20nm particle compartmentalisation after a 10 minute exposure (figure 4.2B), which is earlier than that experienced within the other cell types. The co-localisation of 20nm particles with the tubulin cytoskeleton was observed to occur at all time points within the hepatocyte couplets, which is in contrast to the pattern of uptake observed within the cell lines. The uptake of 200nm particles was limited and apparent only at later time points (30 minutes onwards) in all cell types, with the majority of cell-associated particles being present at the cell surface (figure 4.3). Z stacks (and their associated projections) were used to confirm that the particles were evident within C3A cells (figure 4.4). Again, it was demonstrated that 20nm particles were co-localised with the cytoskeleton at 10 and 30 minute exposure times, that NPs formed compartments within cells at 30 and 60 minutes, and that 200nm particles were internalised to a limited extent, with the majority evident at the cell surface. It was also apparent that the projections gave a better indication of the level of particle uptake by cells, whereby particles present within the cell interior were visualised more clearly, and again it was apparent that there was a size and time dependent uptake of polystyrene particles by hepatocytes. Furthermore, the xz and yz micrographs (created from z stacks) allowed a distinction between internalised and non-internalised particles to be gained, which arises from the knowledge that within

the xz or yz micrographs that were constructed contain information from one y or x ‘plane’ but all z sections (i.e. the whole cell) are visualised, so that particles contained within or outside the cell can be observed. It was clearly indicated that the internalisation of polystyrene NPs occurred to a greater extent than that of their larger counterparts, and that the uptake of particles increased with time (figure 4.5).

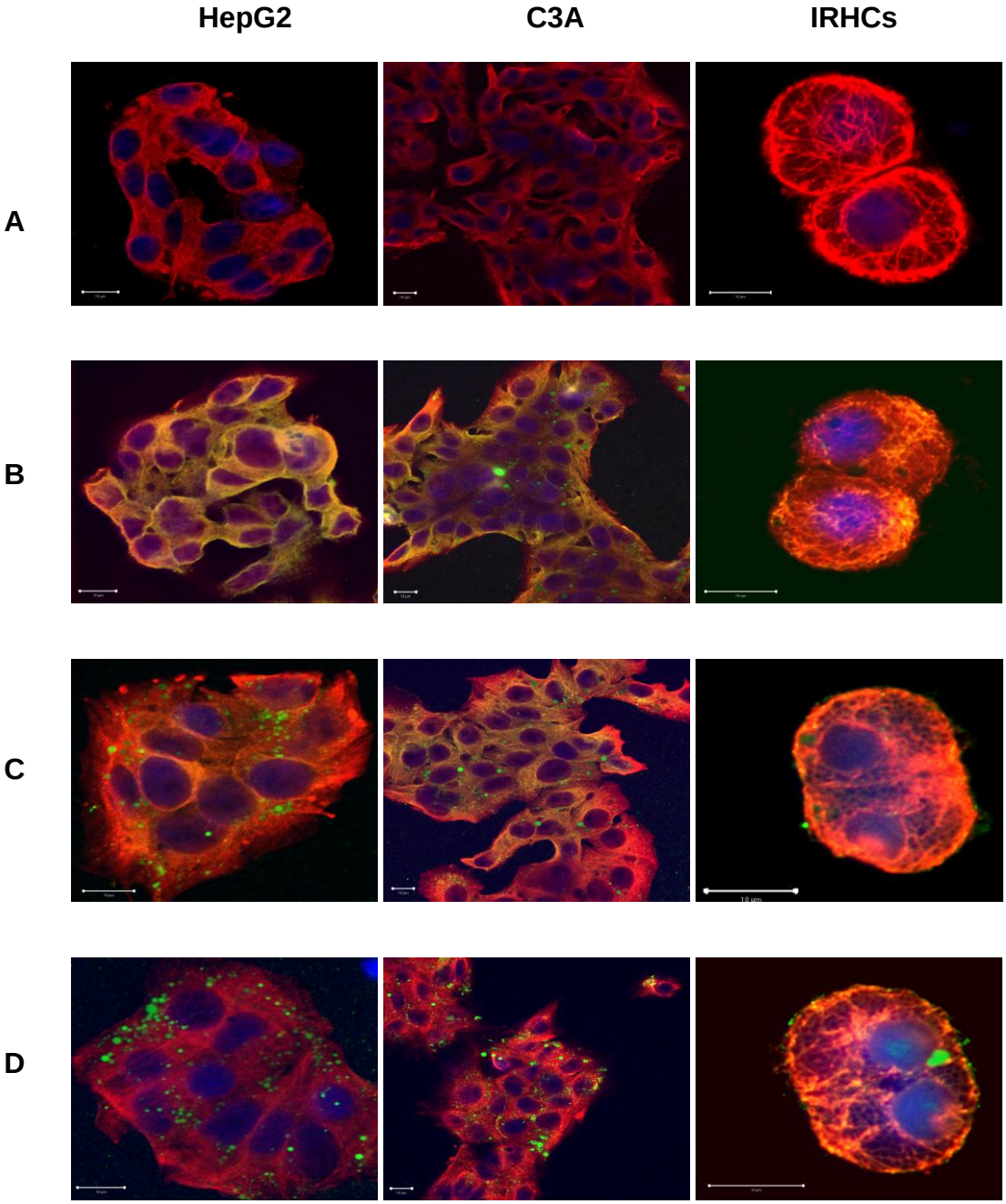


Figure 4.2 The time dependent uptake of 20nm fluorescent polystyrene NPs by hepatocyte cell lines and IRHCs. Cells were treated with cell medium (**A**) or 125µg/ml polystyrene 20nm NPs for 10 (**B**), 30 (**C**), or 60 (**D**) minutes, and then fixed, permeabilised and stained for the tubulin cytoskeleton and nucleus. Red represents the tubulin cytoskeleton, green the 20nm polystyrene particles and blue the nucleus (magnification x63). Appearance of the yellow/orange colour arises as a consequence of the co-localisation of the green polystyrene NPs with the red cytoskeleton. The images are representative of 3 experiments. Scale bar = 10µm

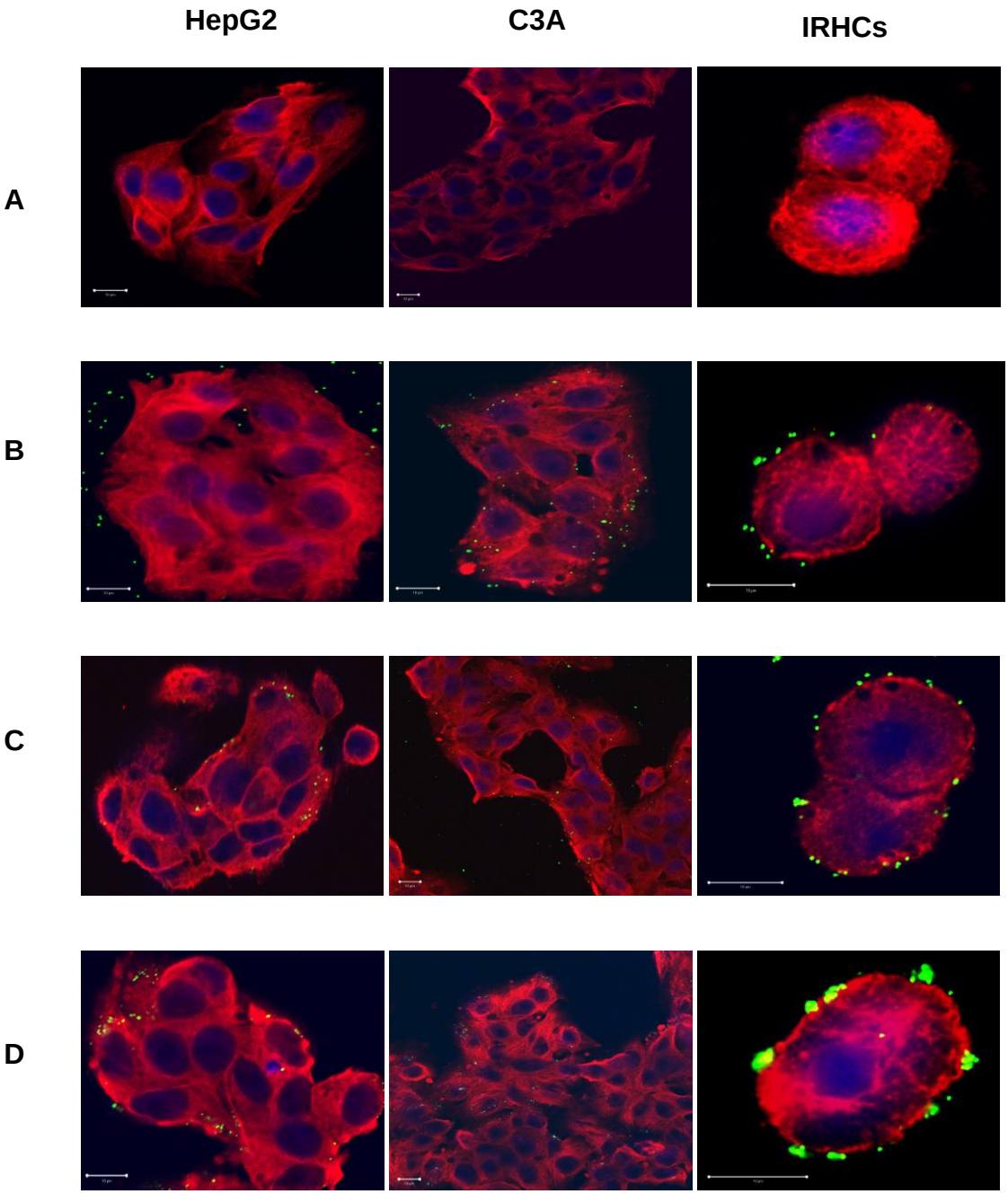


Figure 4.3 The time dependent uptake of 200nm fluorescent polystyrene particles by hepatocyte cell lines and IRHCs. Cells were treated with cell medium (**A**) or 125µg/ml 200nm polystyrene particles for 10 (**B**), 30 (**C**), or 60 (**D**) minutes, and then fixed, permeabilised and stained for the tubulin cytoskeleton and nucleus. Red represents the tubulin cytoskeleton, green the 200nm polystyrene particles and blue the nucleus (magnification x63). Appearance of the yellow/orange colour arises as a consequence of the co-localisation of the green polystyrene NPs with the red cytoskeleton. The images are representative of 3 experiments. Scale bar = 10µm

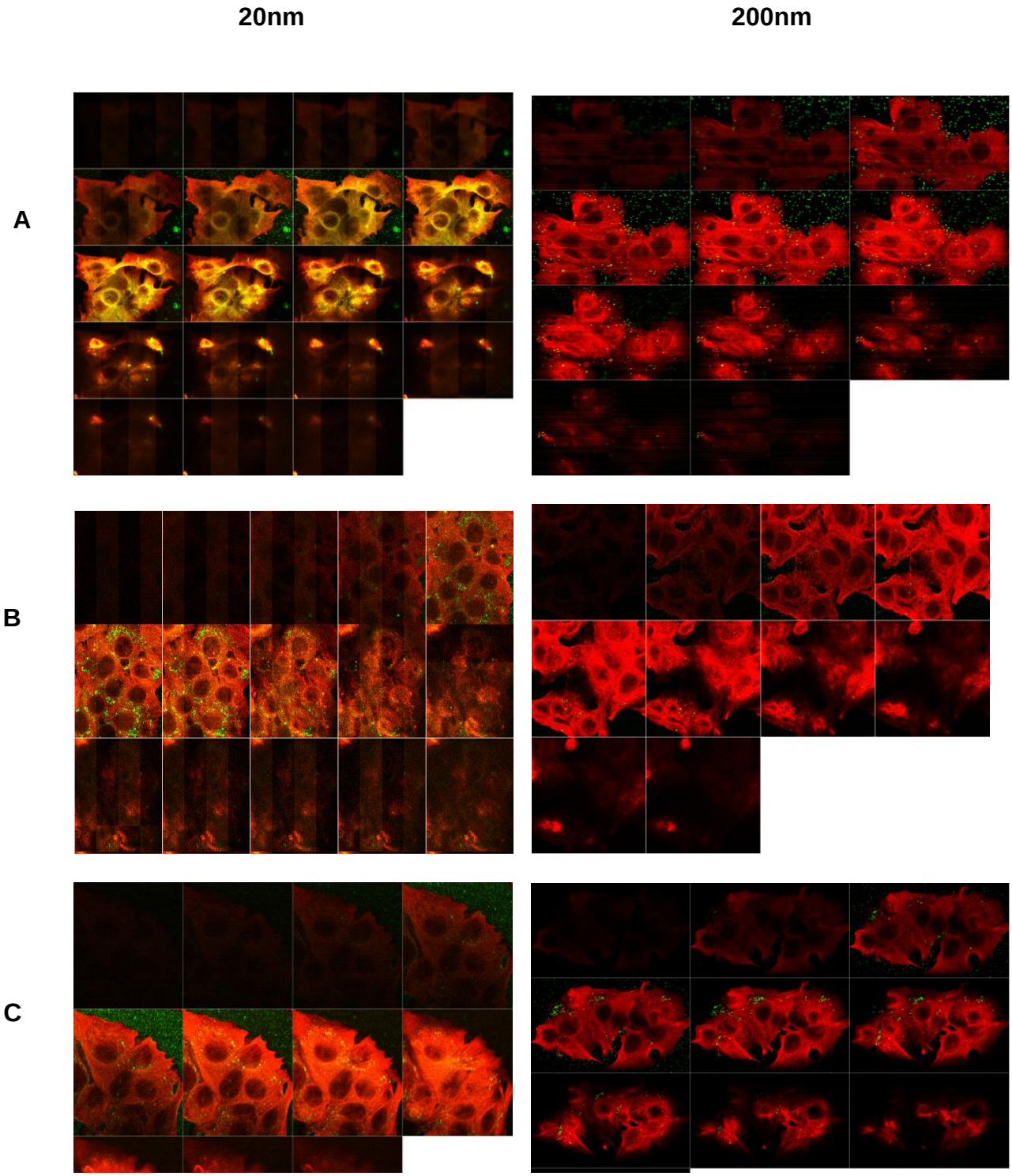


Figure 4.4 Confirming the uptake of 20 or 200nm polystyrene particles by C3A cells using Z stacks. For animations and projections please see the supplementary DVD (if the movie is too fast, pause the animation and the different optical slices can be visualised by changing the 'clip position'). Cells were exposed to 125µg/ml polystyrene particles (20 or 200nm diameter) for 10 (**A**), 30 (**B**), or 60 (**C**) minutes, and then fixed, permeabilised and stained. Red represents the tubulin cytoskeleton, and green the particles (magnification x63). Appearance of the yellow/orange colour arises as a consequence of the co-localisation of the green particles with the red cytoskeleton. A number of optical slices were taken through the cells to determine if particles were evident within the cells.

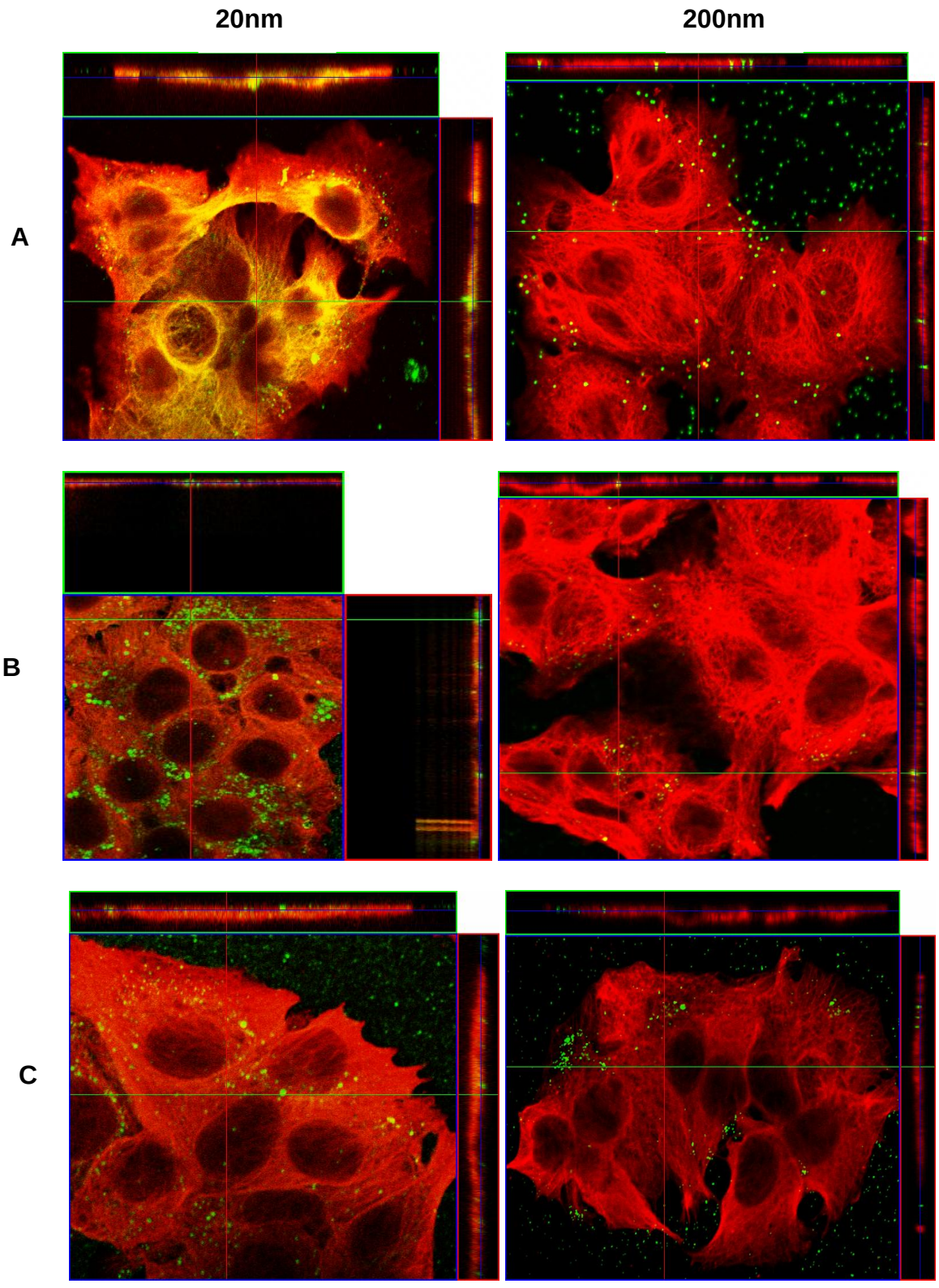


Figure 4.5 Confirming the uptake of 20 or 200nm polystyrene particles by C3A cells with xz and yz micrographs (composite of full z stacks) Cells were exposed to 125µg/ml polystyrene particles (20 or 200nm diameter) for 10 (**A**), 30 (**B**), or 60 (**C**) minutes, and then fixed, permeabilised and stained. Red represents the tubulin cytoskeleton, and green the particles (magnification x63). Appearance of the yellow/orange colour arises as a consequence of the co-localisation of the green particles with the red cytoskeleton. An optical slice of a z stack is used to create a xz and yz micrographs so that the internalisation of particles by cells could be confirmed

4.3.2 Investigating the uptake of polystyrene particles by hepatocyte cell lines using live Imaging

The live imaging of polystyrene particle uptake by the hepatocyte cell lines was carried out in the presence (10% FCS in cell culture medium) and absence of serum. The uptake of 20nm NPs by hepatocyte cell lines was observed to gradually increase over the observation time when dispersed within serum containing medium, and that towards the end of the observation period NPs were observed to preferentially accumulate in compartments located within and between adjacent cells (Figure 4.6). In contrast it was found that in the absence of serum, 20nm polystyrene NPs formed large aggregates which were associated with reduced NP uptake by hepatocytes (Figure 4.6). The uptake of 200nm particles by hepatocytes was less extensive than that of the smaller particles and occurred to a limited extent, with particle uptake evident towards the end of the observation time. The absence of serum from the 200nm particle suspension did not appear to impact on particle aggregation, with the uptake of particles being minimal, and comparable in both exposure scenarios.

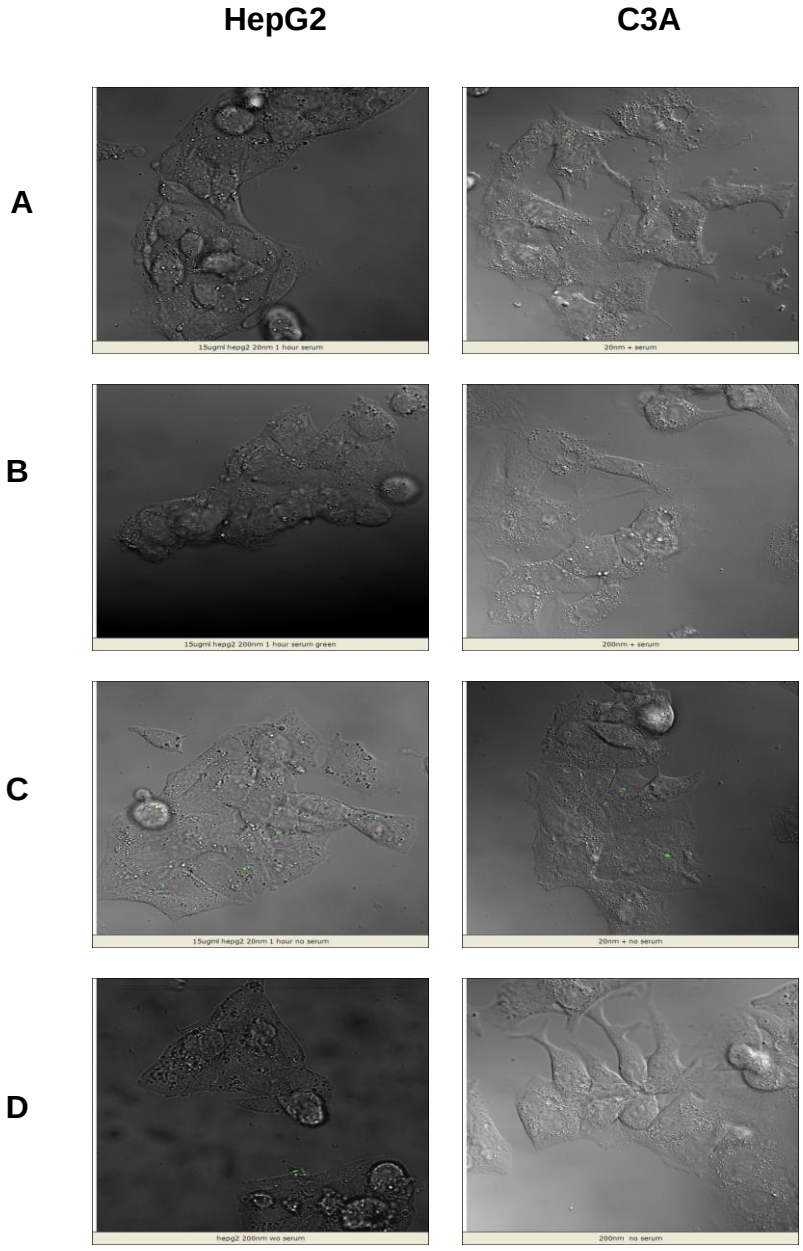


Figure 4.6 The live imaging of fluorescent polystyrene particle uptake (20nm and 200nm) by C3A and HepG2 cells, in the presence and absence of serum. The images above represent the initial image of the observation, with the full animations available within the supplementary data DVD. Cells were imaged for one hour, and administered 15µg/ml 20nm (**A**) or 200nm (**B**) particles dispersed in serum containing cell culture medium and 20nm (**C**) or 200nm (**D**) particles in the absence of serum, after a control observation time of 2 minutes.

4.3.3 Revealing the fate of internalised 20nm fluorescent polystyrene NPs

NPs (20nm) were internalised at all time points, by all hepatocyte cell types, and therefore their intracellular fate requires addressing. As the internalisation of 200nm polystyrene particles was limited, their fate within hepatocytes was not considered further.

4.3.3.1 Investigating the Incorporation of 20nm fluorescent polystyrene NPs within early endosomes

Early endosomes were identified by staining for the early endosome antigen-1 (EEA-1), and were observed within all cell types investigated (figure 4.7A), and this fluorescence was not a consequence of cell autofluorescence (figure 4.7E). The uptake of 20nm NPs was observed to increase progressively over time, however the NPs were not co-localised with EEA-1, at any of the time points, in all cell types investigated (figure 4.7B-D).

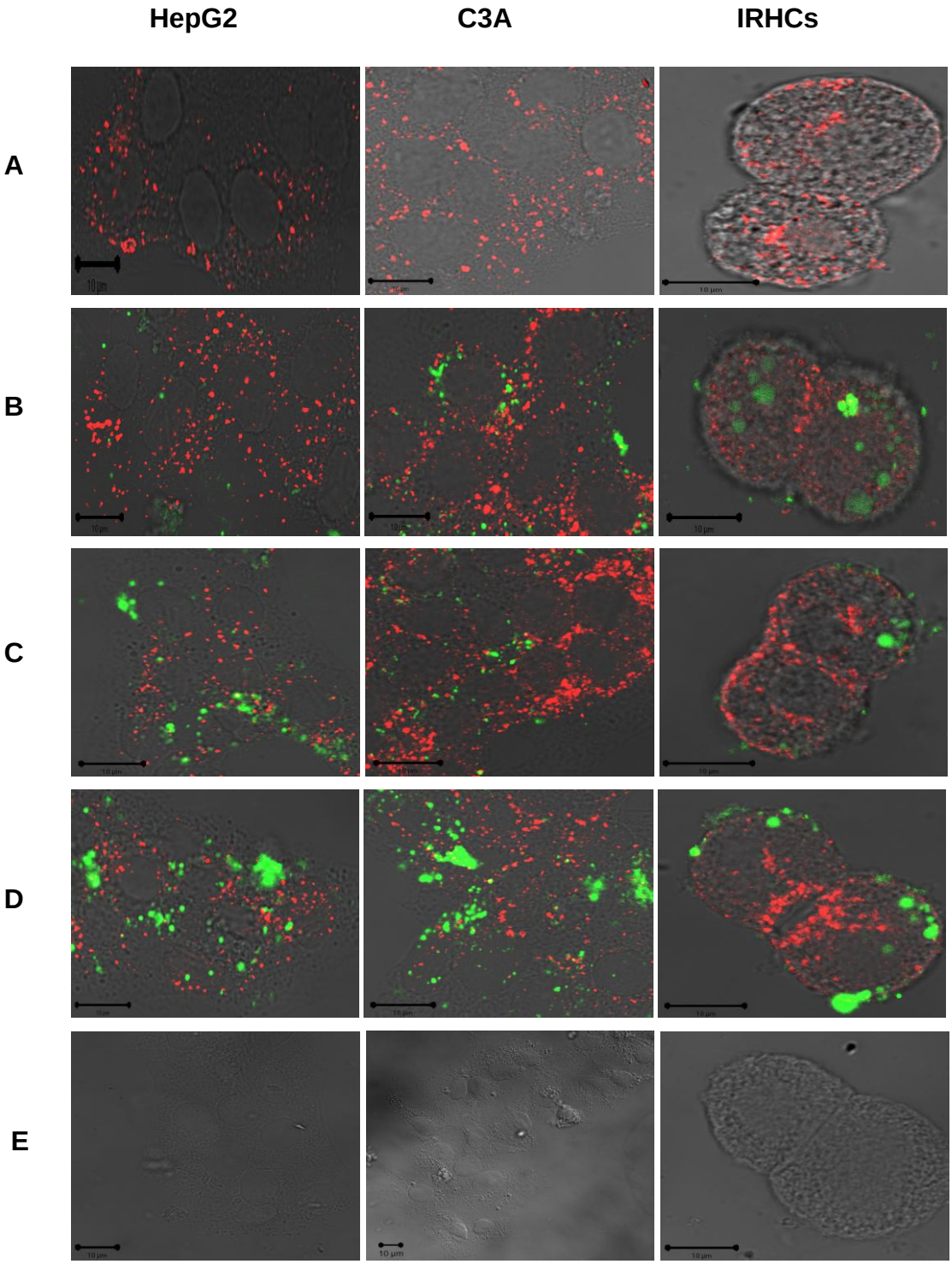


Figure 4.7 Investigating the incorporation of 20nm fluorescent polystyrene NPs into early endosomes of hepatocyte cell lines and IRHCs. Cells were treated with cell medium (**A**) or 125µg/ml polystyrene NPs (20nm) for 10 (**B**), 30 (**C**), or 60 (**D**) minutes, and then fixed, permeabilised and stained for EEA-1. Red represents EEA-1, the early endosome marker, and green the 20nm polystyrene NPs (magnification x63). Image **E** represents cells only (so that no antibodies were exposed to cells but images were taken using same confocal scanning parameters) The images are representative of 3 experiments. Scale bar = 10µm

4.3.3.2 Evaluating the localisation of 20nm fluorescent polystyrene NPs within lysosomes

LysoTracker is a fluorescent probe that labels acidic organelles within cells including lysosomes, and to a lesser extent late endosomes (Rodriguez-Enriquez *et al.*, 2006). LysoTracker was therefore used to determine if NPs were incorporated into degradative organelles. It was found that lysosomes were identifiable within all hepatocyte cell types (figure 4.8A), and that this fluorescence was not a consequence of cell autofluorescence (figure 4.8E). However the NP containing compartments that were evident within hepatocytes were distinct from the lysosomal structures, at all time points and within all cell types investigated (figure 4.8B-D).

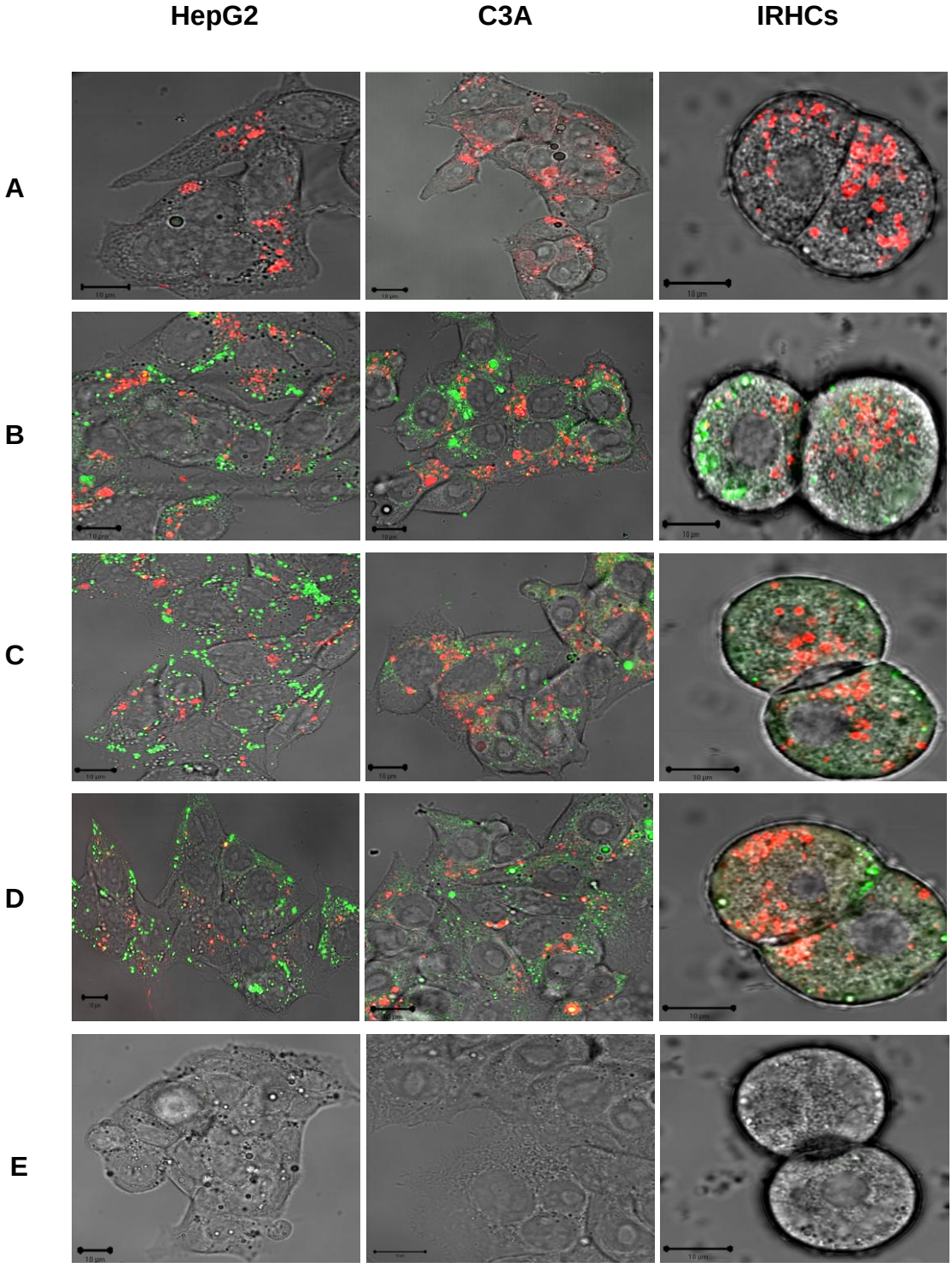


Figure 4.8 Investigating the incorporation of 20nm fluorescent polystyrene NPs into the lysosomes of hepatocyte cell lines and IRHCs. Cells were treated with cell medium (**A**) or 125µg/ml polystyrene NPs (20nm) for 10 (**B**), 30 (**C**), or 60 (**D**) minutes and then exposed to 50nM LysoTracker for 10 minutes. Red represents lysosomes stained with the probe LysoTracker, and green the 20nm polystyrene NPs (magnification x63). Image **E** represents cells only (so that no antibodies were exposed to cells but images were taken using same confocal scanning parameters) The images are representative of 3 experiments. Scale bar = 10µm

4.3.3.3 Determining the accumulation of 20nm fluorescent polystyrene NPs within mitochondria

The fluorescent probe MitoTracker allowed the visualisation of mitochondria within all cell types (figure 4.9A), and that this fluorescence was not a consequence of cell autofluorescence (figure 4.8E). After a 10, and 30 minute exposure, NPs were not located within the mitochondria of any of the cell types investigated (figures 4.9B-C). The confocal images indicated that the 20nm NPs were colocalised within mitochondria after a 60 minute exposure time in the hepatocyte cell lines, but this finding was not reproduced within the primary hepatocyte couplets (figure 4.9D).

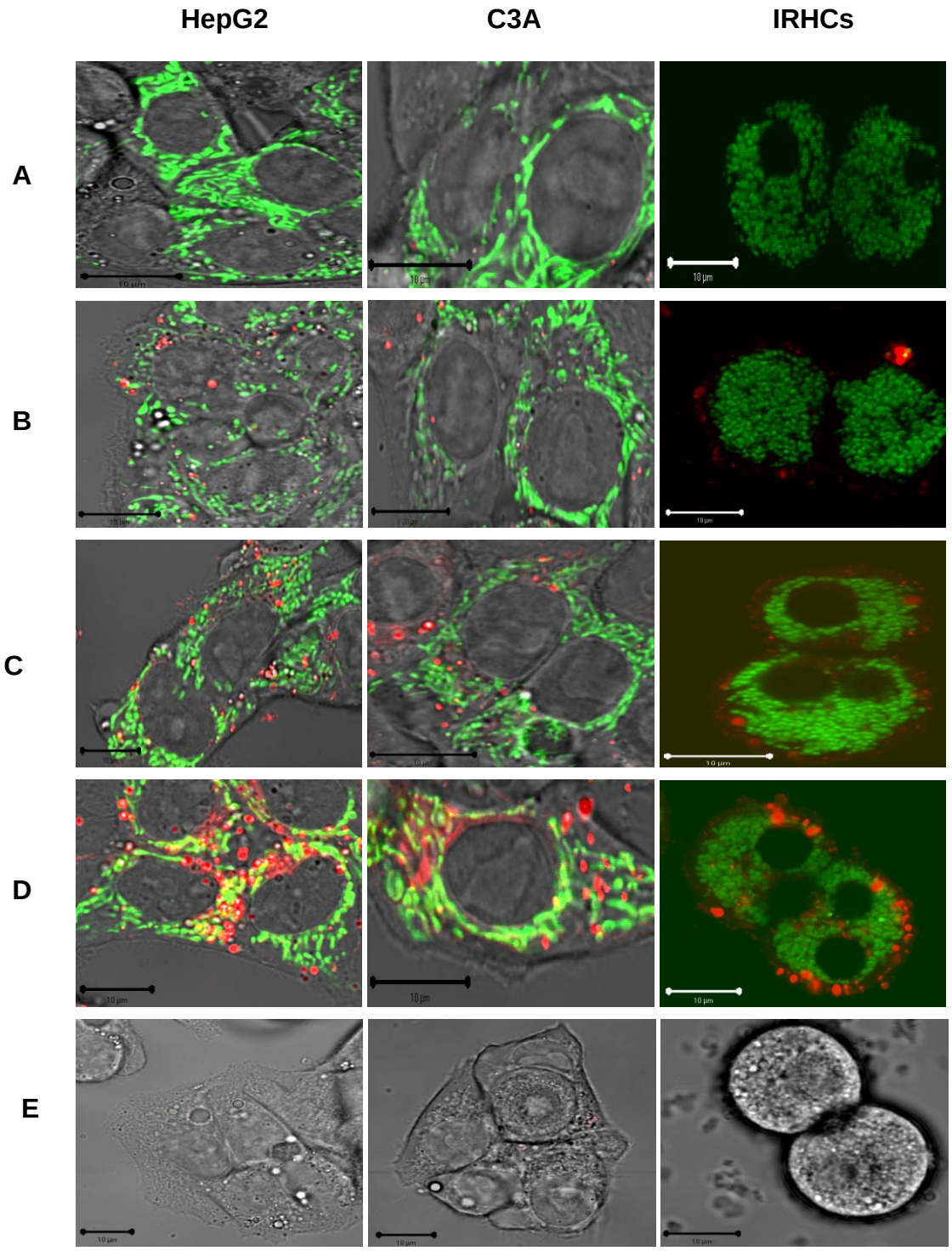


Figure 4.9 Investigating the accumulation of 20nm fluorescent polystyrene NPs into the mitochondria of hepatocyte cell lines and IRHCs. Cells were pre-treated with 500nM MitoTracker for 30 minutes and then exposed to cell medium (**A**) or 125µg/ml polystyrene NPs (20nm) for 10 (**B**), 30 (**C**), or 60 (**D**) minutes. Green represents mitochondria, and red the 20nm polystyrene NPs (magnification x63). Appearance of the yellow/orange colour arises as a consequence of the co-localisation of the red polystyrene NPs with the green mitochondria. Image **E** represents cells only (so that no antibodies were exposed to cells but images were taken using same confocal scanning parameters). The images are representative of 3 experiments. Scale bar = 10µm

4.3.3.4 Investigating the elimination of 20nm fluorescent polystyrene NPs within bile

The administration of CLF was utilised to identify bile canaliculi within all cell types (figure 4.10A). It was found that 20nm NPs were evident within cells, and that NPs were able to accumulate within bile canaliculi to a limited extent after a 30 minute exposure in all cell types (figure 4.10B). It was observed that bile canaliculi were most easily, and reproducibly identifiable within the hepatocyte couplets. To more clearly visualise the incorporation of NPs within the bile canaliculi the zoom function on the confocal microscope confirmed the limited elimination of particles within bile (figure 4.10Ci-iii).

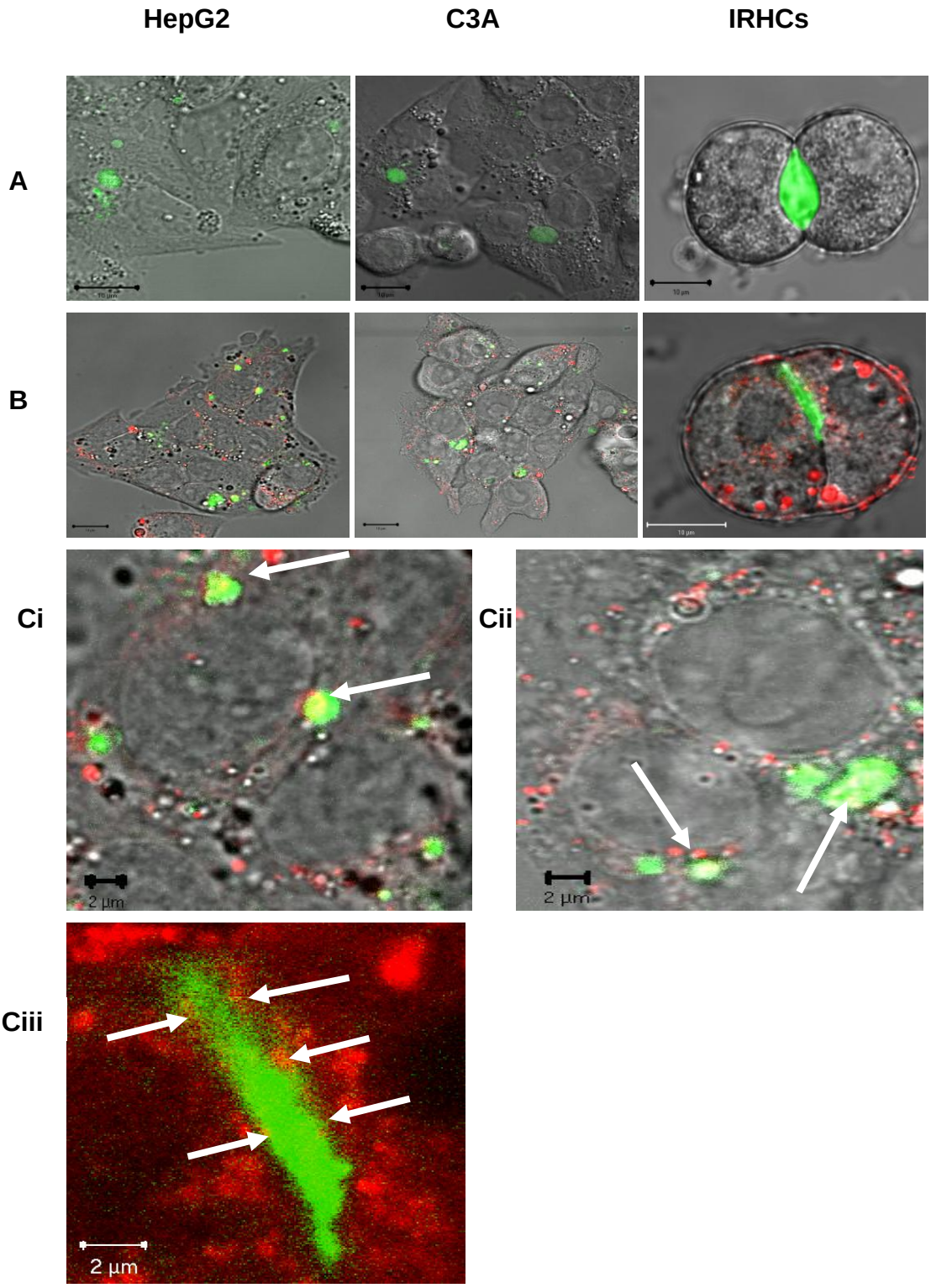


Figure 4.10 The accumulation of the fluorescent bile acid CLF and 20nm fluorescent polystyrene NPs within the canalicular structures of hepatocyte cell lines and IRHCs. Cells were treated with CLF (5µM) for 15 minutes to reveal canalicular structures (**A**). The accumulation of polystyrene NPs (20nm, 62µg/ml, 30 minutes) within bile canaliculi was investigated (**B**). As it was difficult to reveal areas of CLF and NP co-localisation within the canaliculi of HepG2 (Ci), C3A (Cii) and IRHCs (Ciii), a zoom function was used to more clearly visualise bile accumulating structures, so that areas of NP accumulation can be identified by the white arrows. Red represents the 20nm polystyrene NPs, and green the CLF (magnification x63). Appearance of the yellow/orange colour arises as a consequence of the co-localisation of the green CLF with the red polystyrene NPs. The images are representative of 3 experiments. Scale bar = 10µm, unless otherwise indicated.

4.4 DISCUSSION

It is necessary to highlight that the fluorescent polystyrene particles used as experimental tools to establish and develop protocols for testing the toxicity of the PARTICLE_RISK panel to hepatocytes. The data that was produced provided an insight into the potential for NP uptake and their intracellular fate within hepatocytes, so that interesting preliminary results were achieved that provided the opportunity for discussion regarding the future development of this work. The proposed future studies could not be completed within the budget or time-frame of this particular project.

4.4.1 Justification for the use of fluorescent polystyrene particles

Polystyrene particles are a useful research tool, available in fluorescent forms in a variety of sizes, and allow the relationship between particle size and uptake by hepatocytes to be evaluated without the need to consider additional variables, such as composition. Fluorescent, negatively charged, carboxylated polystyrene particles (20nm and 200nm diameter) were used as model particles to evaluate the uptake of NPs by hepatocytes and were chosen as a consequence of their ease of detection, their purity (lack of contamination), narrow size distribution, and their size relevance to the field of nanotechnology. To represent NPs, 20nm diameter particles were utilised, due to the size dimensions to which particles are constrained within the field of nanotechnology. The 200nm diameter particles, of identical composition, were used as they are out-with the NP size range but are still physiologically relevant, in terms of human exposure, due to the fact that particles of this size are present within a number of currently available products, including for example sunscreens and medicines. It is worth noting that the role of charge was not investigated within this study, but needs to be considered in the future.

4.4.2.1 Size and time dependent uptake of fluorescent polystyrene particles by hepatocytes

Imaging by confocal microscopy suggests that particle uptake is time and size dependent, within all hepatocyte cell types tested. Specifically, 20nm NPs appeared to be internalised by hepatocytes to a greater extent, and at earlier time points when compared to the uptake of 200nm particles. The uptake of 200nm polystyrene particles was observed to be limited, with the majority of

cell-associated particles apparent at the cell surface, perhaps awaiting entry into the cells. It is proposed that the presence of 200nm particles at the cell surface could arise as a consequence of the initiation of particle internalisation via CME. This is suggested due to the knowledge that receptors involved in CME are concentrated in specific regions of PM (see section 4.1.4.1), and when substances bind, the ligand-receptor complexes evident on the cell surface, termed patches, are thought to gather together and coalesce (into a tight cap), which is a process known as capping and is thought to initiate the assembly of a clathrin coat to enable the internalisation of a large region of the PM (Salisbury, Condeelis and Satir, 1980, Dammer and O'Halloran, 2000). Therefore perhaps 200nm particles are bound to receptors on the PM, which have been incorporated into caps and are awaiting internalisation into the cells via CME. The internalisation of 200nm particles via capping could thereby explain why the uptake of these particles is more limited than that of their smaller counterparts, as capping is a relatively long process, and further work would be required to test this hypothesis further.

To confirm whether particles were present within cells, z stacks were performed. This was based on the knowledge that the images produced by the confocal microscope are taken from one 'slice' of the cell and therefore information regarding the cell as a whole was missing. However, z stacks allow for the optical sectioning of cells so that images are captured as a series, at specific focus depth intervals, to visualise if NPs are evident throughout the whole cell, and to confirm the uptake of particles into the cell interior (and to prove that they were not merely apparent on the cell surface). In addition, their formation allowed the generation of projections and xz and yz micrographs that allowed a clearer indication of particle uptake to be obtained. The size dependent uptake of particles by HepG2 cells (and other cell lines) has also been observed by Zauner, Farrow and Haines, (2001) who demonstrated that large polystyrene particle (93nm or greater in diameter) uptake was hardly detectable in comparison to that of 20nm NPs, which were 'avidly taken up' by HepG2 cells. Furthermore, Rothen-Rautishauser *et al.,* (2006) observed that <0.1µm fluorescent microspheres (non- charged, positively charged and negatively charged) were able to enter non-phagocytic RBCs, but fine particles (0.2-1µm) were found at the cell surface, and were not internalised. Therefore,

the finding that larger polystyrene particles (out-with the nano size range) were evident at the surface of hepatocytes complements the findings of Rothen-Rautishauser *et al.,* (2006), so that this may be a common property exhibited by larger particles in non-phagocytic cells. All of these studies have used imaging alone to generate this information and the conclusions, so that none of the studies were quantitative. The size dependence of particle uptake by hepatocytes has also been observed *in vivo* where hepatocytes internalised 50nm polystyrene NPs to a much greater extent than 500nm particles of identical composition (Ogawara *et al.,* 1999a).

To confirm the findings observed within the fixed cell imaging, the uptake of particles by live cells was considered, due to the fact that the fixing of cells can cause alterations in cell morphology. Again, it would appear that the uptake of 200nm particles was more limited than that of the smaller particles. The results thereby verified the size and time dependent uptake of particles by hepatocytes. The findings highlight that by considering the uptake of particles in fixed and live hepatocytes, a more realistic portrayal of particle uptake by hepatocytes is gained. The concentrations of particles used to identify the uptake of particles via fixed and live cell imaging varied; a lower particle concentration of particles (15µg/ml) was used within the live imaging studies, the pattern of response was equivalent, and was necessary due to the fact that the visualisation of cells was compromised by a higher particle concentration (125µg/ml) that was equivalent to that used within the fixed cell imaging of particle uptake.

Therefore, overall, the data suggests that a time and size dependent uptake of polystyrene particles by hepatocytes occurred, which is supported by the generation of single plane images, z stacks, projections, xz and yz micrographs and live imaging analysis. However, information regarding the quantification of the process is lacking, which derives from the fact that the design of the study followed a qualitative approach, and as such would seem inappropriate to carry out such quantification of the uptake process retrospectively. However this could be a focus of further investigations in future studies, using, for example, flow cytometry or fluorimetry.

4.4.2.2 Importance of serum to particle uptake

It was observed that the inclusion of serum within the particle dispersing solution seemed to be instrumental to the uptake of 20nm polystyrene particles by live hepatocytes. The enhanced uptake of 20nm NPs, when contained in serum containing medium, could be explained by the improved dispersion of polystyrene NPs within the cell culture medium, which allows them to be more efficiency taken up by cells, due to the smaller size of the particle agglomerates within the cell culture medium. Therefore the live imaging results were able to further suggest that particle size was fundamental to particle uptake, perhaps due to particle size influencing the mode of particle-cell interaction and/or a smaller particle surface area for serum protein adsorption. Alternatively, serum presence may encourage NP internalisation by hepatocytes due to serum protein absorption onto the NP surface to enable uptake via an active receptor mediated mechanism. The adsorption of serum proteins onto the surface of NPs has been previously shown to be integral to NP uptake, to enable cell uptake via receptor mediated endocytosis by hepatocytes *in vivo*, and *in vitro* (Furumoto *et al.*, 2001, Chithrani, Ghazani and Chan, 2006).

It is of note that the serum used was heat inactivated, suggesting that uptake of particles was not mediated via complement activation, as complement proteins would have be denatured by the heat inactivation. This is important as carbon NPs have been demonstrated to stimulate chemoattractant generation within serum (Barlow *et al.*, 2005). These chemoattractants were thought to be complement components which were able to induce macrophage chemotaxis, which suggested that complement generation was important to NP toxicity.

The results discussed highlight the importance of considering the medium in which NPs are dispersed, and how this may impact on their subsequent toxicity. Specifically, if NPs exhibit toxicity *in vitro* that requires their internalisation by hepatocytes, both uptake and therefore toxicity will be facilitated by the presence of serum within the NP suspension. The serum used within the experiments contains a variety of components, and the individual constituents responsible for the increased dispersal of NPs within the experiments are unknown. However, it is physiologically relevant to consider the use of these dispersants due to the fact that their presence is reflective of the situation that

arises when NPs gain access to blood and hence the liver, *in vivo*. Foucaud *et al.,* (2007) used a number of physiologically relevant agents (including DPPC, a component of lung surfactant, and BSA) to disperse ufCB particles. It was found that the addition of BSA and/or DPPC reduced the extent of particle agglomeration so that ufCB was better dispersed than particles suspended in saline. It was also found that the ability of ufCB to induce ROS production (measured using DCFH) within monocytic MM6 cells was dependent upon the composition of the dispersing solution. When ufCB was suspended in saline, no increase in ROS production was observed, however when ufCB was suspended in BSA and/or DPPC intracellular ROS production by MM6 cells increased. Furthermore Sager *et al.,* (2007) demonstrated that BAL fluid was the most effective vehicle in which to suspend NPs, as it was able to substantially decrease the size and number of particle aggregates, when compared to particles suspended in PBS. Furthermore, it was evident that protein and DPPC mixtures (at concentrations present within BAL fluid) were able to reduce the aggregation of NPs, so that the decreased aggregation of NPs may enhance their toxic potency. Therefore these findings highlight that the composition of dispersing solutions are able to impact on NP toxicity, and so it is important that their composition is relevant to the expected exposure scenario. The attractions between individual NPs that promote the formation of larger structures is therefore an obstacle when conducting *in vitro* and *in vivo* toxicity, and ensuring NPs are adequately dispersed will lead to a more accurate assessment of NP toxicity, as NP agglomerates and aggregates are likely to be less toxic than the individual NPs that make them up (Sager *et al.*, 2007). In addition, reducing the tendency of NPs to agglomerate or aggregate is important to maintaining consistent exposure concentrations *in vitro* and *in vivo* and to maintaining the attributes of particles that are associated with their small size. However, as discussed previously, NP aggregates have been demonstrated to be more toxic than aggregates of their larger counterparts (Ferin *et al.*, 1992).

4.4.3 The fate of internalised particles

Therefore as the internalisation of polystyrene NP by hepatocytes was suggested by the imaging analysis, their subsequent fate within the cells was considered, and as the internalisation of 200nm polystyrene particles was

appeared to be limited, their fate was not considered further.. It seemed that a prominent feature associated with the internalisation of NPs at early time points (10-30 minutes) was their co-localisation with the tubulin cytoskeleton. Due to the known functions of the tubulin cytoskeleton, it is hypothesised that it is central to the transport and organisation of internalised NPs within cells, and that this is essential in promoting NP elimination from the cells, by facilitating their transport to the most appropriate cellular location, such as lysosomes or bile canaliculi. At the later time points NP associated fluorescence appears as punctate spots located either within or between cells. This compartmentalisation of NPs, evident within all cell types, suggested that 20nm polystyrene NPs were either arranged into structures that had enabled NP uptake or encouraged the removal of internalised NPs from the cell. It is noteworthy that internalised NPs were contained predominantly within the cytosolic compartment, and within suspected canalicular-like structures but were not observed within the nucleus. This is in contrast with the findings of Geiser *et al.,* (2005) who found that TiO_2 particles were located within the nucleus of lung cells (including fibroblasts), subsequent to inhalation exposure.

Revealing the sub-cellular fate of internalised NPs is of interest due to the ability of their localisation to impact on mechanisms, or extent of NP toxicity. However, there are certain circumstances, for example within the field of molecular biology where fluorescent NPs are targeted to specific sub-cellular organelles to enable their detection. Hoshino *et al.,* (2004b) conjugated different peptides to the surface of QDs to enable their targeting to specific cellular compartments (nucleus, and mitochondria) within live Vero kidney epithelial cells. It was found that surface modifications were able to designate the intracellular site that QDs home to, thereby adding an element of specificity to their fate after internalisation.

4.4.3.1 Determining the incorporation of NPs into early endosomes

Identification of the early endosome surface marker EEA-1 allowed the location of early endosomes within the cells to be visualised. The accumulation of NPs within early endosomes was conducted to determine if an endocytic mechanism of uptake was involved in NP internalisation by hepatocytes. The findings suggested that polystyrene NPs (20nm) were not co-localised with EEA-1, at

any of the time points, in all cell types, suggesting that they were probably not contained within early endosomes. This is in agreement with the finding that NPs (polystyrene beads, 78nm) have been demonstrated to enter macrophages and RBCs by a non-endocytic process that may be mediated via diffusion or another unknown mechanism (Geiser *et al.*, 2005). Furthermore Rothen-Rutishauser, (2006) also found that gold NPs (25nm) were internalised by RBCs but were not membrane bound, suggesting that an endocytic mechanism did not contribute to their uptake. However, on the contrary, it has been found that endocytic mediated entry is accountable for gold NP entry into HeLa cells (Chithrani, Ghazani and Chan, 2006) and TiO_2 particles (mainly in an aggregated form) by A459 epithelial cells (Stearns, Paulauskis and Godleski, 2001). These results highlight the need to test a number of different NP and cell types within toxicity tests as it may be unrealistic to make generalisations about the uptake characteristics of different NPs in different cell types.

The apparent lack of incorporation of internalised particles into early endosomes is therefore suggestive that NPs are not taken up by hepatocytes by an endocytic mechanism. However uptake of NPs by endocytosis cannot be ruled out solely on a lack of evidence of NP localisation within early endosomes. This is due to knowledge that some mechanisms of endocytic uptake do not rely on the involvement of early endosomes; for example caveolin mediated endocytosis does not utilise early endosomes, and instead internalised cargo may be delivered to other cell compartments such as the ER (see section 4.1.4.4) which warrants investigation. The imaging of NPs within early endosomes is also limited by the imaging technique used, whereby evidence of the integration of NPs into endosomes relied on the co-localisation of particles within the image, which was indicated by colour (see section 4.4.4). Therefore to irrefutably exclude the involvement of endocytosis within the uptake of NPs, a number of other techniques could be considered; for example assessing the uptake of particles at $4^{o}C$, determining if NPs are contained within membrane bound vesicles using TEM, or by utilising pharmaceutical inhibitors of uptake (see section 4.1.4.6). If NP uptake is not mediated by endocytosis, other uptake mechanisms will have to be considered, for example there is some evidence that hepatocytes exhibit a phagocytic ability under some

circumstances (Soji *et al.*, 1992), which could contribute to NP internalisation, or other uptake processes such as diffusion may participate.

4.4.3.2 Determining the potential for polystyrene NP accumulation within lysosomes

The incorporation of polystyrene NPs into lysosomes was considered as these degradative organelles could represent a potential route of NP elimination from hepatocytes. The findings suggested that it was likely that NPs did not accumulate within the lysosomes in all cell types, however this is paradoxical to the behaviour of other NPs. Goya *et al.,* (2008) demonstrated that iron oxide NPs were internalised by primary human dendritic cells, and primarily localised within lysosomes. The consequences of NP accumulation within lysosomes requires addressing, including the ability of lysosomes to degrade NPs, which will inevitably be affected by NP composition. It is expected that lysosomes could be capable of degrading a polymer based NPs, such as the polystyrene NPs utilised within the experiments, on internalisation, however it would be unlikely to occur within the short exposure times investigated, so that their lack of accumulation within lysosomes is unlikely to be explained by their degradation. These findings therefore demonstrate that the ability of NPs to accumulate within lysosomes may be dependent on the NP and cell type investigated, which are capable of impacting on the fate of NPs within cells. In addition, the processing of NPs within cells will inevitably be affected by the function of the target cell in question, for example dendritic cells are immune cells that are responsible for the recognition of foreign material and equipped with the necessary attributes that facilitate the degradation of substances (including hydrolytic enzymes and ROS), whereas hepatocytes have different functional capabilities that will inevitably affect the way they interact with, internalise and process particles. Alternatively, the imaging technique provided limitations in providing information regarding the fate of internalised NPs, that are considered in more detail later (section 4.4.4).

There are circumstances where it would be preferential for NPs to escape lysosomal accumulation, such as the delivery of NP based therapeutics to the cell interior. The importance of NPs entering a non-degradative pathway was exemplified by the findings of Lai *et al.,* (2008). It was observed that 24nm

polymer NPs were able to enter the human epithelial HeLa cells via clathrin and caveolin independent endocytosis which enabled them to avoid their transfer to lysosomes, which would ordinarily terminate any beneficial attributes that were intended by their administration. It was observed that this was a size dependent phenomenon, as 43nm particles were internalised by endocytic processes that encouraged their accumulation within acidic organelles. Rejman *et al.,* (2004) demonstrated that the size of latex NPs dictated the mechanism of endocytic uptake that was responsible for their internalisation by the non-phagocytic B16 cells, which impacted on the subsequent fate of NPs. Specifically, It was found that particles smaller than 200nm were internalised by CME and found within lysosomes, and 500nm particles taken up by caveolin mediated endocytosis and absent from lysosomes. These findings therefore suggest that the size of particles, method of internalisation and fate of particles are inherently linked; thus illustrating the complexity of the processes that contribute to the uptake and intracellular fate of particles. The findings also highlight the importance of considering the design of NPs to ensure that NPs are able exert their action within the cell interior. Paradoxically, if NPs are able to bypass the degradative pathway this will prolong the time that NPs are able to interact with sub-cellular components, to potentially increase their likelihood of exerting toxicity.

4.4.3.3 Evaluating the localisation of polystyrene NPs within mitochondria

It has previously been demonstrated that ultrafine particles are able to enter mitochondria *in vitro*, within RAW 264.7 (macrophage) and BEAS-2B (bronchial epithelial) cells using TEM analysis (Li *et al.*, 2003). This finding correlated with the observation that polystyrene NPs were suggested able to accumulate within the hepatocyte cell lines. This is of relevance as the localisation of NPs in mitochondria has been demonstrated to be integral to their mechanism of toxicity as Li *et al.* (2003) found that NPs (contained within particulate air pollution) were able to accumulate within the mitochondria of macrophage and epithelial cells, to elicit major structural damage that was thought to be responsible for oxidative stress generation. It was not known if NPs were able to accumulate within mitochondria directly due to their small size, or whether accumulation within this organelle was an indirect consequence of NP elicited toxicity, and therefore resulted due to particle mediated oxidative stress which

was able to induce mitochondrial damage that was able to permit the entry of NPs. The fixed imaging analysis indicated that 20nm polystyrene NPs were potentially contained within a proportion of the mitochondria in the cell lines, but this finding was not reproduced within the primary hepatocyte couplets, highlighting that the cell lines may not always be predictive for the effects of NPs in primary cells. However, at the time points observed in the cell lines, not all internalised NPs were found within the mitochondria so that they may be organised into as yet unknown structures, so that the NPs have more than one intracellular fate. The use of TEM analysis could provide a better indication of the incorporation of NPs within mitochondria or other sub-cellular structures. Therefore, it is necessary to evaluate the reliability of the finding, by perhaps using fluorescent probes that do not bleach so easily, or other methods of cell imaging (such as TEM) to confirm this finding, accordingly if it transpires that NPs are contained within mitochondria, then it would be of interest to determine if their accumulation impacts on their function, and how they gain access within the mitochondria (including the possibility that they are transported through pores within the mitochondrial membrane).

It is noteworthy that the appearance of mitochondria within primary cells and the cell lines was different. However, TEM confirmed that the distribution and morphology of mitochondria apparent within primary cells was comparable to that achieved when the MitoTracker probe was used (see figure 5.12). In addition, the imaging of mitochondria within hepatocyte cell lines was comparable to that apparent within the literature (Tirmenstein *et al.*, 2002). Therefore differences in mitochondria appearance within the different cell types may derive from species differences.

4.4.3.4 Determining the elimination of polystyrene NPs within bile

Fixed cell imaging of polystyrene 20nm NP uptake suggested that within the hepatocyte cell lines there were distinct areas located between adjacent cells, where NPs appeared to accumulate, and were hypothesised to represent bile canaliculi. It was observed that there were regions located between adjacent cells where the fluorescent bile acid CLF accumulated, which were therefore assumed to represent canalicular-like structures However it was evident that the polystyrene NPs and CLF were not co-localised to a great extent within

these structures of the cell lines, and that the appearance of canalicular structures within cell lines was not evident with high reproducibility. Therefore isolated rat hepatocyte couplets were used to further investigate the accumulation of NPs within bile canaliuli due to the fact that they have more defined canalicular structures than cell lines. Within hepatocyte couplets it was apparent that there was limited accumulation of 20nm polystyrene NPs within the canaliculus, so that their elimination from the liver within bile is expected to occur to a relatively low extent, although this is difficult to conclude as only one relatively short time point was investigated.

It is also necessary to consider the mechanism responsible for the elimination of NPs within bile. Generally, substances cannot be directly secreted into the canaliculus (and therefore be excreted in bile) without first entering hepatocytes, due to the presence of tight junctions (Meier and Stieger, 2000). However, there is evidence of the paracellular transport of substances (between adjacent hepatocytes, directly into the canaliculus) when the permeability of tight junctions is compromised by toxicant exposure (Lowe *et al.*, 1985). Horse radish peroxidase (HRP) is a large protein and its elimination within bile has two components; initially HRP accumulation within bile canaliculi occurs via the paracellular route, (Roma *et al.,* 1997, Lora *et al.*, 1997) or HRP can undergo transcellular transport via endocytic vesicles into bile canaliculi, which occurs more slowly and is reliant on an intact cytoskeleton (Lora *et al.*, 1997, Kawahara *et al.*, 1989). The increased access of HRP to the bile canaliculus can be indicative of damage to the tight junctions which, for example can be induced by oxidative stress (Ahmed-Choudhury *et al.*, 1998). As NPs are equivalent to HRP in terms of size, and may exert toxicity via oxidative stress (as observed with ultrafine particles), NPs may access the bile canaliculus through the paracellular route to be eliminated within bile.

From the results it was observed that NPs were eliminated within bile canaliculi, to a limited extent. However this may be a consequence of the high level of fluorescence associated with CLF detection by the confocal microscope. Therefore analysis by TEM may provide a better indication of the elimination of particles within bile. Evaluating the accumulation of 20nm polystyrene NPs within bile canaliculi is of relevance as it may provide evidence of a potential

route of NP elimination from the body within bile (and then ultimately faeces), and thereby account for the accumulation of NPs within the liver observed *in vivo*. In fact, there is already evidence of polystyrene NP elimination from the body within bile in rats (Furumoto *et al.*, 2001). In addition Kreyling *et al.,* (2002b) demonstrated that iridium NPs were found within the faeces after pulmonary exposure; this could arise as a consequence of their clearance via the mucociliary escalator, however there was also evidence of accumulation of the NPs within the liver so that the NPs could have been secreted into bile, to allow their elimination from the body within faeces. This has been demonstrated by Kreyling *et al.,* (unpublished observations) within the PARTICLE_RISK project, who observed a size dependent elimination of radiolabelled gold particles (ranging from 1.4nm to 200nm) within bile. It was found that smaller particles were eliminated within bile 24 hours after i.v. administration, to a greater extent than their larger counterparts and were found within the faeces.

4.4.4 Limitations of confocal microscopy and improvement of the experimental set up

Making assessments about the fate of internalised particles was made possible by the use of fluorescent probes and NPs that had distinct excitation and emission properties, which allowed their identification using confocal microscopy. Therefore NPs and sub-cellular structures were routinely identified by confocal microscopy and were apparent on the generated images by giving them a green or red colour. Consequently, when NPs and the sub-cellular structure of interest were located within the same region of the cell, a yellow colour became apparent; and was termed co-localisation. This co-localsation indicates that the NP was apparent within the same sub-cellular location, and therefore potentially the same organelle. However, the limit of resolution of the confocal microscope is greater than 20nm allowing only aggregates to be observed, and for localisation to be an estimate rather than definitive. To further investigate the co-localisation of NPs with sub-cellular structures, z stacks were completed, where possible. However this was not always possible due to the bleaching of some of the fluorescent probes used including CLF, LysoTracker and MitoTracker. Therefore TEM analysis would perhaps provide a more realistic approach when considering the intracellular fate of particles

within hepatocytes, providing that the NPs can be adequately distinguished from other structures within the cell.

In addition, it would have been of interest to conduct quantitative studies in parallel to the qualitative experiments that were carried out, to confirm the findings and conclusions made, specifically the time and size dependency of particle uptake by hepatocytes. In addition, it is of great interest and relevance to confirm the observation that NPs were contained within mitochondria by further imaging analysis using different fluorescent probes (that would not bleach and thereby allow 3D projections to be completed), and by determining if mitochondria function was affected by NP localisation. However, time was the major limitation to the completion of these investigations, which in addition to the knowledge that this aspect of the thesis was not the major focus of the work conducted within the PARTICLE_RISK project, meant that the quantification of the uptake processes was not attempted, but could have involved the use of flow cytometry or fluorimetry. In addition, confirmation of the localisation of NPs within organelles could be achieved using density gradient centrifugation, despite the fact that differential centrifugation was attempted to evaluate the *in vivo* distribution of the fluorescent polystyrene particles. It is therefore accepted that there were limitations to the studies described, and that these could be addressed in future studies. Accordingly, it is necessary to emphasise that investigating particle uptake by hepatocytes was intended to be a qualitative study that utilised fluorescent polystyrene particles (as research tools) and quantification of the process was not an anticipated endpoint,. Therefore although it is of interest to assess the reliability of some of the findings, (which ultimately derived from limitations within the experimental set up and analysis). this was beyond the scope of the study, and not possible retrospectively.

4.4.5 Importance of developing *in vitro* models when assessing NP toxicity

HepG2 and C3A cells are hepatocyte carcinoma derived cell lines. IRHCs are isolated from the rat liver and retain the structural and functional polarity that hepatocytes exhibit *in vivo* and therefore are thought to be a more realistic and relevant model of hepatocyte behaviour. If cell lines exhibit similar behaviour to primary cells, animal use can potentially be minimised. This is of particular

importance when considering the large number of different NP types (in terms of composition, size etc) that will require assessment of toxicity. Therefore due to the large number of different types of NPs under use and development, combined with the range of potential targets, it is necessary to develop relevant *in vitro* models for investigating their toxicity. Cell lines are often used to investigate the effects of potential toxicants to cells that are specific to the target organ of interest, and are often assumed to be representative of the response of cells exposed *in vivo*. In general, the response of both cell line types was comparable to that of primary cells. However, one aspect where the behaviour of the different cell types was variable was when the accumulation of NPs within mitochondria was assessed. Other problems were also encountered when using primary cells, for example when conducting staining of hepatocyte couplets they had to be plated at a higher concentration, and poly-D-lysine had to be used to increase their likelihood of cells attaching to coverslips.

4.4.6 Revealing the sub-cellular fate of fluorescent NPs using differential centrifugation

Although not previously described, differential centrifugation was also used in this study in order to determine whether fluorescent polystyrene NPs accumulated within the liver *in vivo*, and their sub-cellular fate, subsequent to intratracheal (i.t.) instillation and intravenous (i.v.) injection (see appendix section A1). Following tissue homogenisation, differential centrifugation was carried out to separate the rat liver into distinct sub-cellular fractions (nuclear, mitochondrial, lysosomal, microsomal and cytosolic), using sequentially higher speeds of centrifugation. Firstly, a control rat liver was fractionated into the five distinct sub-cellular compartments and the purity of the fractions was then determined by assessing the activity of enzyme markers, specific to particular organelles, within the different liver fractions. To determine if fluorescent polystyrene NPs could be detected within the tissue homogenate and different liver fractions, an increasing particle concentration (ranging from 0-320µg/ml) was added to each liver fraction, and the associated fluorescence detected using a fluorimeter. As a result it was hoped that particle concentrations within treated samples could be quantified if the fluorescent intensity of the liver fractions was known (see appendix section A1). It was apparent that fluorescent polystyrene particles (20nm diameter), administered by i.t.

instillation (62 or 125ug, 18 hour exposure) did not accumulate within the liver, at a detectable level and at the time point investigated. This conclusion was based on the fact that no increases in fluorescence of the tissue homogenate or liver fractions were associated with particle exposure (see appendix section A1). Consequently the lack of particle translocation to the liver after i.t. exposure is thought to derive from the knowledge that when inhaled, the delivery of particles to the liver is restricted due to the presence of clearance mechanisms within the lung, so that barriers act to restrict the passage of particles into the systemic circulation and thereby prevent their transport to other targets, such as the liver. Therefore, 20nm fluorescent polystyrene particles were administered i.v. via a tail vein injection (1mg, 2 or 4 hour exposure) to determine if this resulted in the accumulation of NPs within the liver. However, it was again found that the particles did not accumulate within the liver at a detectable level (see appendix section A1). Therefore, despite the fact that fluorescent polystyrene NPs could be detected within each liver fraction, using a fluorimeter, when added directly fractions obtained from control animals, particle presence could not be detected after i.t. or i.v. administration. Alternatively, it is possible that the detection of particles may be limited by the sensitivity of the fluorimeter; so that the small concentrations of particles that are likely to accumulate within the liver cannot be detected via the method used. Therefore radiolabelled NPs could be used in future experiments, which could be more easily identified and more precisely quantified. However, the work conducted, highlighted that an attempt was made to quantify the uptake of NPs by liver cells, despite being unsuccessful.

4.4.7 Conclusion

In summary, it is important to assess whether cell lines mimic the response of primary cells and *in vivo* models, in order to ensure that a relatively realistic prediction of NP behaviour can be obtained. This study was conducted in order to assess the possible usefulness or limitations of the HepG2 and C3A hepatocyte cell lines (Boess *et al.*, 2003), by comparing their response to that of primary rat hepatocytes. Further studies are required to address their comparability with *in vivo* models. Particle uptake by hepatocytes (primary and cell lines) was observed to be size, time and serum dependent. The uptake and intracellular fate of polystyrene NPs in HepG2 and C3A cell lines and

IRHCs was found to be in the main, comparable; with the imaging analysis suggestive of little or no evidence of accumulation of the 20nm polystyrene NP in early endosome or lysosome structures, which indicates that an endocytic mechanism of uptake was not paramount to their internalisation, and that they are not degraded by lysosomes. There was some evidence of NP accumulation within the mitochondria of cell lines but not in the primary hepatocytes, and this finding warrants further investigation, for confirmation. There was some suggestion of excretion into bile canaliculi, but this was limited which suggests that these NPs may not be extensively eliminated within bile. Polystyrene NPs were evident within the mitochondria of the cells lines but not within the primary hepatocyte mitochondria, however, not all NP compartments within the cells were contained within mitochondria so that they could have another, as yet unknown fate within the cells which could concur to the fate of internalised NPs within the primary hepatocyte couplets.

The potential for NPs to accumulate within the liver has, as yet, unknown consequences. Nano-sized particles have been observed to elicit an inflammatory response in the rat lung (see for example Ferin *et al.*, 1992) and to stimulate pro-inflammatory marker production *in vitro* (Brown *et al.*, 2004). These effects are dependent on oxidative stress (Brown *et al.*, 2004), and such a response requires investigation in hepatocytes. In addition, hepatocytes have been used within this study, however Kupffer cells, the resident liver macrophages, are likely to facilitate the removal of NPs from the circulation within the liver and their contribution to NP uptake requires investigation.

Finally, the results suggest that hepatocyte cell lines are a good, but not perfect, model to assess particle uptake patterns of primary cells. Further investigations are necessary to determine whether these observations are true for other particle types, and to investigate whether the impact of different NPs on hepatocyte function and viability is comparable between the cell lines and primary cells. Due to the fact that the response of the two cell lines was comparable, C3A cells will only be used to represent the cell lines when assessing the impact of NP exposure on hepatocyte function.

Chapter Five

Determining the impact of NP exposure on hepatocyte function

5.1 The importance of determining the functional consequences of hepatocyte exposure to NPs

The increased manufacture, and continued discovery of the novel, exploitable properties exhibited by NPs makes it necessary to consider the health implications associated with engineered NP exposure, which is information that is currently lacking. This will be facilitated by unravelling the ability of NPs to interfere with processes at a cellular level, to determine the underlying mechanisms responsible for the contribution of NPs to the initiation, progression or exacerbation of disease. It is therefore important to consider the functional consequences of NP exposure, as this will provide insight into the ability of NPs to exert toxicity, and is important as partners within the PARTICLE_RISK project (Jacobsen *et al*., 2008) observed that NPs contained within the panel were capable of inducing hepatic necrosis, highlighting that the liver is a serious contender for NP toxicity, within the body.

Inflammation and oxidative stress have been proposed to be fundamental to the toxicity of ultrafine particles (Stone, 2000), so much so that it was assumed to be the common response subsequent to particle exposure, with extensive background information available to support this contention (for a review see Donaldson and Stone, 2003). Consequently, a vast array of evidence has revealed the mechanisms responsible for executing the toxicity of ultrafine particles, and provided the impetus for assessing the toxicity of engineered NPs at a cellular level. However the generalisations made, regarding the mechanisms responsible for driving ultrafine particle toxicity, were based on findings conducted within the lung or within lung cell models. This was relevant due to the knowledge that the lung was considered to be the primary target of ultrafine particulate toxicity, as the route of particulate air pollution exposure occurred predominantly via inhalation. Therefore the extrapolation of these findings to other tissues requires assessment, as it has become apparent that NPs are able to translocate from their site of exposure. In addition the studies conducted used a limited number of particles, specifically concentrating on those contained within PM_{10}, or surrogate particles that were used to represent PM_{10} composition. Therefore, although these particles were within the NP size range, it is evident that engineered NPs are more diverse in nature (in terms of chemical composition, shape etc) and so the generalisations that were made

about NP toxicity may be irrelevant or less clear. Consequently, previous studies have provided the basis for concern surrounding the increased exploitation of engineered NPs, and provided the foundation for research, by directing the most appropriate experimental approaches when evaluating NP toxicity, as little is currently known about the potential of engineered NPs to elicit adverse effects on exposure (Kipen and Laskin, 2005). It is therefore necessary to evaluate whether the diverse nature of engineered NPs available will confer to different mechanisms of toxicity becoming apparent, and to dissect out whether engineered NPs are able to induce toxicity via these mechanisms within the liver or whether NP toxicity has to be evaluated on a site, and NP by NP basis.

Evidence supporting or confounding the ability of NPs to exert toxicity via oxidative stress and inflammation will be considered, and will concentrate on studies conducted using the particle types contained within the PARTICLE_RISK panel, but will also be extended to include other NP types to determine what attributes of NPs are most influential in dictating their toxicity.

5.1.2. Inflammation: a potential pathway for NP toxicity?

5.1.2.1 What is inflammation, and when does it cause damage?

Under normal circumstances an inflammatory response is beneficial and is initiated as a protective measure to enable the removal of harmful, or foreign material, and to promote the repair of damaged tissue (Driscoll *et al.*, 1997). However if inflammatory responses are persistent, or activated inappropriately tissue damage can occur and for this reason NP mediated inflammation has been implicated in the toxicity of NPs.

Attention has focused on the pro-inflammatory effects induced by NPs, due to the characteristic neutrophil driven inflammatory response observed *in vivo* (see for example Shvedova *et al.*, 2005,) and associated increase in pro-inflammatory cytokine production by 'target' cells *in vitro* and *in vivo* (see for example Brown *et al.*, 2000). Therefore an important component of any inflammatory response is the activation and accumulation of inflammatory cells, which is orchestrated by cytokines. Cytokines are signalling molecules released from inflammatory or tissue cells that are responsible for

communicating and directing an inflammatory response, by altering the behaviour of the cell from which it was released (autocrine), the activity of neighbouring cells (paracrine) or cells located at a distance to the cell where they originate (endocrine) (Andus, Bauer and Gerok 1991). The cytokines that are produced and released from cells can dictate the type of response that is implemented, specifically whether it is pro- or anti- inflammatory in nature. Pro-inflammatory cytokines include interleukin-1 (IL-1), tumour necrosis alpha (TNFα), and IL-8 that are responsible for encouraging the removal of NPs, by directly, or indirectly stimulating the recruitment of inflammatory cells from the circulation to the site of NP exposure. Anti-inflammatory cytokines include IL-4, and IL-10, and are able to suppress or terminate inflammatory responses, and to restore normal tissue function, in order to limit the injurious effects associated with sustained, inflammatory reactions (Tilg, Kaser and Moschen 2006). A cascade type response is associated with cytokine activity, due to the fact that cytokines produced by one cell type are able to influence the activity of a number of surrounding cells, and that the response therefore persists once the initial signal has ceased. Furthermore a single cytokine can be produced by a number of cell types, in response to different stimuli and are able to carry out multiple biological functions. Consequently, there is often redundancy, with regards to cytokine function, so that if one cytokine is present in insufficient quantities then another can take over its role (Andus, Bauer and Gerok 1991). Cytokines that are able to dictate what cell types are involved in responses are termed chemokines as they are responsible for the recruitment of inflammatory cells from the circulation via chemotaxis. This is reliant on the ability of cells to detect and respond to these signals, so that inflammatory cells can be organised appropriately and function effectively to overcome the inflammatory insult.

Ordinarily, acute inflammatory responses are triggered by the activation of resident inflammatory cell populations such as macrophages, which release inflammatory mediators such as cytokines and chemokines to promote the recruitment and stimulation of inflammatory cells such as neutrophils from the circulation (Lawrence and Gilroy 2007). When the inflammatory stimulus is no longer considered a threat to normal tissue function, stimuli that were responsible for promoting inflammatory cell recruitment are no longer produced

or are neutralised, and the recruited cells are removed (via the activation of apoptosis and disposal by recruited monocytes or resident macrophages), and anti-inflammatory mediators are produced (which enables a switch from neutrophil to monocyte recruitment) to result in the efficient resolution of the inflammatory response (Lawerence and Gilroy 2007). It is important that inflammatory responses are resolved as inflammatory cells are designed to combat inflammogenic agents, and are therefore equipped with hydrolytic and proteolytic enzymes and reactive oxygen species (ROS) that are released within the inflamed site, and can enhance injury if their activity is unchecked due to the non selective nature of these mediators (Lawerence and Gilroy, 2007). It is relevant that inflammatory responses stimulated by NPs may be persistent in nature, due to the fact that although macrophages are able to internalise particles, they have a limit to the number of particles that can be taken up (termed overload), which can increase NP interactions with other cell types to cause the excessive release of mediators to make the response long lasting (Ferin *et al.*, 1992, Oberdorster *et al.,* 1992).

5.1.2.2 Detecting inflammatory responses

As inflammation is regarded as being central to the toxicity of NPs, it is relevant to consider what characterises the response that is implemented. Exposure to NPs is typified by the increased production of pro-inflammatory cytokines such as IL-8. There are a number of techniques available to evaluate the pro-inflammatory response elicited by NPs; cytokine production can be measured using enzyme linked immunosorbant assays (ELISAs) or Western blots, or by detecting increases in cytokine mRNA expression *in vivo* and *in vitro*. The infiltration of inflammatory cells can be assessed *in vivo* through the collection of biological fluids such as blood or bronchoalveolar lavage (which contains cells that are collected from the lungs) to provide differential cell counts. Alternatively histological analysis of the tissue of interest enables a visualisation of the inflammatory cell infiltration or tissue damage to be accomplished.

5.1.2.3 NP mediated inflammation

As inflammation is known to be central to the toxicity of ultrafine particles within the lung, it is important to overview the evidence that supports this claim. Firstly the recruitment of neutrophils to the site of exposure has been illustrated to

typify the exposure of the lungs to NPs; including ufCB (Li *et al.*, 1999), TiO_2 (Ferin *et al.*, 1992), polystyrene NPs (Brown *et al.*, 2001) and SWCNTs (Shvedova *et al.*, 2007). It is thought that neutrophils are recruited to sites of NP exposure to promote the destruction of NPs, and thereby accomplish their role within host defence. However, paradoxically, neutrophils are also capable of inflicting tissue damage, due to the fact that neutrophils are equipped with the necessary tools (including proteases, and ROS) to destroy pathogens, but when released can cause tissue damage due to the non selective nature of these mediators (Jaeschke, 2006).

It is now necessary to outline what causes the characteristic inflammatory cell recruitment associated with NP exposure. Increased pro-inflammatory mediator and neutrophil chemoattractant production, is thought to drive the inflammatory response mediated by NPs, and has been a common finding within *in vitro* and *in vivo* studies. ufCB has been observed to induce an increase in TNFα production within the rat lung with a concomitant increase in neutrophil infiltration (Li *et al.,* 1999). IL-8 production was increased in A549 epithelial cells when exposed to polystyrene NPs (Brown *et al.* 2001) or TiO_2 NPs (Singh *et al.* 2007). Carbon, nickel and cobalt NPs increased macrophage inflammatory protein-2 (MIP-2) and TNFα production in the rat lung, which was associated with a neutrophil influx (Dick *et al.*, 2003). The recruitment of inflammatory cells to tissues also requires the increased expression of cell adhesion molecules on the surface of endothelial cells that line blood vessels which supply the target tissue of interest. Oesterling *et al.,* (2008) observed that aluminium oxide NPs (10-20nm) were able to increase the expression of cell adhesion molecules (including vascular cell adhesion molecule (VCAM-1) and inter-cellular adhesion molecule 1 (ICAM-1)) on primary vascular porcine endothelial cells and endothelial HUVEC cells, which caused the increased adhesion of activated monocytes. These findings demonstrate that exposure of blood vessels to NPs encourages the recruitment of inflammatory cells from the circulation to favour the development of a pro-inflammatory response within the tissue supplied by the affected blood vessel. However, there is also the potential for NP exposure to contribute to the progression of inflammatory diseases within blood vessels, including conditions such as atherosclerosis

(Oesterling *et al.,* 2008). However, these are *in vitro* findings and their extrapolation to an *in vivo* scenario requires assessment.

Despite the fact that inflammation has been observed to be central to NP mediated toxicity, it does not always transpire. It has been found that the composition of NPs is a major determinant in the propensity of NPs to cause inflammation, so that iron oxide NPs (45nm) failed to elicit an inflammatory response, but zinc oxide particles (47nm) induced considerable cell death and resulted in the increased expression of ICAM, IL-8 and monocyte chemotactic protein (MCP-1) in HAEC endothelial cells (Gojova *et al.,* 2007). However, as there is considerable evidence that supports the pro-inflammatory potential of a variety of NPs (including those contained within the PARTICLE_RISK panel) *in vitro* and *in vivo*, exposing the ability of NPs to elicit inflammation within the liver is of great interest and relevance.

5.1.3.1 Oxidative stress: a common cellular response to NP exposure?

Oxidative stress occurs when there is an imbalance between oxidants and antioxidants within cells, so that oxidant presence is favoured due to the excessive production of oxidants and depletion of antioxidants (Li and Karin, 1999, MacNee and Rahman, 2001). Examples of reactive oxygen species (ROS) include superoxide (O_2^-), and the hydroxyl radical ($^{\bullet}OH$) (Ryter *et al.*, 2007).

ROS are present within cells at a low concentration, and are produced as a consequence of normal cell activity; for example cytochrome P450 activity within hepatocytes is known to generate ROS, and oxidative metabolism (responsible for ATP generation) by mitochondria has been identified as a major intracellular source of ROS (Ryter *et al.*, 2007). It is also important to recognise that ROS generation has beneficial consequences for the cell, as for example, they are involved in cell signalling (which allows the regulation of gene expression) and are required for the degradation of foreign material by immune cells and so participate within host defence (Valko *et al.*, 2007, Ryter *et al.*, 2007). However increases in cellular ROS can induce oxidative stress, which constitutes a major threat to normal cell function, as ROS are able to oxidise cellular constituents such as nucleic acids, proteins and lipids within cells, which

causes their modification or degradation to such an extent that cell function is compromised, and cell death ensues (Ryter *et al.*, 2007, Valko *et al.,* 2007). Consequently 'normal' levels of ROS within cells are low and their existence is dependent on their rate of production and rate of clearance, suggesting that there is a delicate balance between the beneficial outcomes associated with oxidant activity, and the harmful consequences related with their excessive production and termed oxidative stress (Valko *et al.,* 2007). It is therefore imperative that cells are able to overcome any increases in ROS production to sustain normal cell function.

5.1.3.2 Cellular defences against increased ROS production

To limit the damage inflicted by increased ROS presence within cells there are defence mechanisms in place that aim to limit the persistence, and therefore deleterious effects of ROS. It is thought that increases in ROS generation stimulate a protective response within cells that includes the participation of antioxidants (which aspire to remove ROS from the cell), the implementation of repair mechanisms (to restore oxidised molecules to their original state), and preventative measures (due to oxidant mediated expression of genes, the products of which have antioxidant capabilities, see sections 5.1.3.4 and 5.1.4). These measures (stimulated by oxidants) therefore aim to eliminate ROS from cells to re-establish the original redox state [so that the levels of ROS are 'reset' to normal after a temporary increase in their levels (Valko *et al.,* 2007, Droge, 2002, Li, Xia and Nel, 2008, Ryter *et al.*, 2007)] and thereby limit their propensity to inflict damage. Antioxidants are present in enzymatic (such as superoxide dimutase, and catalase) and non-enzymatic (including ascorbic acid, tocopherol, and glutathione) forms, and may be specific to a particular oxidant species, or may enable the removal of a number of different ROS. Therefore it is important to highlight that the increased generation of ROS does not necessarily have a detrimental impact on normal cell function due to the existence of protective measures within cells that can be implemented to avoid the persistence of ROS. However, if the production of ROS overwhelms the defences present, the damaging effects of ROS transpire. This includes responses that are pro-inflammatory in nature (due to the activation of ROS sensitive signalling cascades), tumour development (due to the ability of ROS to increase the frequency of gene mutations), reduced cell viability due to, for

example, the oxidation of cell components such as lipids present within the cell membrane which are able to compromise plasma membrane integrity (Li, Xia and Nel, 2008, Driscoll, 1996). It is therefore essential to evaluate the ability of NPs to induce oxidative stress and to consider the potential consequences of its manifestation.

5.1.3.3 How are ROS and oxidative stress detected?

Increases in NP mediated ROS generation are detected through the use of a number of different techniques which measure increases in oxidant production, or antioxidant depletion from cells. An increase in ROS production can be detected using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA). Depletion of cellular antioxidants is indicative of increased ROS generation, for example decreases in glutathione (GSH) concentration can be determined using fluorescent probes, such as *o*-phthaldialdehyde, or by carrying out Western blot analysis to determine protein levels. Furthermore the contribution of oxidative stress can be suggested if treatment with antioxidants (such as ascorbic acid) or free radical scavengers (such as mannitol) can diminish the observed NP mediated toxicity *in vitro* or *in vivo*. In addition as ROS are known to oxidise cellular components, and so a common way of detecting oxidative stress development is by looking for markers of the process, for example when DNA is 'attacked' by ROS, 8-hydroxyguanosine (8-OH-G, an oxidised form of the DNA nucleotide guanosine) is formed (Valko *et al.,* 2007).

5.1.3.4 Evidence of NP mediated oxidative stress

Due to the fact that oxidative stress is thought to be fundamental to NP mediated toxicity it is necessary to review evidence of NP induced oxidative stress in different cell types, as it has been a focus of many toxicological assessments. It is of particular importance to outline previous demonstrations of oxidative stress within the PARTICLE_RISK panel, in addition to other particles to determine the universality of oxidative stress manifestation to NP toxicity. As cell lines have been routinely used to evaluate NP mediated oxidative stress, their tissue and cell type of origin is demonstrated in table 1.1. The ability of NPs to induce oxidative stress, subsequent to particle exposure, must also encompass the possibility that oxidants can be generated by activated phagocytic cells (that aspire to eliminate inhaled particles from the

lungs), introduced to the body due to their generation at the particle surface (in cell free conditions), or a consequence of interruption with normal cell function (MacNee and Rahman, 2001).

Stone *et al.,* (1998) observed that 14nm ufCB (and not 260nm CB) caused a time dependent depletion of cellular GSH, and increase in GSSG concentration within the A549 epithelial cell line, and that ufCB induced cytotoxicity was preventable through pre-treatment with mannitol (a free radical scavenger) which provided confirmation for the involvement of oxidative stress within the toxicity of NPs. Koike and Kobayashi (2006) investigated the ability of CB NPs (14, 56, 95nm diameter) to induce oxidative stress in primary rat alveolar macrophages and alveolar epithelial SV40T2 cells. It was observed that all sizes of CB NPs were able to elicit oxidative stress (in a dose dependent manner) within cells using the dithiothreitol (DTT) assay (where DTT is consumed by ROS presence), with the smallest particles producing the greatest effect. The levels of the antioxidant enzyme heme oxygenase-1 (HO-1) were also observed to increase with NP treatment, so that NP induced oxidative stress was able to stimulate a protective response within cells, highlighting the attempt of cells to overcome NP induced oxidative stress. Silver NPs (7-20nm) have been observed to cause a dose dependent depletion in GSH content in A431 skin carcinoma cells, with a concurrent increase in lipid peroxidation, and reduction in cell viability evident (Arora *et al.*, 2008). SWCNTs have been observed to induce oxidative stress in HaCaT keratinocytes (Shvedova *et al.,* 2003), and within the lungs of mice after intratracheal exposure (Shvedova *et al.,* 2007) which was determined by the detection of decreased GSH content and accumulation of lipid peroxidation products. Hussain *et al.,* (2005) observed that silver NPs (15nm) were able to cause an increase in ROS generation in BRL3A hepatocytes, which resulted in a depletion in GSH cell content, so that oxidative stress was suggested to mediate the observed reduction in cell viability. It has therefore been frequently observed that there is an inverse relationship between ROS and GSH levels, which is known to be a marker of oxidative stress development. Sayes *et al.,* (2005) found that C_{60} exerted toxicity in HDF fibroblasts, HepG2 hepatocytes, and NHA astrocytes through an increase in ROS generation that caused lipid peroxidation, which implied that oxidative stress mediated membrane damage was responsible for

cell death. It was also observed that the administration of the antioxidant ascorbic acid could prevent the development of oxidative damage and therefore the appearance of C_{60} mediated toxicity, providing further evidence for the importance of oxidative stress to C_{60}P toxicity in a variety of cell types. Lovric *et al.,* (2005a) demonstrated that QDs (CdTe) were able to induce cytotoxicity within MCF-7 human breast cancer cells. The cytotoxic effect was associated with an increase in ROS production, and could be prevented via pre-treatment with the antioxidant N-acetyl cysteine (NAC), thereby implicating oxidative stress within the toxicity of QDs. Park *et al.,* (2008) demonstrated that cerium dioxide NPs (15, 25, 30 and 40nm diameter) were able to induce oxidative stress within BEAS-2B bronchial epithelial cells; measured by the intracellular oxidation of DCFH. The observation that a dose dependent increase in ROS production was apparent on exposure to cerium dioxide NPs, correlated to a dose dependent decrease in glutathione concentration, and again insinuated that oxidative stress is involved in the toxicity of NPs, and that GSH depletion is a relevant marker of oxidative stress. Lin *et al.,* (2006) evaluated the ability of silica dioxide particles (15nm) to induce oxidative stress within A549 epithelial cells, with DCFH oxidation used as an indicator of ROS production. Oxidative stress has therefore been observed to manifest on exposure of a variety of NPs within a number of different cellular targets. Wilson *et al.,* (2002) demonstrated that ufCB (14nm) was able to generate oxidants in a cell free (indicated by DCFH fluorescence) environment, whereas CB (260nm) was unable to elicit such a finding, thus suggesting that particle physicochemical properties (such as size) influence their reactivity. This finding was also replicated in the presence of MM6 and J774 macrophage cells (indicated by DCFH fluorescence and GSH depletion) but the oxidative potential of ufCB was amplified when compared to that observed in cell free conditions, and so it was suggested that ufCB initiated an oxidative burst within phagocytic cells. The results therefore demonstrate that NPs do not require the participation of cells to generate ROS, so that ROS generation could conceivably inflict damage to cells within the vicinity of exposure. However the oxidative potential of NPs is increased by cell presence, so that not only particle properties (such as size) influence oxidative stress development, but the ability of cells to respond to particle presence.

Although there is a vast amount of evidence that suggests oxidative stress is central to NP induced toxicity, and it is evident that NPs vary in their ability to elicit increases in ROS within cells. For example, contrary to findings that have demonstrated that C_{60} induces oxidative stress to mediate its toxicity, there is evidence that C_{60} can act as a free radical scavenger, and thus have antioxidant properties (Gharbi *et al.*, 2005). Limbach *et al.,* (2007) observed that iron, cobalt and manganese NPs were not able to induce oxidative stress, when exposed to A549 epithelial cells. Therefore despite being comparable, in terms of size, shape, extent of agglomeration, experimental set up and morphology their ability to induce oxidative stress was composition dependent, following the pattern manganese>cobalt>iron. It is therefore evident that NPs are able to exert oxidative stress to varying extents. Oxidative stress development also appears to be dependent on the experimental set up, so that exposure conditions, such as methods of NP dispersal are influential in NP toxicity. Foucaud *et al.,* (2007) found that BSA and DPPC were able to enhance the dispersion of ufCB, which lead to increased ROS production in cell free conditions, and when ufCB was exposed to macrophage MM6 cells. Furthermore, oxidative stress has been suggested to be a multi-tier response, whereby protective responses elicited by ROS transition to damaging effects as particle concentration, and therefore ROS levels increase (Li *et al.*, 2003). The cytoprotective response, induced by sub-lethal concentrations of ROS is suggested to be mediated by the transcription factor Nrf2, whose activation results in the translocation of Nrf2 to the nucleus where it binds to transcriptional regulatory elements, termed antioxidant response elements (ARE) contained within antioxidant genes (Baulig *et al.*, 2003). ARE activation then increases the expression of genes that that encode antioxidant proteins (such as superoxide dismutase, catalase, and heme-oxygenase-1) which is integral to combating, and protecting against rises in ROS, and contrary to the notorious role of ROS within the toxicity of particles. However, if this protective response fails (due to an excessive burden of ROS), oxidative stress leads to the activation of transcription factors, (see section 5.1.4) to elicit an inflammatory response (Li *et al.*, 2004). Consequently cytoprotective responses are implemented to prevent against the damaging effects of ROS, and if ineffectual (due to an excessive burden of ROS), may escalate to an inflammatory, and potentially cytotoxic response, highlighting the delicate

balance that exists between the ability of ROS to trigger protective or harmful effects. Baulig *et al.,* (2003) observed the increased binding of Nrf2 to ARE to instigate a cytoprotective response, driven by the increased production of antioxidants, on exposure of 16-HBE epithelial cells to diesel exhaust particles (DEP). Li *et al.,* (2004) demonstrated that DEPs were able to activate an antioxidant driven protective pathway via Nrf2 and ARE in RAW264.7 macrophages and BEAS-2B epithelial cells. It is therefore necessary to determine if the Nrf2/ARE pathway is active on engineered NP exposure due to the critical role they play to the toxicity of DEP, which is information that is currently lacking. However, as the toxicity of DEP is known to be driven by the particulate matter it contains, in addition to organic material and metals that adsorb onto the particle surface, the relevance of the findings to engineered NPs requires assessment. However as engineered NPs have been demonstrated to be capable of stimulating the production of ROS (see section 5.1.3.4), a cytoprotective response may be initiated at sub-lethal concentrations.

The available data therefore suggests that engineered NPs are able to exert oxidative stress on exposure, however this may be dependent upon the target cell of interest, NP under investigation, intrinsic ability of NPs to generate ROS, and experimental set up. Therefore despite the fact that it is not a universal finding within all NP types and cells investigated, its recurring manifestation provides compelling evidence that NPs have the potential to exert toxicity via this mechanism, and necessitates that NP mediated oxidative stress development is considered as a potential outcome of NP exposure to the liver.

5.1.4 Involvement of transcription factor activation in NP mediated toxicity

Transcription factors are DNA binding proteins that interact with promoter regions of genes to control the expression of the gene products. Nuclear factor kß (NF- kß) is a transcriptional regulator, which on activation translocates from the cytoplasm to the nucleus to regulate the expression of a repertoire of genes. The gene products generated are principally pro-inflammatory in nature, and therefore play an important role in the development of an inflammatory response (Li and Karin, 1999). Pro-inflammatory gene products, whose production is increased by the activation of NF- kß include; cytokines (including TNFα, and IL-1), chemokines (such as IL-8), adhesion molecules (for example

ICAM-1), and immunoregulatory molecules (such as cyclo-oxygenase and nitric oxide synthase products) all of which are capable of initiating or sustaining an inflammatory response (Li and Karin, 1999).

A number of stimuli including oxidative stress, pro-inflammatory mediators (including TNFα) and increases in cytosolic calcium have been implicated in the activation of NF- kß (Rahman and MacNee, 1998). Therefore as NPs can induce oxidative stress (Li *et al.*, 1999) and increases in intracellular calcium (Stone *et al.*, 2000), the activation of transcription factors has been suggested to play a pivotal role in mediating NP toxicity, and illustrates that a cascade of events are involved within NP toxicity, so that cellular responses to NP exposure are multi-faceted. Shukla *et al.,* (2000) highlighted the causal relationship between particle induced ROS production and activation of NF- kß, whereby exposure to particulate matter resulted in increases in mRNA levels of NF- kß regulated genes including TNFα and IL-6 in C10 alveolar epithelial type 2 cells, which was mediated by particle induced oxidative stress. Furthermore, Brown *et al.* (2004) demonstrated that the exposure of macrophages to carbon NPs resulted in the activation of transcription factors (such as NF-kß). This was suggested to be mediated by rises in intracellular calcium and ROS, which induced the increased expression and production of pro-inflammatory proteins such as TNFα, to exert a pro-inflammatory response. The processes (inflammation and oxidative stress) that are critical for driving the toxicity of NPs have therefore been identified, along with the signalling pathways (NF-kß) that are of particular relevance.

In addition to eliciting damaging responses, particulate exposure also has the capability of evoking the activation of transcription factors that are able to increase the activation of genes that encode proteins that partake in cytoprotective responses (Ishii *et al.*, 2000) that counteract any potentially toxic events, such as oxidative stress (see section 5.1.3.4). This is exemplified by the transcription factor Nrf2, which on activation translocates from the cytoplasm to the nucleus to co-ordinate the increase expression of drug metabolising enzymes and antioxidants via AREs. The increased expression of a battery of genes by the Nrf2/ARE pathway therefore constitutes an important mechanism of cellular defence against oxidative stress to enable the

detoxification of compounds capable of generating ROS, or by facilitating ROS removal (Ishii *et al.*, 2000).

5.1.5 The NP toxicity hypothesis

The mechanism by which particles impart their pro-inflammatory response within the lungs is thought to be a consequence of the increased transcription of pro-inflammatory mediators, mediated by increases in oxidative stress (Stone *et al.*, 2000). Oxidative stress and inflammation are therefore thought to act in concert to provide the basis for the toxicological outcomes, such as cell death, associated with NP exposure. The ability of oxidative stress to initiate an inflammatory response that underlies the toxicity of NPs has been experimentally proven. Monteiller *et al.,* (2007) demonstrated that ufCB and TiO_2 NPs mediated oxidative stress (indicated by GSH depletion), and IL-8 gene expression and production were key events involved in NP toxicity to A549 alveolar epithelial cells. Manna *et al.,* (2005) demonstrated that SWCNTs induced oxidative stress in HaCaT keratinocytes, which correlated with the activation of NF-kß, which was suggested to be responsible for eliciting an inflammatory response that compromised cell viability. Singh *et al.,* (2007) demonstrated that TiO_2 (20-80nm or 40-300nm) particles induced a size dependent expression of IL-8 on exposure to A549 epithelial cells, and was related to their ability to stimulate ROS generation. Furthermore Shvedova *et al.,* (2007) illustrated that SWCNTs elicited a robust inflammatory response within mouse lungs that was virtually eliminated by pre-treatment with the antioxidant ascorbic acid, thereby implicating oxidative stress.

Oxidative stress is consequently recognised as a hallmark of NP exposure, and is paramount to the inflammogenic and cytotoxic outcomes associated with NPs, so that there is a unifying hypothesis surrounding the potential consequences of NP exposure. Therefore a complex cascade of events are involved in mediating ultrafine particulate toxicity, and it is necessary to determine if similar phenomena are exhibited by a wider range of engineered NPs. The NP toxicity hypothesis; that NP mediated toxicity is reliant on oxidative stress and inflammation development has therefore been confirmed within wide ranging studies that have used a variety of NPs, and diverse cell

types, so that these endpoints are a relevant consideration when assessing NP toxicity to the liver.

5.1.6 The ability of NPs to compromise cell viability

5.1.6.1 Mechanisms of cell death

Cell death can be described as an irreversible loss of cell function and structure, and can occur via apoptosis or necrosis (Patel and Gores, 1995). Necrosis is a passive form of cell death which results from an abrupt loss of plasma membrane integrity, a defect which allows the release of cytoplasmic contents of cells to trigger an inflammatory reaction which has the potential to damage surrounding cells (Patel and Gores, 2005). In contrast, apoptosis is a form of programmed cell death, which regulates cell populations within tissues, in addition to occurring as a defence mechanism to enable the removal of damaged cells, so that both physiological and pathological stimuli can trigger apoptosis (Patel and Gores, 2005). Apoptosis is a tightly regulated, energy dependent process that involves the activation of caspases (proteolytic enzymes) to initiate a complex signalling cascade that commits the cell to death by cleaving key cellular proteins and ultimately apoptotic cells are recognised by phagocytic cells to permit their removal (Elmore, 2007). Apoptosis is associated with a number of morphological modifications including cell shrinkage (which causes organelles to become more tightly packed), loss of cell contact with neighbouring cells, nuclear fragmentation, and cytoplasm vacuolation (Patel and Gores, 2005, Elmore, 2007). In apoptosis, the cell ultimately fragments, so that cells are broken up into membrane bound fragments which contain intact organelles and nuclear material, and are referred to as apoptotic bodies (Patel and Gores, 1995, Elmore, 2007). Therefore the mechanisms and morphologies associated with apoptosis and necrosis are distinct, so that necrosis is a passive process which has the potential to affect large numbers of cells, whereas apoptosis is a controlled, process which can affect individual cells (Elmore, 2007).

5.1.6.2 Detecting cell death

Determining the ability of NPs to impact on cell viability can be identified by detecting the number of alive (viability assay) or dead cells (cytotoxicity test) subsequent to the exposure of cells to NPs. The lactate dehydrogenase (LDH)

assay is utilised to evaluate cytotoxicity, and is based on the knowledge that under normal conditions the enzyme LDH is located within the cytoplasm of cells, but on membrane damage, cytoplasmic components are released into the extracellular environment, which is indicative of cell death. The release of LDH from cells, indicates that damage to the plasma membrane of the cell has occurred, thus implying that a greater proportion of cells are not viable. The MTT assay ascertains the number of cells that are viable and measures the mitochondrial succinate dehydrogenase activity within cells, and works on the principle that only the mitochondria of viable cells are able to function, so that a decrease in mitochondrial activity is suggestive of cell death. The live/dead assay utilises fluorescent probes, to simultaneously distinguish between the number of viable, and non-viable cells within a sample. The Trypan blue assay is reliant on the knowledge that Trypan blue is usually excluded from cells due to its large size and negative charge, but when cells are damaged, to an extent that is sufficient in severity to cause cell death, the cell membrane becomes permeable to the dye and dead cells appear blue when observed using light microscopy (Lewinski, Colvin, and Drezek, 2008). Evaluating cell morphology can give an indication of the type of cell death involved in a cytotoxic response.

5.1.6.3 NP mediated cytotoxicity

Inflammation and oxidative stress induced by NPs can be overcome with time due to the defence systems present within cells, for example within an experimental setting ufCB has been observed to deplete GSH with A549 epithelial cells at 4 hours, but the cells are able to recover GSH levels after a 6 hour exposure (Stone *et al.*, 1998). However if NP exposure is prolonged, cells are unable to recover and the damage to cell components and loss of cell function, can trigger cell death, due to the ability of ROS to attack, modify and degrade cell components, which is known to perturb normal cell function when present at elevated, non-physiological concentrations (Ryter *et al.*, 2007). Inflammation can also directly contribute to cell death, as cytokines, such as TNFα are known to be cytotoxic at high concentrations, or indirectly through the attraction of inflammatory cells that release damaging mediators (such as proteases) on activation (Jaechske, 2006). Therefore if exposure levels, or duration of exposure to NPs induces toxicity that exceeds the protective mechanisms evident within cells, normal cell function cannot be sustained, and

cell viability is compromised. Cell death is therefore an important endpoint to consider when evaluating NP toxicity as processes known to contribute to cell death are known to be related to NP exposure.

SWCNTs have been demonstrated to cause a reduction in cell viability in HaCaT keratinocytes (Shvedova *et al.,* 2003), and A549 epithelial cells (Davoren *et al.* 2007) however this was apparent at high concentrations of 400 and 800ug/ml]. Barlow *et al.,* (2005) demonstrated that ufCB and TiO_2 NPs were able to elicit a greater release of LDH from the L-2 alveolar type 2 cells when compared to their larger counterparts, so that exposure to these particles is able to elicit a size dependent cytotoxic response. Silver NPs have been observed to cause cytotoxicity with skin (Arora *et al.,* 2008) and liver (Hussain *et al.,* 2005) cells. Therefore NPs have been shown in a number of studies to compromise cell viability despite being diverse, in terms of size, composition, exposure times and concentrations.

It must also be recognised that not all NPs are able to induce cytotoxicity, elicit different extents of cytotoxicity on exposure, or induce cell specific toxicity. Jia *et al.,* (2005) compared the cytotoxicity of SWCNTs, MWCNTs and C_{60} when exposed to primary guinea pig alveolar macrophages, and observed that the different carbon based NPs did not elicit comparable levels of cytotoxicity, so that SWCNTs produced the greatest and fastest cytotoxicity when compared to MWCNTs, but C_{60} did not induce a cytotoxic response (when administered at an equal mass dose), illustrating that NPs composed of the same material do not behave similarly. Park *et al.,* (2008) found that cerium dioxide particles were able to induce cell death when exposed to bronchial epithelial BEAS-2B cells, but not other cell types, including rat cardiomyocytes (H9C2) and brain fibroblasts (T98G), thus highlighting that NP toxicity may be site, and cell specific within the body. Sayes *et al.,* (2004) found that the cytotoxicity of C_{60} to the HDF dermal fibroblasts and HepG2 hepatocytes was dependent on its surface coating (that aimed to make C_{60} more water soluble, such as $C_{60}(OH)_{24}$). Specifically, it was found that the introduction of surface attachments decreased the cytotoxicity of $C_{60,}$ so that the greatest extent of toxicity was evident when C_{60} was in its pure form. This is an important finding, as if surface modifications are able to reduce the cytotoxicity of C_{60} whilst

retaining the properties that make it attractive for exploitation then adverse outcomes associated with its use could be avoided. Ryman-Rasmussen *et al.* (2007) observed that the charge of QDs (CdSe/ZnS) was able to influence their toxicity, specifically, neutral QDs did not affect HEK keratinocyte cell viability whereas positively, and negatively (produced greatest toxicity) charged QDs were able to induce a cytotoxic response.

Despite the knowledge that not all NPs are capable of eliciting cytotoxicity, the finding that cytotoxicity has been a common observation, suggests that this endpoint must be considered when evaluating NP toxicity within the liver. However it is important to highlight that the liver has an enormous regenerative capacity whereby it is able to initiate a proliferative response to replace lost cells, to maintain normal liver function however this will depend on the extent of damage evident (Jaeschke *et al.*, 2002).

5.1.7 Liver mediated bile secretion

Bile formation is an exclusive function of the liver, and allows the elimination of substances from the body. The ability of particles to affect the canalicular accumulation of CLF within isolated rat hepatocyte couplets will be used as an indicator of cholestasis, as the impact of NPs on bile secretion has not been previously investigated. Hepatocyte couplets have been proven to be a useful *in vitro* model when evaluating the known *in vivo* capabilities of cholestatic agents such as the immunosuppressant drug Cyclosporin A (Roman *et al.*, 2003) tertiary-butylhydroperoxide (Ahmed-Choudhury, Orsler and Coleman 1998), menadione (Stone *et al.*, 1994) and alpha-naphthylisothiocyanate (Orsler *et al.,* 1999). These findings highlight the predictive capability of this *in vitro* model of bile secretion, due to the fact that they are capable of reproducing the cholestatic capabilities that substances exhibit *in vivo,* and also provide an opportunity to investigate the underlying mechanisms responsible for cholestasis at a cellular level which cannot be attained when *in vivo* models of bile secretion are used.

5.1.8 Aims and objectives

To investigate the ability of the PARTICLE_RISK particle panel to interfere with normal hepatocyte cell function. The ability of NPs to induce oxidative stress

was determined by detecting a decrease in the cellular antioxidant glutathione. The impact of particle exposure on cell viability was assessed using the LDH and MTT assays. The ability of particles to affect bile secretion was investigated by determining the accumulation of CLF within the bile canaliculi of hepatocyte couplets (CVA of CLF) subsequent to NP exposure. The gross (using SEM) and ultrastructure (using TEM) morphology of hepatocytes exposed to particles was determined, as this can be indicative of toxicity.

5.2 Materials & Methods

5.2.1Materials

5.2.1.1Equipment

The following equipment was employed: Dynex Magellan Biosciences MRX Revelation plate reader, FLUOstar Optima Microplate Reader, Bibby Stuart Scientific Orbital microplate shaker, Hitachi S-4800 Type II Field Emission Gun-Scanning Electron Microscope and Tecnai 12 Transmission Electron Microscope. All other equipment used has been described previously.

5.2.1.2 Chemicals

The chemicals used included: Potassium hydroxide, potassium bicarbonate, tetrasodium ethylenediaminetetraacetic (Na_4EDTA), *o*-phthaldialdehyde (OPT), β-nicotinamide adenine dinucleotide, (NADH, reduced form), sodium pyruvate, 2,4-dinitrophenylhydrazine, Perchloric acid, Hydrochloric acid, sodium hydroxide (NaOH), 2,5-diphenyltetrazolium bromide, 2-propanol, sulphuric acid, sodium cacodylate, dipotassium hydrogen orthophosphate, postassium dihydrogen orthophosphate, sodium succinate, and Tween 20 were all purchased from Sigma-Aldrich (Poole, UK).

BioRad protein reagent was purchased from BioRad Laboratories (Hertfordshire, UK) and gluteraldehyde was obtained from Agar scientific (Stanstead, UK)

All other chemicals used were purchased from sources previously stated.

5.2.2 Exposure of the C3A hepatocyte cell line and IRHCs to the PARTICLE_RISK panel

5.2.2.1Standardisation of particle concentrations

To fulfil a screen like test of particle toxicity to hepatocytes it was decided that particle concentrations of 31, 125 and 250µg/ml would be used to represent low, medium and high particle concentrations respectively. It was also necessary to standardise the concentration of particles used in the different exposure set ups; specifically due to the necessity of conducting experiments within 24 well plates (diameter of 15mm and SA=176.6mm^2) or Petri dishes (diameter of 55mm and SA=2374.6mm^2), which was required within different

protocols, and due to equipment limitations and particle availability. Consequently NP concentrations were expressed as the mass concentration of particles per unit surface area within the exposure 'site' (namely µg/mm^2). This was important as the exposure of hepatocytes to particles within 24 well plates was carried out at a volume of 250µl, and within Petri dishes particles were exposed at a volume of 3ml, which if occurred at a concentration that was expressed as µg/ml, would result in cells contained in Petri dishes being exposed to a greater concentration of particles. Therefore, particle concentrations of 0.04, 0.18 and 0.35 µg/mm^2 were used for the exposure of cell lines to the PARTICLE_RISK particle panel and 0.04 and 0.08µg/mm^2 for the exposure of hepatocyte couplets.

5.2.2.2 Exposure of hepatocytes to particles

C3A cells were plated at a concentration of 3x10^5 cell/ml in a 24 well plate (at a volume of 1ml) and incubated overnight at 37^{o}C, 5% CO_2. The next day the cells were exposed to the PARTICLE_RISK particle panel (or equivalent control) for 2,4,6, or 24 hours at 37^{o}C, 5% CO_2, at concentrations of 31, 125 or 250µg/ml (250µl, in complete C3A cell culture medium), which was equivalent to particle concentrations of 0.04, 0.18 and 0.35µg/mm^2 respectively.

Hepatocyte couplets were isolated as previously described (see section 3.6) and plated in 24 well plates at a concentration of 2x10^5 cells/ml and incubated for 4 hours at 37^{o}C, 5%CO_2. The cells were then exposed to the PARTICLE_RISK particle panel (or appropriate control) for 2, 4, and 6 hours at concentrations of 31, or 62µg/ml (250µl, in L-15 medium and 10% FCS), which were equivalent to 0.04 and 0.08ug/mm^2 respectively.

5.2.3.1 Determining the impact of NP exposure on the C3A hepatocyte cell line and IRHC intracellular GSH content

Reduced GSH is a tripeptide (glutamate–cysteine-glycine) intracellular antioxidant and in the presence of ROS, is oxidised to GSSG. Depletion of cellular GSH, and increases in GSSG content are therefore indicative of oxidative stress. The method used to measure intracellular GSH levels was adapted from Hissin and Hilf, (1976). In order to assess the intracellular GSH content a 'cell extract' is generated, whereby cells are lysed to release the

intracellular contents (in which GSH is contained) and was achieved through the treatment of cells with Perchloric acid. Once GSH is extracted from cells its concentration was measured using the fluorochrome OPT, which binds to sulphydryl groups (-SH), found within the amino acid cysteine of GSH to yield a fluorescent complex that enables the detection of GSH (Stevenson *et al.*, 2002).

5.2.3.2 Extraction and measurement of GSH from hepatocytes

The following solutions (made fresh on the day of the experiment and stored at 4^{o}C until required) were required for the extraction and measurement of GSH:

Extraction buffer: 9mM tetra sodium EDTA in 14% Perchloric acid (10ml) and PBS (pH 7.4, 10ml).
Neutralising solution: 1M potassium hydroxide and 1M potassium bicarbonate (25.03g) in 250ml dH_2O.
GSH Buffer (pH 8.0): 0.1M sodium dihydrogen orthophosphate, 0.005M tetra sodium EDTA, in 100ml dH_2O.

Subsequent to particle exposure (see section 5.2.2.2), C3A cells and hepatocyte couplets were washed with ice cold PBS (pH 7.4), before ice cold PBS (250µl) was added to the wells, and the cells scraped off using a plastic cell scraper, on ice. For protein determination 40µl of the cell suspension was removed, and stored on ice until required. Extraction buffer (500µl) was added to the wells for 15 minutes on ice. The solution was then transferred to an eppendorf and centrifuged at 2370g for 5minutes at 4^{o}C. The supernatant was then combined with neutralisation buffer (500µl) and centrifuged at 2320g for 5 minutes at 4^{o}C. The supernatant, termed the cell extract, was removed and stored on ice until the concentration of GSH could be determined, on the same day.

The concentration of GSH within the cell extracts was extrapolated from a GSH standard curve. A GSH stock solution of 0.05M GSH (in GSH buffer) was diluted to provide a 0.5mM GSH working solution. A top standard of 50µM was generated and a standard curve created by carrying out a 1 in 2 serial dilution to obtain a concentration range from 50 to 3.25µM. GSH buffer (180µl) was added to the wells of a 96 well clear bottomed plate (Porvair sciences,

Middlesex, UK) and standards or samples (10µl) added to the appropriate wells in triplicate. OPT (10µl, 1mg/ml in methanol) was then added to the 96 well plate and incubated for 15 minutes at room temperature. The fluorescence was determined using the FLUOstar Optima Microplate Reader with an excitation wavelength of 350nm and emission wavelength of 420nm. The concentration of GSH within the samples was extrapolated from the standard curve, and the concentrations adjusted for any dilutions made within the experiment. To standardise the results, protein measurements from the cell suspensions were determined using the BioRad protein assay (see section 5.2.4), so that the expression of GSH concentration was expressed as µMoles GSH/mg of protein.

5.2.4 Protein BioRad Assay

The BioRad protein assay is based on the principle of the Bradford protein assay, whereby protein levels are quantified colourimetrically using the dye Coomassie Brilliant Blue G-250 (which has a red/brown colour). On protein binding the dye undergoes a shift in absorbance from 465 to 595nm, so that the complex formed attains a blue colour. BSA is used as a protein standard, to obtain a standard curve, in order to quantify the protein levels within the samples.

BioRad stock reagent was diluted 1 in 5 with distilled water and filtered. Triplicate groups of sample or standard (10µl) were added to the appropriate wells of a 96 well plate and the diluted reagent was pipetted into wells (200µl). The absorbance was measured using the Dynex Magellan Biosciences MRX Revelation plate reader, at a wavelength of 595nm. The standards were prepared using BSA (in distilled water) with concentrations ranging from 1 to 0.03mg/ml.

5.2.5.1 The LDH assay

LDH catalyses the inter-conversion of lactate and pyruvate and is located within the cytoplasm of cells. When cells are damaged, LDH is released, due to a decrease in cell membrane integrity, which is associated with cell death. Determining LDH release from cells, as a consequence of particle exposure, therefore gives an indication of cell viability. Within the experimental protocol used, the supernatants are collected from particle exposed cells and LDH contained within the cell supernatant then converts pyruvate to lactate. The

pyruvate that is not converted by LDH then attaches to 2,4-dinitrophenylhydrazine to result in the formation of a brown complex. Consequently, increased brown colour is indicative of low LDH activity as there is more pyruvate present that has not been converted by LDH to lactate, thereby indicating that the LDH content of the sample is low. Triton X-100 is used as a positive control, as it lyses cells, and therefore represents the total releasable LDH. As Triton X-100 is assumed to cause 100% cell death, expressing results as a percentage of the positive control allows the level of cytotoxicity to be quantified and standardised.

5.2.5.2 Determining the impact of NP exposure on C3A cell viability, using the LDH assay

The plating of C3A cells and exposure to the PARTICLE_RISK particle panel, was conducted as described (see section 5.2.2.2). Positive control samples were prepared by lysing cells with 0.1% Triton X-100 (250µl, prepared in complete C3A cell culture medium). Subsequent to particle treatments, the cell supernatants were collected, and frozen at -80^{o}C until LDH activity was measured.

The activity of LDH was extrapolated from a standard curve which was created by preparing pyruvate standards. NADH (1mg/ml) contained within 0.75mM sodium pyruvate (in dH_2O) was prepared and diluted so that pyruvate concentrations that were equivalent to LDH activity ranging from 0 – 2000 Units/ml were obtained.

To measure LDH activity, standards (60µl) or samples (10µl) were transferred in triplicate, to the appropriate well of a 96-well plate. NADH (50µl, 1mg/ml in 0.75mM sodium pyruvate) was then added to the sample wells only and the plate incubated at 37^{o}C, 5% CO_2 for 30 minutes. Next, 2,4-dinitrophenylhydrazine (0.2mg/ml, in 1M Hydrochloric acid, 50µl) was added to all wells of the plate and incubated for 20 minutes at room temperature. NaOH (4M, 50µl) was added to each well on the plate and the absorbance read at 540nm in a plate reader.

5.2.5.3 Investigating the adsorption of LDH onto the surface of NPs

It is important to determine if particles are able to interfere with the measurement of LDH within cell supernatants, as it is known that biological molecules are able to bind to the surface of particles (Brown *et al.*, 2001). Therefore the ability of LDH to adsorb onto the surface of all PARTICLE_RISK particles was determined, as if this occurred LDH activity would be under-estimated.

C3A cells were plated at a concentration of $3x10^5$ cells/ml (1ml) in a 24 well plate and incubated overnight (37^oC, 5% CO_2). The next day cells were exposed to 0.1% Triton-X100 (250µl, in complete C3A cell culture medium) and incubated for one hour (37^oC, 5% CO_2). The supernatants were collected and then used to disperse the PARTICLE_RISK particles at a concentration of 250µg/ml. The particle treatments (250µl) or equivalent control (0.1% Triton X-100 supernatant) were then incubated (37^oC, 5% CO_2) for 4 hours in a fresh 24 well plate. After the incubation time the supernatants were centrifuged at 32700g for 5 minutes and LDH activity measured, as described (see section 5.2.5.2).

5.2.6.1 The MTT assay

The MTT assay is a colourimetric assay used to determine mitochondrial function, and therefore cell viability (as only the mitochondria of viable cells will function) and was first described by Mossman (1983). The MTT reagent (a yellow coloured tetrazolium salt) is taken up by cells and exclusively reduced by the mitochondrial enzyme succinate dehydrogenase to an insoluble purple coloured formazan product. Product formation is quantified spectrophotometrically, subsequent to its solubilisation within the solvent 2-propanol, with the amount of product formed being directly proportional to the number of viable cells. A decrease in absorbance, relating to a decrease in product formation from control levels is indicative of a reduction in cell viability.

5.2.6.2 Determining the impact of particle NP on C3A cell and IRHC viability, using the MTT assay

The plating of C3A cells and hepatocyte couplets into 24 well plates, and exposure to the PARTICLE_RISK particle panel, was conducted as described

previously (see section 5.2.2.2). After particle treatments, plates were centrifuged at 140g for 5 minutes, the supernatant was removed and fresh complete cell culture medium added to each well (300µl). The MTT reagent, 2,5-diphenyltetrazolium bromide (5mg/ml, in PBS) was then added to each well (75µl) and incubated at 37^{o}C, 5% CO_2, for 3 hours. After the incubation, the plate was centrifuged at 140g for 5 minutes, the supernatant removed and 2-propanol (300µl) added to each well, mixed with a pipette, and the plate immediately centrifuged at 140g for 10 minutes. The supernatants were transferred to a 96 well plate in triplicate (100µl per well) and the optical density of each well determined at a wavelength of 570nm using a Dynex Magellan Biosciences MRX Revelation plate reader.

5.2.6.3 Determining the interference of NPs with the MTT assay

It is important to determine if particles are able to interfere with the MTT assay in order to avoid the collection of misleading or false results. This arises due to the fact that particles may contribute to the absorbance of the MTT assay to make it appear that cells are more viable than they actually are, or particles bind to assay components (such as the MTT reagent, or product) to prevent their participation in the assay to mistakenly infer that the particles are toxic.

To determine the ability of the PARTICLE_RISK particle panel to interfere with the MTT assay appropriate controls were used, namely; 1) the MTT reagent and particles were added simultaneously to cells (to determine if the particles were able to bind to the MTT reagent) as if particles were observed to bind to the MTT reagent, it would be unavailable to cells and cell viability would be underestimated, 2) the MTT protocol was followed and then particles incubated (30 minutes) with the solubilised MTT product to evaluate the ability of particles to attach to the coloured product, which would lead to an underestimation of cell viability 3) the absorbance of the particles alone (at the wavelength used to quantify the formation of the coloured MTT product) was investigated to determine if particles were able to contribute to MTT absorbance readings. All controls were also performed in the absence of cells, to determine if the particles themselves were able to reduce MTT. Particle treatments were conducted for 4 hours, at a concentration of 0.35µg/mm^2 (at a volume of 250µl).

5.2.7 Effect on NP exposure on bile secretion

Hepatocyte couplets were isolated as previously described (see section 3.6) and plated onto sterilised 42mm glass coverslips (in 55mm Petri dishes), at a concentration of $1x10^5$ cells/ml (volume of 4ml) and incubated at 37°C, in atmospheric air for a minimum of 4 hours. Cells were then exposed to the PARTICLE_RISK particle panel (in L-15 cell culture medium with 10% FCS), or appropriate control, at a concentration of 31µg/ml (3ml, equivalent to 0.04 $\mu g/mm^2$) for 30 minutes or 2 hours. During the last 15 minutes of the particle incubation 5mM CLF (3µl) was added to the Petri dish so that cells were exposed to a concentration of 5µM CLF (excitation of 485nm and emission of 515nm). The cells were then washed with complete cell culture medium, the coverslip transferred to a POC-R observation chamber, L-15 cell culture medium with 10% FCS (2ml), and the chamber placed on the heated stage (37°C) of the confocal microscope. Hepatocyte couplets were identified using the green reflected light on the confocal microscope, using the x20 objective (see figure 5.1). The total number of couplets, and number of couplets able to accumulate CLF within their bile canaliculi (termed the canalicular vacuole accumulation (CVA) of CLF) was determined using cell counters. The results are expressed as the percentage of couplets able to accumulate CLF.

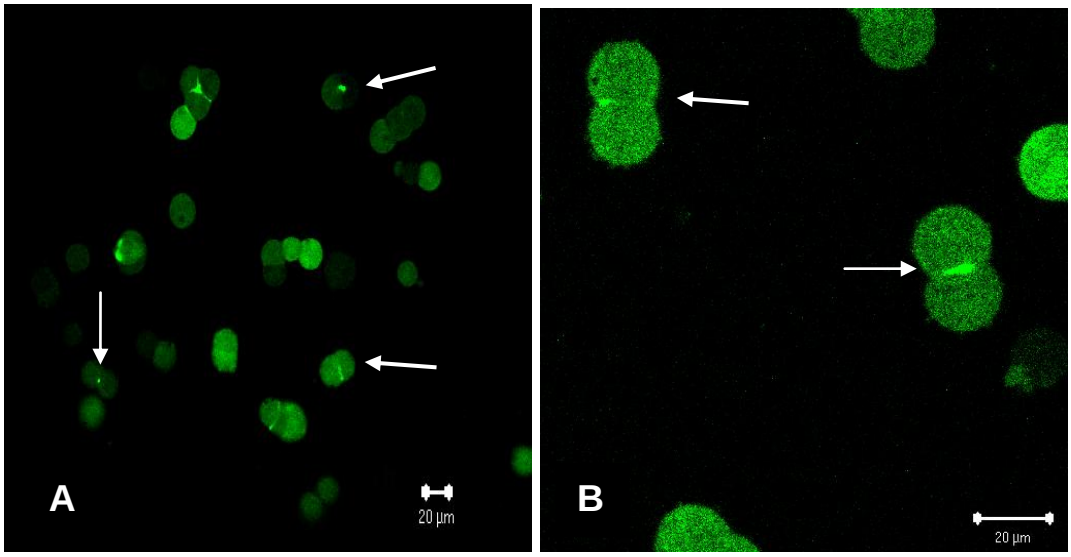


Figure 5.1 Determining the CVA of CLF. Couplets were exposed to CLF (5µM) for 15 minutes then the total number of couplets, and number of couplets accumulating CLF was determined (using cell counters). CLF accumulation within bile canaliculi can be identified by the arrows. CLF accumulation within couplets visualised using confocal microscopy with a x20 (**A**) or x63 (**B**) objective. Scale bar = 20µm

5.2.8 Determining cytokine levels within hepatocyte supernatants

5.2.8.1 Detection of multiple cytokines simultaneously: Multiplex analysis

Analysis of cytokine production, within cell supernatants, conducted using the Bio-plex human 17-plex kits allowed the simultaneous detection of 17 cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 IL-10, IL-13, IL-17, G-CSF, GM-CSF, IFNγ, MCP-1, MIP-1 β and TNFα, The rat 9 Bio-plex kit permitted the detection of 9 cytokines at one time; IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GMCSF, IFNγ and TNFα. The utilisation of these kits is of benefit as they provide the opportunity to detect the production of a number of different cytokines at one time, from a limited sample volume, to give an indication of the cytokine profile associated with NP exposure to hepatocytes. This is of relevance to the *in vivo* situation, where cell responses to toxicants rarely involve the activity of only one cytokine, so that these kits encompass the reality of cytokine cascades within an inflammatory response.

The kits work on the following principle (summarised in figure 5.2). Fluorescently dyed microspheres have (capture) antibodies attached to their surface that are able to recognise the cytokine of interest, and each individual cytokine that is detected within the kit is associated with a particular bead colour. The (capture) antibody conjugated microspheres are incubated with the sample, to allow the recognition and binding of the cytokines present within the sample to the appropriate antibody. A detection antibody, specific for each cytokine within the kit is then added to recognise successful binding of sample to the capture antibody, which are then conjugated to the fluorescent tag streptavidin-phycoerythrin (strep-PE). The constituents of each well are identified (based on bead colour), and cytokine quantities (based on intensity of strep-PE fluorescence) using a Bio-Plex suspension array system machine, and cytokine quantities within samples are extrapolated from a standard curve. A Bio-Plex Suspension Array System, functions like a flow cytometer, and has two lasers which are used to detect what cytokine the microspheres are associated with (which is dependent on its colour), and then the microspheres that have bound the target cytokine of interest was determined (according to the Strep-PE).

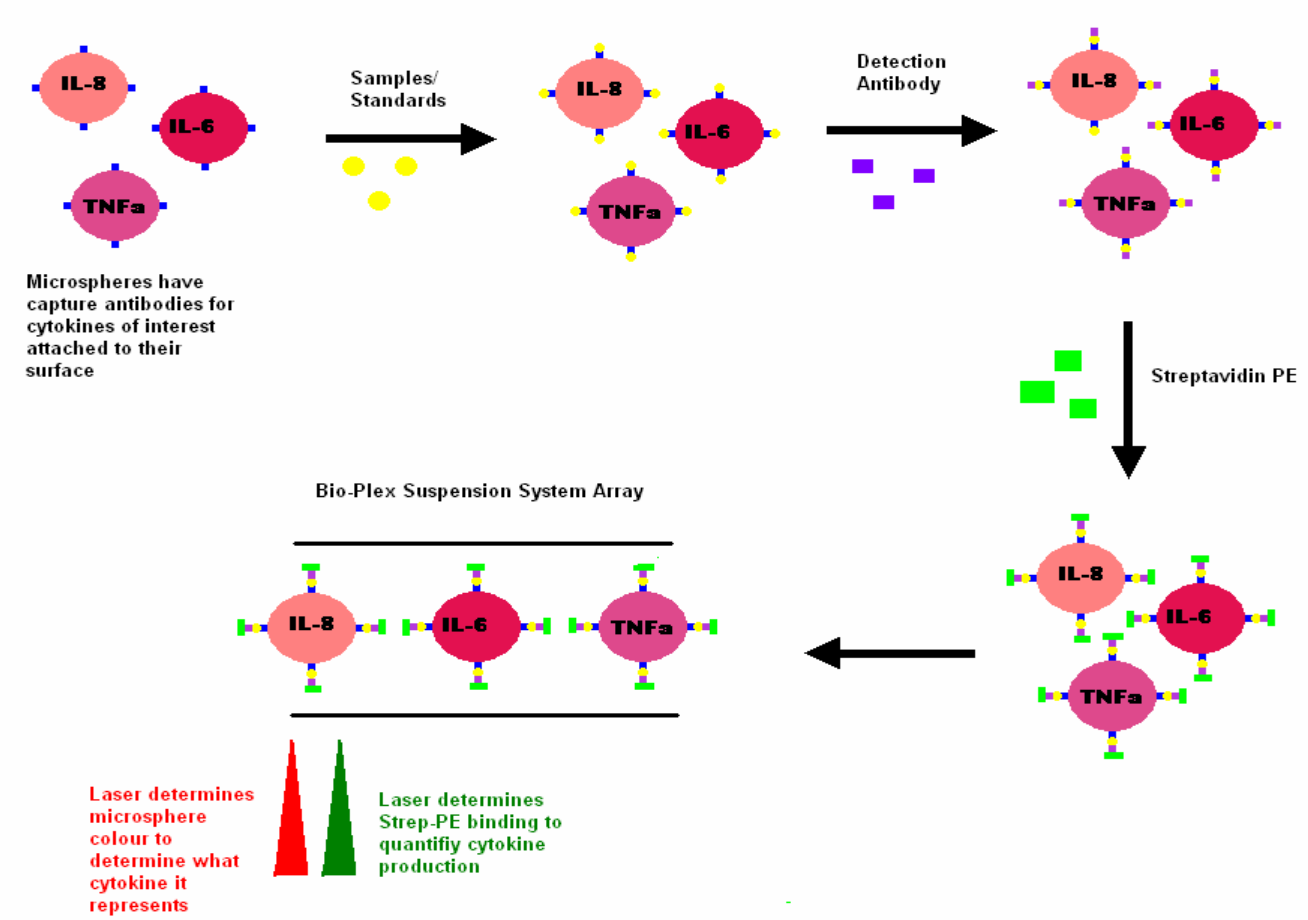


Figure 5.2 The principle of the multiplex cytokine kits. Multiplex analysis allows for the simultaneous detection of a variety of cytokines. Colour coded microspheres that have capture antibodies directed against specific cytokines, bind to cytokines present within the sample and are then recognised through the attachment of the detection antibody that subsequently binds Strep-PE which is a fluorescent tag which allows the cytokines to be quantified.

Multiplex analysis was therefore used to gain an indication of the cytokine profile associated with the exposure of hepatocytes to the PARTICLE_RISK particle panel (in a 'look and see' approach), that could be further investigated using conventional ELISA kits.

5.2.8.2 Determining PARTICLE_RISK particle mediated cytokine release from hepatocytes using multiplex analysis

The plating of C3A cells, and hepatocytes couplets, and their subsequent exposure to the PARTICLE_RISK particle panel was conducted as previously described (see section 5.2.2.2). The collected supernatants were frozen at -80^{o}C until required.

Multiplex analysis was conducted at Scottish Biomedical (Glasgow , UK). Bio-plex human 17-plex (used to analyse C3A cell supernatants), rat 9 plex kits (used to analyse hepatocyte couplet supernatants), and 96 well filter plates were purchased from Bio-Rad Laboratories (Hertfordshire, UK), and cytokine analysis conducted according to the manufacturer's protocol. Briefly, a 96 well filter plate was pre-wet by the addition of Bio-Plex assay buffer (100µl per well). The buffer was removed by vacuum filtration and multiplex bead solution (50µl per well) added to the appropriate wells on the plate. The buffer was then removed using vacuum filtration, so that the beads remained within the wells. Sample supernatant (in singlet) or standards (in duplicate) were added to the wells (50µl) for 30 minutes at room temperature on a microplate shaker. The plate was then washed (x3) with Bio-Plex wash buffer and the detection antibody (25µl per well) added to the plate for 30 minutes. The plate was washed (x3) with Bio-Plex wash buffer and then Streptavidin-PE (50µl per well) added to each well. The plate was then incubated for 10 minutes, in the dark on a microplate shaker. Next, the plate was washed (x3) with Bio-Plex wash buffer, and the beads re-suspended in 125µl Bio-Plex wash buffer. The plate was shaken for 30 seconds prior to being read on the Bio-Plex system.

5.2.8.3 Detecting cytokine production using ELISAs

The most convenient way of detecting the initiation of an inflammatory response is to use an ELISA kit which detects cytokines that are released from cells into the cell medium (termed the cell supernatant). After exposure to the PARTICLE_RISK particles C3A cell and hepatocyte couplet supernatants (see section 5.2.2.2) were collected and cytokine content was determined by ELISA according to the manufacturers protocol. Human IL-8 ELISA kits (for C3A cell supernatant analysis) were purchased from BioSource (Nivelles, Belgium) and rat MIP-2 ELISA kits (for hepatocyte couplet supernatant analysis) were purchased from R&D systems (Abington, UK). Regardless of the manufacturer of the ELISA kit the same protocol was followed, unless stated otherwise. Plates (96 well) were coated with the appropriate concentration of capture antibody (100µl, in PBS), sealed and left in the fridge (BioSource kit) or at room temperature (R&D systems kit) overnight. The next day the plates were washed (x2) with washing buffer (0.1% Tween 20, in saline) using a squirt bottle, and 300µl blocking buffer (0.5% BSA, in PBS) was added to all wells for

2 hours at room temperature on a microplate shaker. The plates were then washed (x2) and the standards (made up in standard diluent (0.5% BSA, 0.1% Tween 20 in PBS)) and samples (100µl) added to the appropriate wells in duplicate for 2 hours at room temperature on a microplate shaker. The plates were washed (x3) and 100µl detection antibody (in standard diluent) added to the plates which were then incubated at room temperature for 2 hours on a microplate shaker. The plates were then washed (x4) and streptavidin-HRP (in standard diluent) added to each well for 20 minutes in the dark, at room temperature (on the microplate shaker). The plates were washed (x4) and 100µl substrate solution (R&D systems, Abingdon, UK) added to each well, and allowed to incubate for 20 minutes, in the dark to make the reaction colourimetric. The reaction was terminated by the addition of 1M sulphuric acid (50µl). The plates were read at 450nm on a Dynex Magellan Biosciences MRX Revelation plate reader, and cytokine levels within the samples extrapolated from the standard curve. The intra- assay coefficients of variation were 5%, 8% and 3% for IL-8, TNFα and MIP-2 respectively. The inter-assay coefficients of variation were 7%, 9% and 4% for IL-8, TNFα and MIP-2 respectively.

5.2.9.1 Investigating the effect of particle exposure on IRHC morphology using SEM analysis

Hepatocyte couplets were isolated as previously described (see section 3.6) and plated onto sterilised poly-D-lysine (0.1mg/ml, in PBS) coated 10mm glass coverslips, in 24 well plates, at a concentration of $2x10^5$cells/ml (volume of 1ml) and incubated at 37^oC, 5% CO_2 for 4 hours. Cells were then exposed to the PARTICLE_RISK panel of particles (in L-15 cell culture medium & 10% FCS), at a concentration of 31µg/ml (0.04µg/mm^2) for 4 hours (250µl). Subsequent to particle exposure, cells were washed with 0.1M sodium cacodylate buffer (pH 7.3) and fixed with 2% gluteraldehyde (500µl, in 0.1M cacodylate buffer) for 2 hours at 4^oC. The samples were then washed and resuspended in 0.1M sodium cacodylate buffer (500µl). The subsequent preparation of samples for SEM analysis was conducted externally at Edinburgh University by Dr Stephen Mitchell. Briefly, the samples were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 45 minutes, prior to being washed in three 10 minute changes of 0.1M sodium cacodylate buffer. The samples were then dehydrated in 50%, 70%, 90% and 100% normal grade acetones for 10 minutes

each, then for a further two 10 minute changes in analar acetone. Dehydrated samples were then critical point dried, mounted on aluminium stubs and sputter coated with gold palladium. A Hitachi S-4800 Type II Field Emission Gun-Scanning Electron Microscope was used to image the cells.

5.2.9.2 Determining the effect of particle exposure on IRHC ultrastructure using TEM analysis

Hepatocyte couplets were isolated as previously described (see section 3.6), plated and exposed to the PARTICLE_RISK particle panel as described for SEM analysis (see section 5.2.9.1) Subsequent to particle exposure cells were washed with complete cell culture medium and the cells collected in cell medium (500µl) by pipetting up and down. The cell suspension was transferred to eppendorfs and centrifuged (490g, 2 minutes) and the cell pellet suspended in 0.1M sodium cacodylate buffer (1ml, pH 7.3), and centrifuged again. Following centrifugation the cells were fixed in 1M gluteraldhyde (500µl, in 0.1M sodium cacodylate buffer) for 3 hours at 4^{o}C. After fixation, the cells were centrifuged (490g, 2 minutes) washed in cacodylate buffer and the pellet resuspended in 500µl 0.1M sodium cacodylate buffer. The final sample preparation was performed externally by Dr Stephen Mitchell at Edinburgh University. Briefly, cells were dehydrated, embedded in araldite resin, sectioned (**60nm thick**), then stained with uranyl acetate and lead citrate. The imaging of cell was performed at Bristol University using a Tecnai 12 Transmission Electron Microscope.

5.2.10 Statistical analysis

The statistical significance of the results was assessed using ANOVA (analysis of variance test) analysis, with a Tukey multiple comparison, using the MiniTab programme. ANOVA analysis was selected as this statistical test allows comparisons to be made between (the means of) several treatment groups. A finding is considered significant if the p value is less than 0.05. The null hypothesis for ANOVA analysis is that the means from the different groups are equal. Therefore, if the null hypothesis is rejected (i.e. if $p<0.05$), it can be concluded that at least one group is different from the others. To reveal what groups are different from one another a Tukey comparison is incorporated within the test. The test determines if there are any significant differences

between the different groups, and if there are significant differences evident it establishes what groups are significantly different from each other. Accordingly within the studies outlined it is relevant to consider if the different particle treatments are different to the control group within each time point.

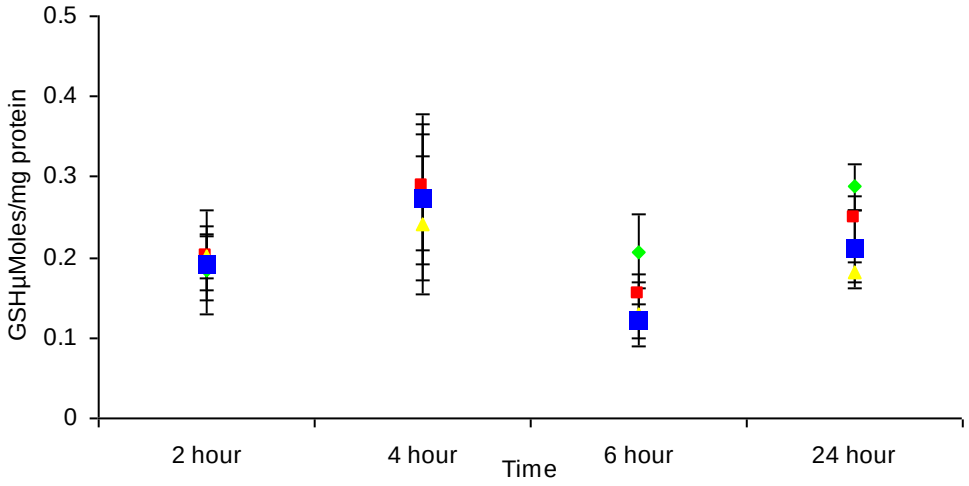
5.3 Results

For all experiments (unless otherwise stated), results represent the means of three separate experiments (conducted on three different days), with the error bars representing the standard error of the mean (SEM).

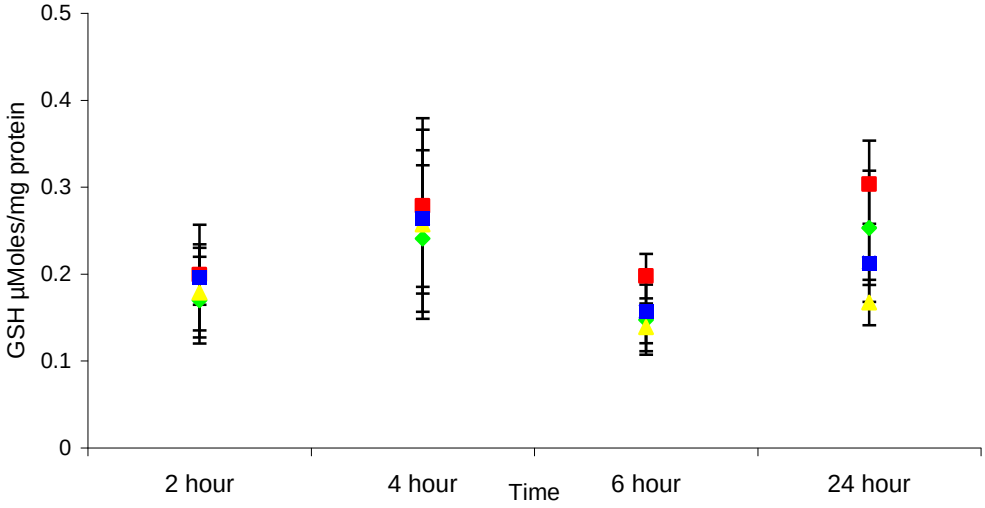
5.3.1.1 Investigating the effect of NP exposure on GSH levels within the C3A hepatocyte cell line

Exposure of C3A cells to ufCB, CB, CNT, QD620 and C_{60}, elicited no significant depletions in cellular GSH (all p values>0.05), at all concentrations and time points investigated (figure 5.3). Although no significant findings were found in C3A cells exposed to ufCB, at the 6 hour time point a slight depletion of GSH was observed, with an apparent recovery of GSH levels to control levels at 24hours (figure 5.3A). It was apparent that exposure to QD621 elicited a dose and time dependent decrease in GSH levels within C3A cells, with the maximal depletion of GSH evident after a 24 hour exposure (figure 5.3E). Accordingly, a significant decrease in GSH levels were induced by QD621 at the 24hour time point, when treated with concentrations of 0.18 $\mu g/mm^2$ ($p<0.05$), and 0.35$\mu g/mm^2$ ($p<0.05$). In addition, at the 6 hour time point it was evident that there was a tendency for a dose dependent depletion of GSH levels when C3A cells were exposed to QD621, as GSH levels decreased from 0.155±0.06 GSH µMoles/mg protein in the control group to 0.069 ±0.03 GSH µMoles/mg protein in cells exposed to 0.35 $\mu g/mm^2$ QD621 (figure 5.3E).

The impact of serum presence, within the cell culture medium in which the particles were suspended was investigated. It was observed that GSH levels were generally higher in serum depleted cells. This is exemplified by the finding that at the 2 hour time point, control cells had a GSH concentration of 0.184±0.06 µMoles/mg protein in the presence of serum, whereas, in the absence of serum GSH levels were 0.324± 0.03 µMoles/mg protein. Furthermore it is important that the pattern of response remained consistent within the two exposure scenarios, so that regardless of serum presence there was a tendency for ufCB to induce a time and dose dependent depletion of GSH (figures 5.3A and G).


0.5
0.4
0.3
0.2
0.1
0
GSHµMoles/mg protein
2 hour
4 hour
6 hour
24 hour
Time

A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0.4
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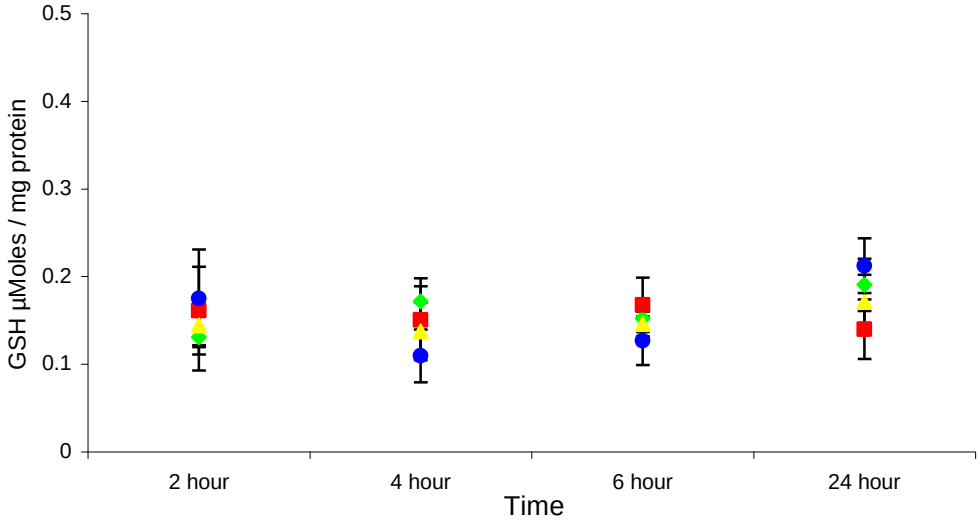
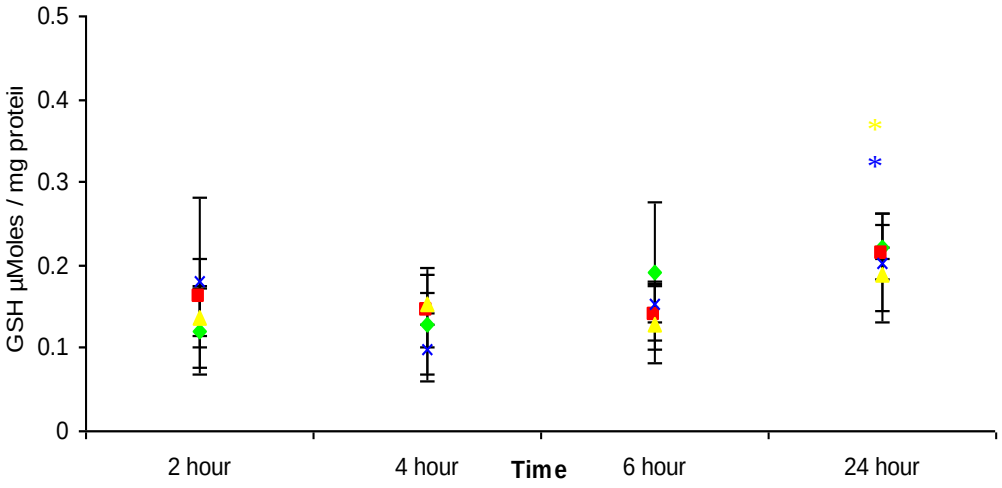
0.2
0.1
0
GSH µMoles/mg protein
2 hour
4 hour
6 hour
24 hour
Time

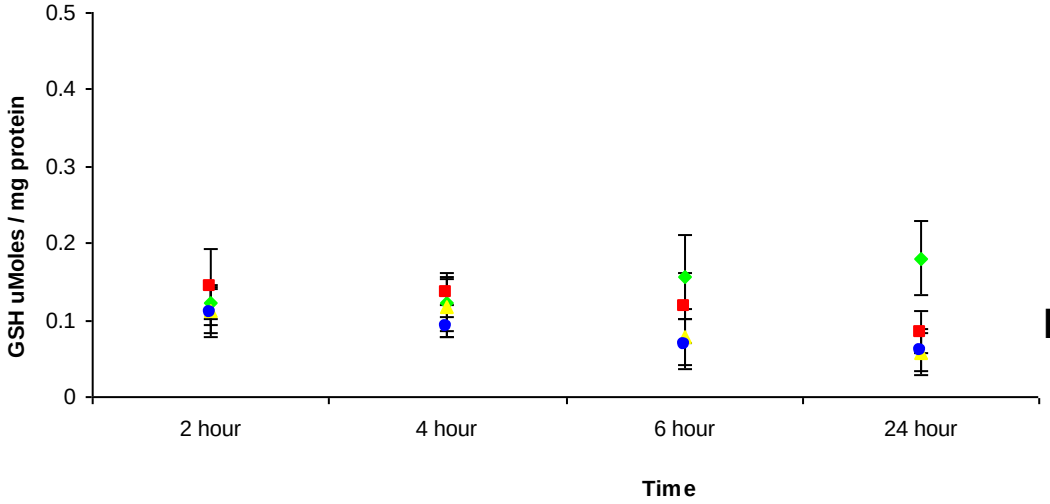
B


0.5
0.4
0.3
0.2
0.1
0
GSH µMoles / mg protein
2 hour
4 hour
6 hour
24 hour
Time

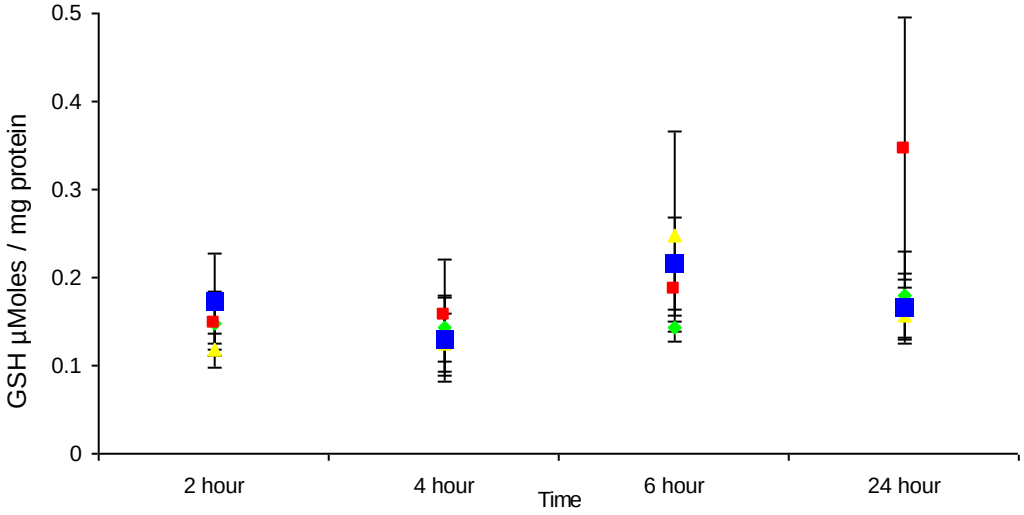
C


0.5
0.4
0.3
0.2
0.1
0
GSH µMoles / mg proteii
2 hour
4 hour
Time
6 hour
24 hour

D


0.5
0.4
0.3
0.2
0.1
0
GSH uMoles / mg protein
2 hour
4 hour
6 hour
24 hour
Time

E


0.5
0.4
0.3
0.2
0.1
0
GSH µMoles / mg protein
2 hour
4 hour
Time
6 hour
24 hour

F

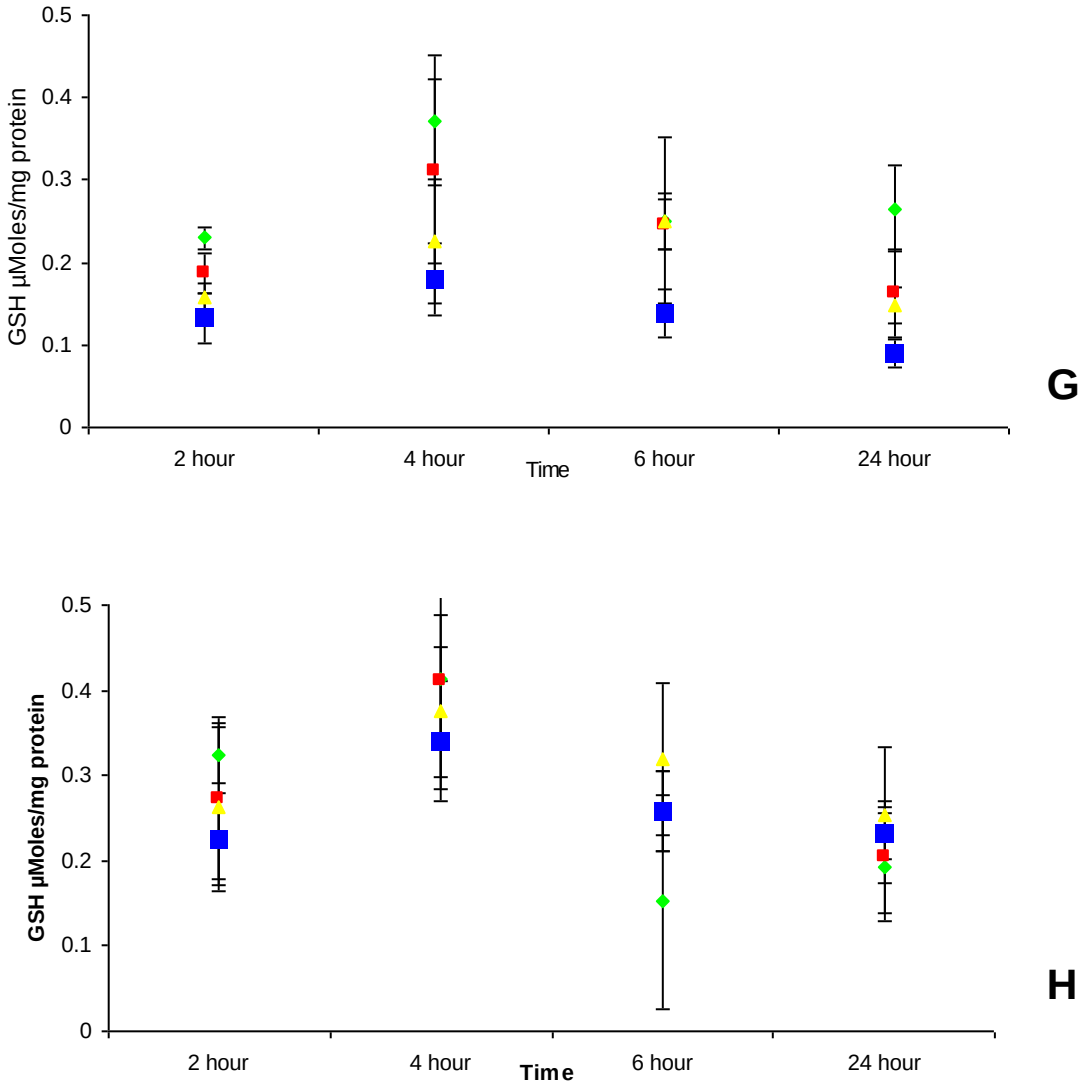
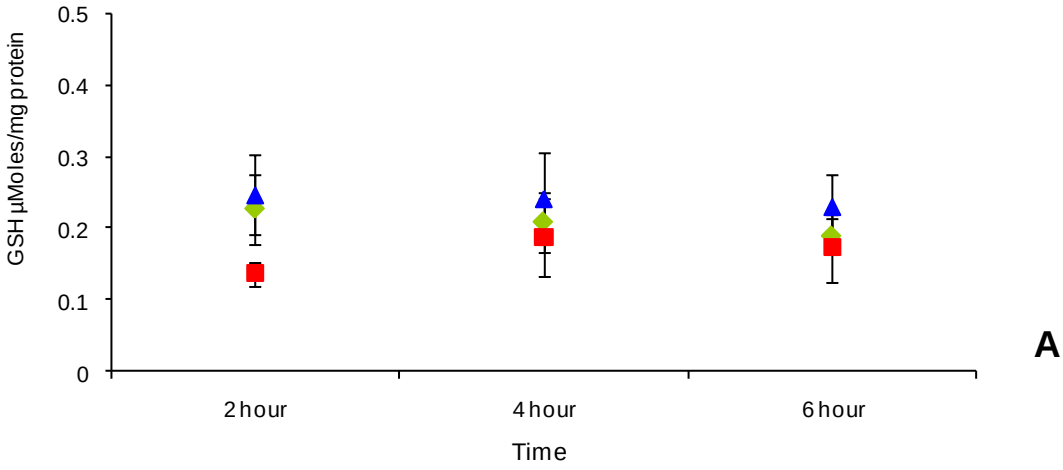


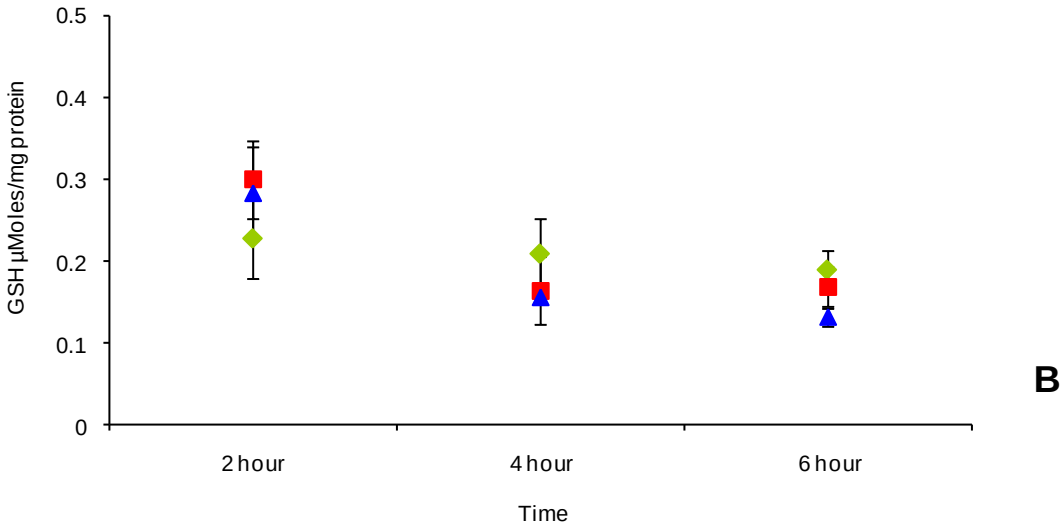
Figure 5.3 C3A cell GSH content after exposure to PARTICLE_RISK particles. C3A cells were treated with cell medium (control), 0.04, 0.18 or 0.35 µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**), ufCB w/o serum (**G**), or CB w/o serum (**H**) for 2,4,6 or 24 hours. Values represent mean ± SEM (n=3), significance indicated by * = p<0.05, ** = p<0.01 when particle treatments are compared to the control group.

5.3.1.2 The impact of NP exposure on IRHC GSH levels

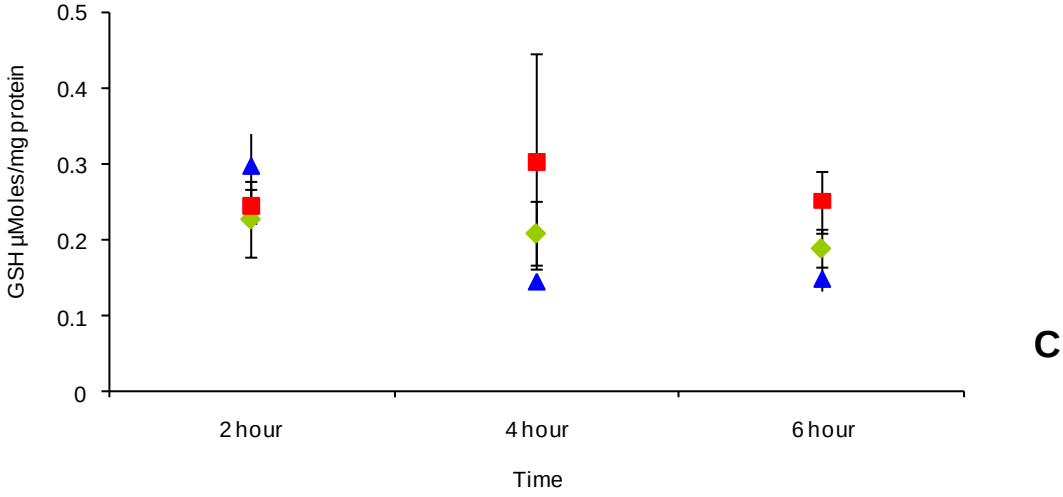
It was observed that no significant decreases in GSH were associated with the exposure of hepatocyte couplets to ufCB, CB, CNT, C_{60}, QD621 or QD620, at all concentrations and time points (figure 5.4). However, there was a tendency for the exposure of hepatocyte couplets to QD621 to decrease GSH levels, for example GSH levels were found to decrease from 0.189±0.03 GSH µMoles/mg protein in the control group to 0.14±0.003 GSH µMoles/mg protein in hepatocyte couplets exposed to 0.08µg/mm^2 for 6 hours, but this finding was not significant.


GSH µMoles/mg protein
0.5
0.4
0.3
0.2
0.1
0
2 hour
4 hour
6 hour
Time

A


GSH µMoles/mg protein
0.5
0.4
0.3
0.2
0.1
0
2 hour
4 hour
6 hour
Time

B


GSH µMoles/mg protein
0.5
0.4
0.3
0.2
0.1
0
2 hour
4 hour
6 hour
Time

C

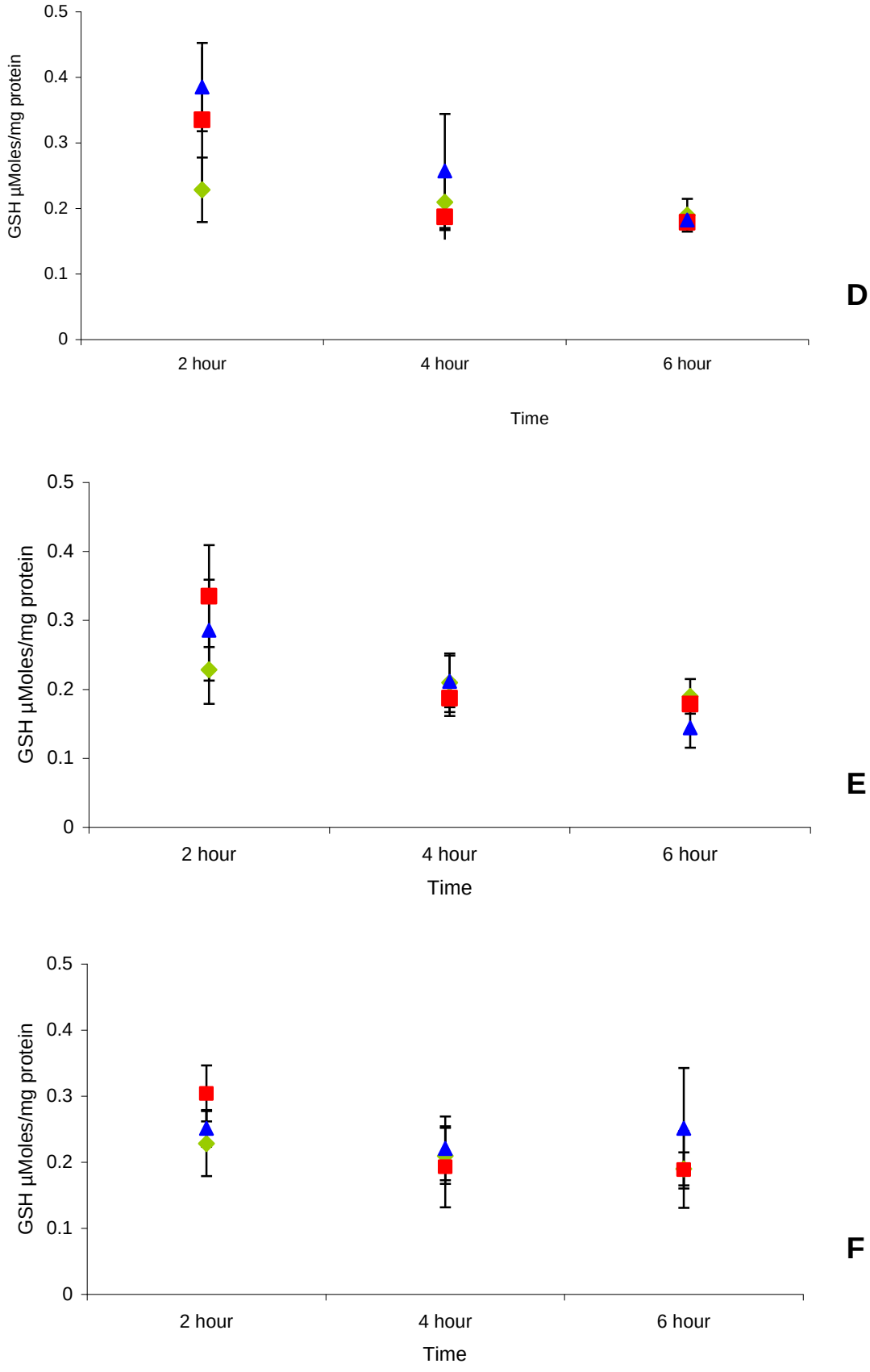
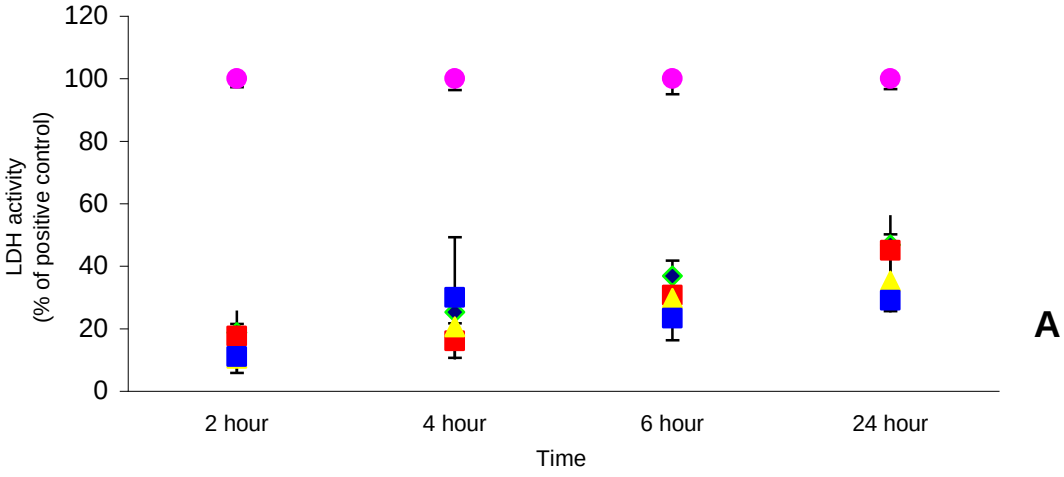


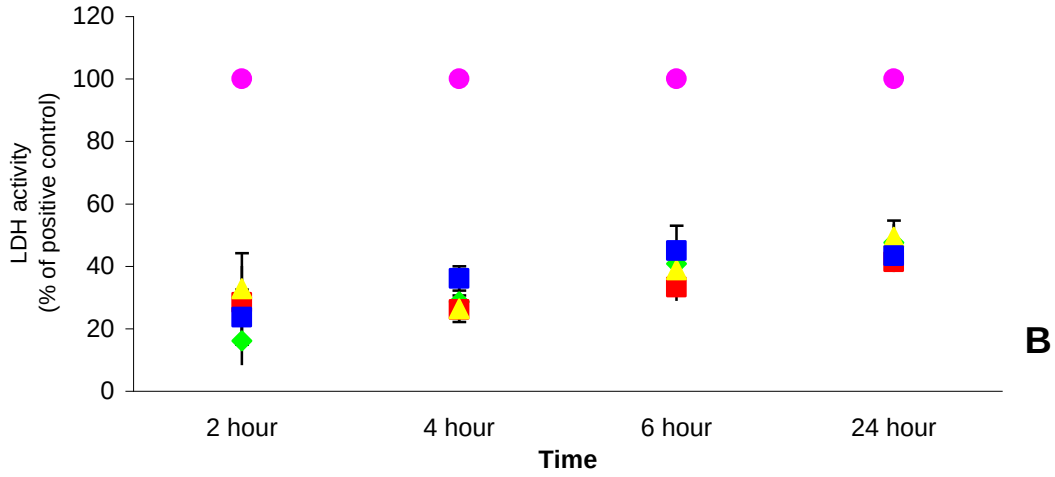
Figure 5.4 Hepatocyte couplet GSH content after exposure to PARTICLE_RISK particles. Hepatocyte couplets were treated with cell medium (control), 0.04, or 0.08 µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**), for 2,4, or 6 hours. Values represent mean ± SEM (n=3).

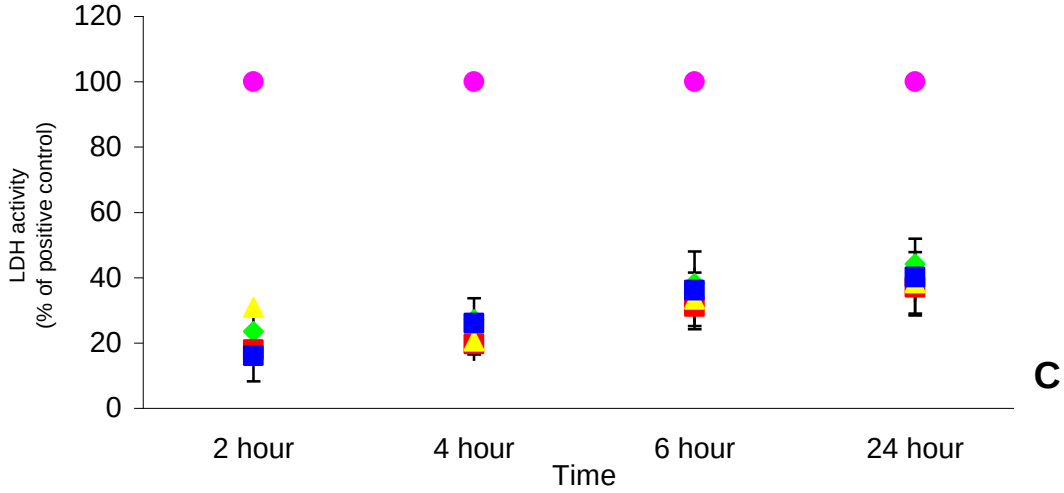
5.3.2 Impact of NP exposure on hepatocyte viability

5.3.2.1 Impact of NP exposure on C3A hepatocyte viability, using LDH release as an indicator

On treatment of C3A cells with ufCB, CB, CNT, and C_{60}, at all concentrations and time points investigated no significant increases in LDH release from C3A cell were apparent which is evident in figure 5.5. QD621 caused the greatest extent of cell death, with significant levels of LDH release observed when cells were exposed to QD621 at all concentrations for 24 hours, when compared to the control. At 6 hours, a significant increase in LDH release was observed in cells treated with 0.18µg/mm^2 QD621 ($p=0.0011$), with a tendency for an increase in LDH activity evident in cells treated with 0.35µg/mm^2 QD621 as 68±7% of cells were dead, compared with 35±5% in the control group. It was demonstrated that QD620 induced cell death after a 24 hour exposure time, for 0.18µg/mm^2 ($p<0.001$) and 0.35 µg/mm^2 ($p<0.001$) treatments, with a tendency for the 0.04 µg/mm^2 treatment to produce a similar effect ($p=0.0562$).


120
100
80
60
40
20
0
LDH activity
(% of positive control)
2 hour
4 hour
6 hour
24 hour
Time
A


120
100
80
60
40
20
0
LDH activity
(% of positive control)
2 hour
4 hour
6 hour
24 hour
Time
B


120
100
80
60
40
20
0
LDH activity
(% of positive control)
2 hour
4 hour
6 hour
24 hour
Time
C

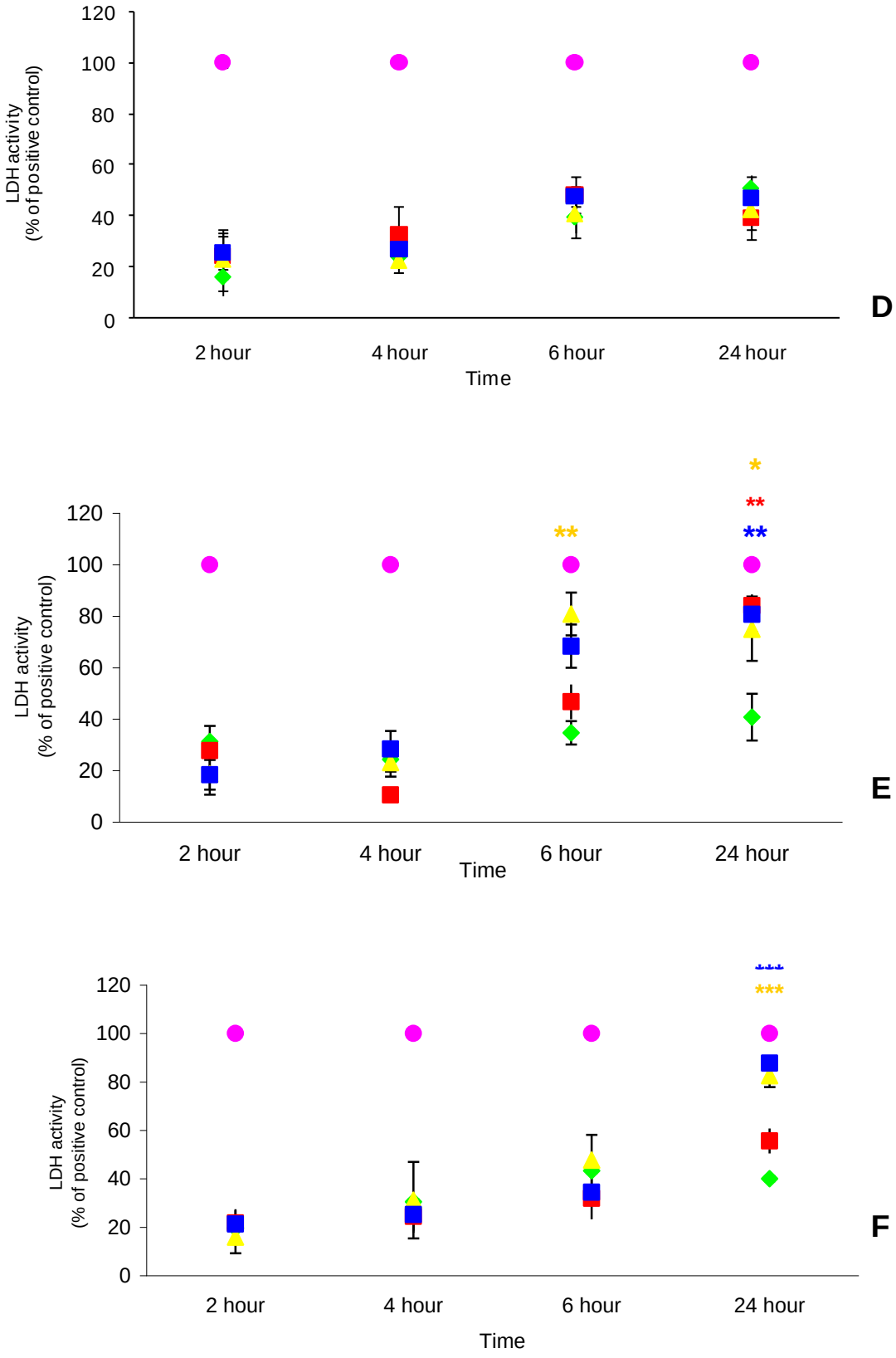


Figure 5.5 C3A hepatocyte LDH release after exposure to the PARTICLE_RISK panel of particles. C3A cells were treated with cell medium (control), 0.04, 0.18 or 0.35 µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**), or triton X-100 (positive control) for 2,4,6 and 24 hours and LDH activity measured. Results are expressed as a % of the positive triton control. Values represent mean ± SEM (n=4), significance indicated by * = p<0.05, ** = p<0.01 *** = p<0.001 when particle treatments were compared to the control group.

5.3.2.2 LDH adsorption onto the surface of NPs

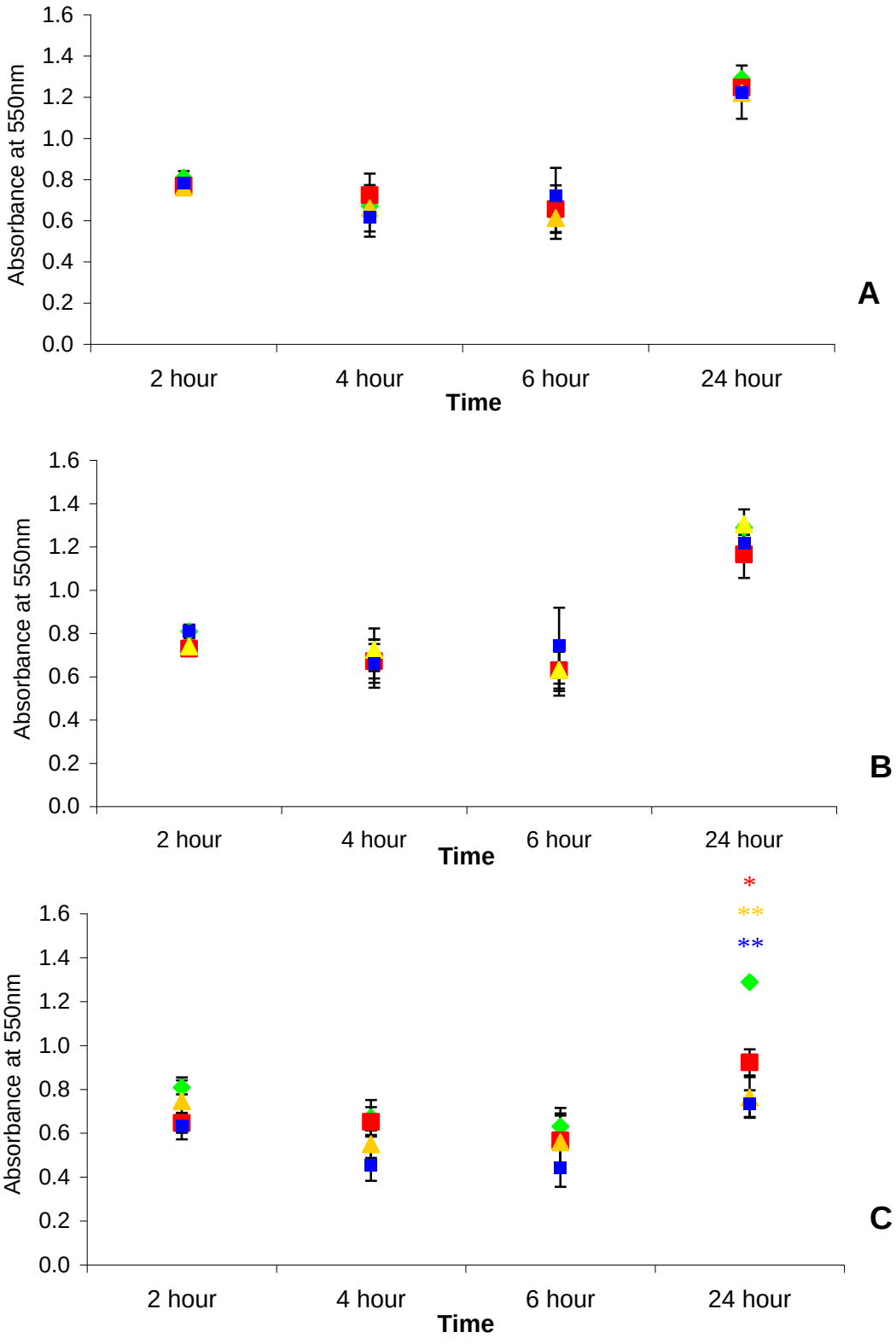
Adsorption of LDH onto the particle surface was investigated, as if this occurred, the activity of LDH from samples would be underestimated and therefore inaccurate results would be collected. Within the experimental protocol used, cellular LDH was released by Triton-X100 exposure and then incubated with particles. Therefore if LDH adsorbed onto the surface of particles then LDH activity would be lower than expected, so that it would not be equivalent to Triton-X100 treated cells. It was found that there was a loss of LDH activity in the presence of ufCB, so that LDH may adsorb onto the surface of ufCB ($p=0.015$, table 5.1). LDH did not bind to the particle surface of all other PARTICLE_RISK particles ($p>0.05$), as LDH activity was equivalent to the Triton-X100 treatment (table 5.1.).

Table 5.1 Determining the adsorption of LDH onto the surface of PARTICLE_RISK particles. C3A cells were exposed to 0.1% Triton-X100 to release all cellular LDH. The PARTICLE_RISK particles were then suspended in the LDH rich supernatants, at a concentration of 0.35µg/mm^2, and incubated for 4 hours at 37^{o}C in a fresh 24 well plate, and LDH activity determined. The LDH activity of the particle treatments were compared to the Triton-X100 supernatants. Values represent mean ± SEM (n=3), significance indicated by *=$p<0.05$.

Particle	LDH activity (units/ml)
Control	1942.74 ± 5.7
ufCB	1907.9 ± 0.57*
CB	1941 ± 3.6
CNT	1934.5 ± 5.8
C60	1938.7 ± 1.5
QD621	1923.932 ± 9.2
QD620	1936.9 ± 1.3

5.3.2.3 Impact of NP exposure on C3A hepatocyte viability, assessed using the MTT assay

Within the control cells, it was observed that there was an increase in MTT absorbance from the 6 hour to 24 hour time point, which is indicative of cell proliferation over the incubation time (figure 5.6). It was evident that no decreases ($p>0.05$) in mitochondrial function (relative to the control) were observed when C3A cells were exposed to ufCB, CB, and C_{60} at all concentrations, and time points investigated (figures 5.6A, B and D). When cells were exposed to CNTs, there was a dose dependent decrease in cell viability at 24 hours (indicated by a decrease in absorbance, figure 5.6C), with a significant cytotoxic effect evident at all concentrations investigated ($p<0.05$). QD620 were able to induce a cytotoxic effect when exposed to C3A cells for 4 (for all concentrations, $p<0.05$), and 24 ($p<0.001$ for all concentrations investigated) hours, when compared to the control group. QD621 were able to induce the greatest reduction in MTT activity within C3A cells. Significant decreases in cell viability were observed at 4 ($p<0.01$ for all concentrations investigated), 6 [0.18µg/mm^2 ($p=0.0196$), 0.35µg/mm^2 ($p=0.03$)], and 24 ($p<0.001$ for all concentrations investigated) hours when cells were exposed to QD621.


Absorbance at 550nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2 hour
4 hour
6 hour
24 hour
Time
A
B
C
*
**
**

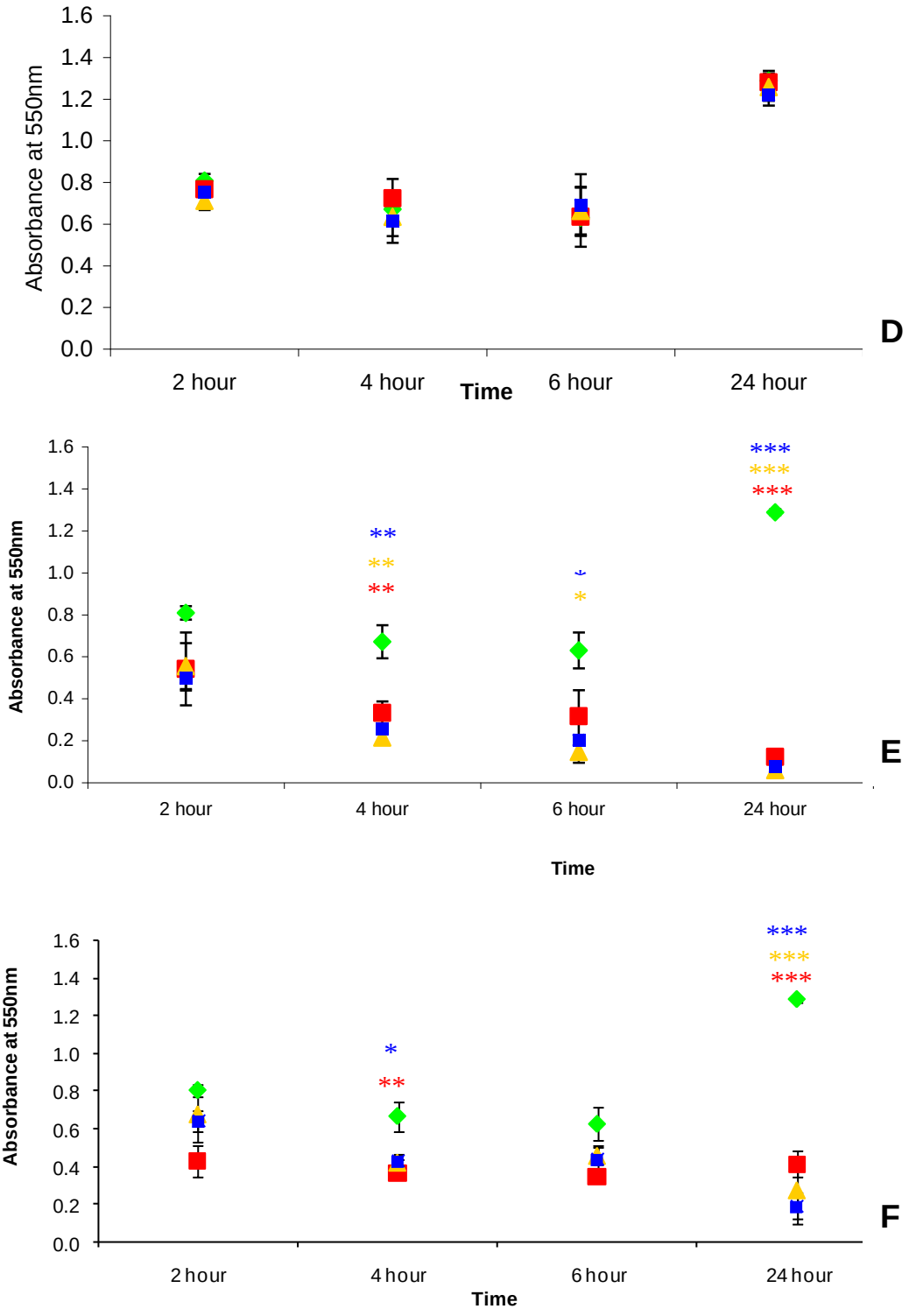


Figure 5.6 C3A hepatocyte MTT activity after exposure to the PARTICLE_RISK panel of particles. C3A cells were exposed to cell medium (control), 0.04, 0.18 or 0.35 µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) or QD620 (**F**), for 2,4,6 and 24 hours and MTT activity measured. MTT activity was quantified by absorbance, as it is representative of the amount of coloured product that is formed due to the activity of mitochondria within viable cells. Values represent mean ± SEM (n=3), significance indicated by * = p<0.05, ** = p<0.01 *** = p<0.001 when particle treatments are compared to the control group.

5.3.2.4 Investigating the ability of NPs to interfere with the MTT assay

To determine if the particles contained within the PARTICLE_RISK panel were able to interfere with the MTT assay, a number of 'control' experiments were performed. If particles were able to bind to the MTT reagent, to prevent its participation within the assay, a decrease in absorbance (at the wavelength that MTT activity was detected) would be expected when particles and the MTT reagent were added to cells at the same time compared to cells treated with MTT reagent but not particles (experiment 1). Alternatively if particles were capable of attaching to the MTT product, a decrease in absorbance (at the wavelength that MTT activity is detected) would be expected when particles were added at the same time as propanol which was used to solubilse the MTT product (experiment 2). Therefore by subtracting the absorbance readings of the 'control' experiments from those obtained from the 'normal' MTT assay (that followed the protocol used to assess the impact of particle exposure on hepatocyte viability, see section 5.2.6.2) it was possible to identify any changes in absorbance caused by particles within the different experimental set ups (relative to the control within each experiment). It was found that ufCB and QDs were able to significantly decrease ($p<0.05$) the absorbance readings within experiment 1, thus suggesting that these particles were able to bind to the MTT reagent, and prevent its participation within the MTT assay (table 5.2). It was also evident that QDs were able to cause an increase in absorbance, when they were added at the same time as propanol ($p<0.05$, table 5.2), so that these NPs do not appear to bind to the MTT product, but perhaps encourage its solubilsation. There was no differences in absorbance observed within the different experimental set ups, in the absence of cells (data not shown).

The absorbance of particles (at the wavelength used to detect the coloured MTT product formation) was also determined, to evaluate the ability of particles to contribute to the observed MTT activity. It was found that ufCB, CB and CNTs were able to contribute to absorbance readings ($p<0.05$). The other particles within the panel were unable to contribute to the absorbance reading.

Table 5.2 Determining the interference of the PARTICLE_RISK panel within the MTT assay. MTT absorbance readings (at 550nm) in the presence of cells, using different controls to assess the interference of NPs within the MTT assay. Experiment one determined the ability of particles to bind to the MTT reagent (by simultaneously incubating the particles and MTT reagent with the cells). Experiment two determined if particles were able to contribute to the solubilisation of the MTT product. The absorbance of particle suspensions was also evaluated. Data represents mean +/- SEM. Significance indicated by *=p<0.05.

Treatment	Normal MTT assay	experiment 1	experiment 2	NP absorbance
Medium only	0.426 ± 0.016	0.392 ± 0.009	0.468 ± 0.008	0.185 ± 0.01
ufCB	0.401 ± 0.005	0.370 ± 0.01*	0.544 ± 0.03	0.362 ± 0.011*
CB	0.426 ± 0.004	0.376 ± 0.01	0.520 ± 0.03	0.303 ± 0.166*
CNT	0.310 ± 0.002	0.299 ± 0.011	0.509 ± 0.06	0.454 ± 0.005*
C_{60}	0.408 ± 0.016	0.384 ± 0.022	0.468 ± 0.008	0.202 ± 0.004
QD621	0.296 ± 0.005	0.139 ± 0.003*	0.54 ± 0.01*	0.200 ± 0.012
QD620	0.432 ± 0.014	0.196 ± 0.009*	0.437 ± 0.034*	0.260 ± 0.012

5.3.2.5 Impact of NP exposure on IRHC viability

It was found that no decreases in mitochondrial function (p values > 0.05, relative to the control) were observed when primary hepatocyte couplets were exposed to all particle types, at all concentrations and time points investigated, (figure 5.7). However it was apparent that exposure to 0.08µg/mm^2 QD621 revealed a tendency for a decrease in cell viability at 2, and 4 hours. This is exemplified by the finding that absorbance decreased from 0.220±0.05 in control cells to 0.103±0.03 in hepatocyte couplets exposed to 0.08µg/mm^2 QD621 for 4 hours, but this was not significant.

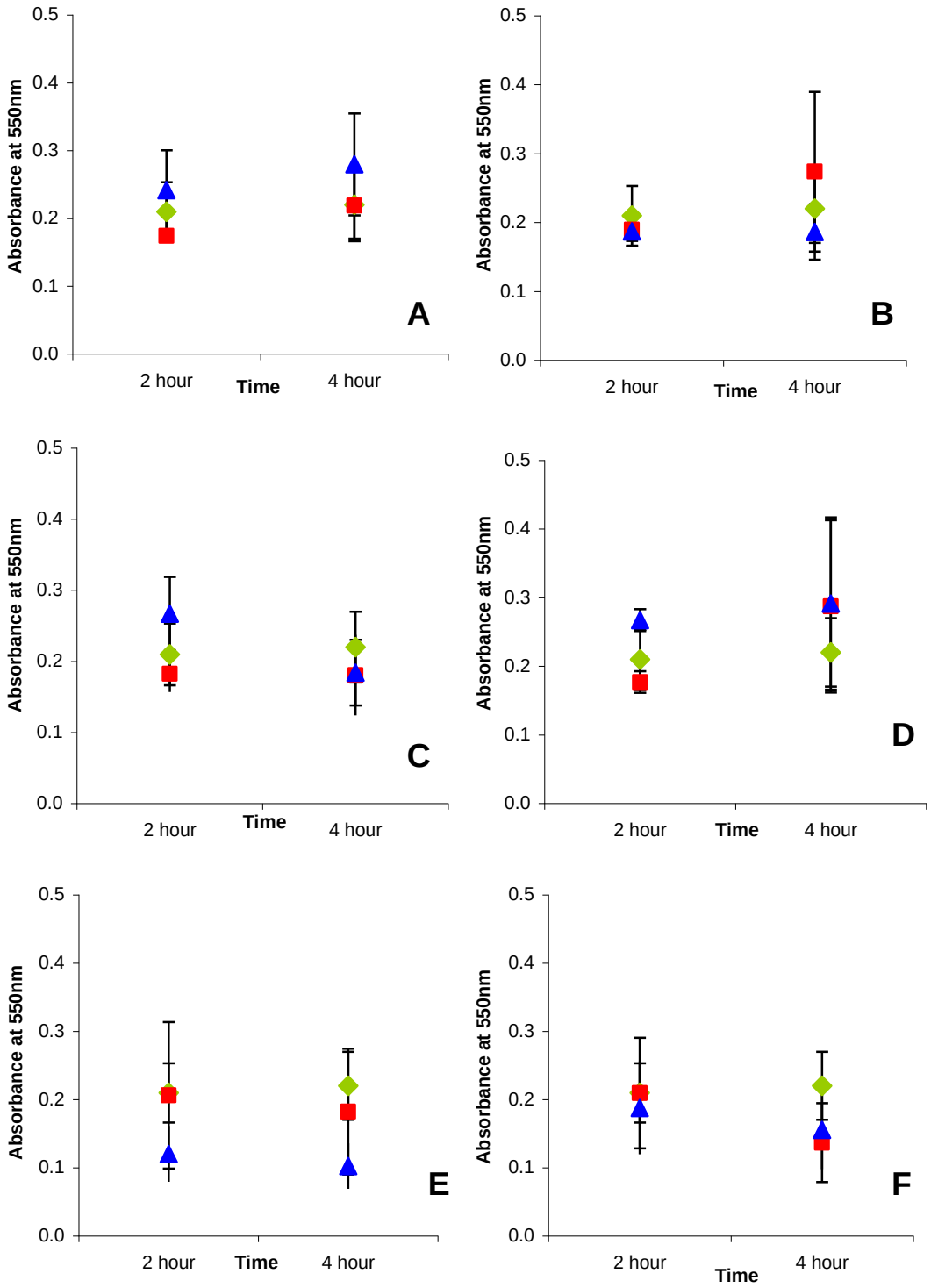


Figure 5.7 Hepatocyte couplet MTT activity after exposure to the PARTICLE_RISK panel of particles. C3A cells were treated with cell medium (control), 0.04, or 0.08 µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) or QD620 (**F**), for 2 or 4 hours and MTT activity measured. The greater the absorbance the greater the mitochondrial activity, therefore a decrease in absorbance is suggestive of cell death. Values represent mean ± SEM (n=3).

5.3.3.1 Impact of NP exposure on C3A hepatocyte cytokine production

Changes in cytokine production, as a consequence of particle exposure, were assessed within the cell supernatants of exposed hepatocytes and determined by multiplex and ELISA analysis. When considering the observations of the multiplex analysis it was evident that out of the 17 cytokines that were looked at within the assay, that only IL-8 production was stimulated by particle exposure, with no increases in the generation of the other cytokines measured. Therefore to confirm the finding that IL-8 levels were increased, conventional ELISA analysis was completed.

IL-8 levels were lower that the detection limit within the 2 and 4 hour time points, in all treatment groups (data not shown). It was observed that ufCB, CB, CNT, and C_{60} were unable to elicit an increase in IL-8 production by C3A cells, at 6 or 24 hours (figures 5.8A-D). However there was a tendency for the carbon based particles to reduce IL-8 levels to lower than control levels when exposed to high particle concentrations, as for example at 24 hours IL-8 production by control cells was 276±77 pg/ml, and 127±15pg/ml in cells exposed to 0.08µg/mm^2 ufCB (figure 5.8A). It was apparent that there was a trend for QD621 exposure to induce an increase in IL-8 production, at a low concentration, as exposure of cells to a concentration of 0.04µg/mm^2 QD621 for 24 hours increased IL-8 levels from 276±77pg/ml to 380±53pg/ml, however these results did not reach significance (figure 5.8E). It was evident that higher concentrations of QD621 reduced IL-8 levels to lower than that of the controls at 24 hours, for example IL-8 levels decreased from 276±77pg/ml in the control group to 54±32pg/ml at a concentration of 0.35µg/mm^2 µg/ml, but this was not a significant finding (figure 5.8E). QD620 caused a dose and time dependent increase in IL-8 production by C3A cells (figure 5.8F). It was apparent that exposure of C3A cells to 0.18µg/mm^2 ($p=0.0204$) and 0.35µg/mm^2 ($p=0.0212$) QD620 particles for 24 hours caused a maximal increase in IL-8 production. There was a trend for QD620 exposure (0.35µg/mm^2) to increase IL-8 production at 6 hours as IL-8 levels increased from 41±9 in the control group to 343±284 (figure 5.8F).

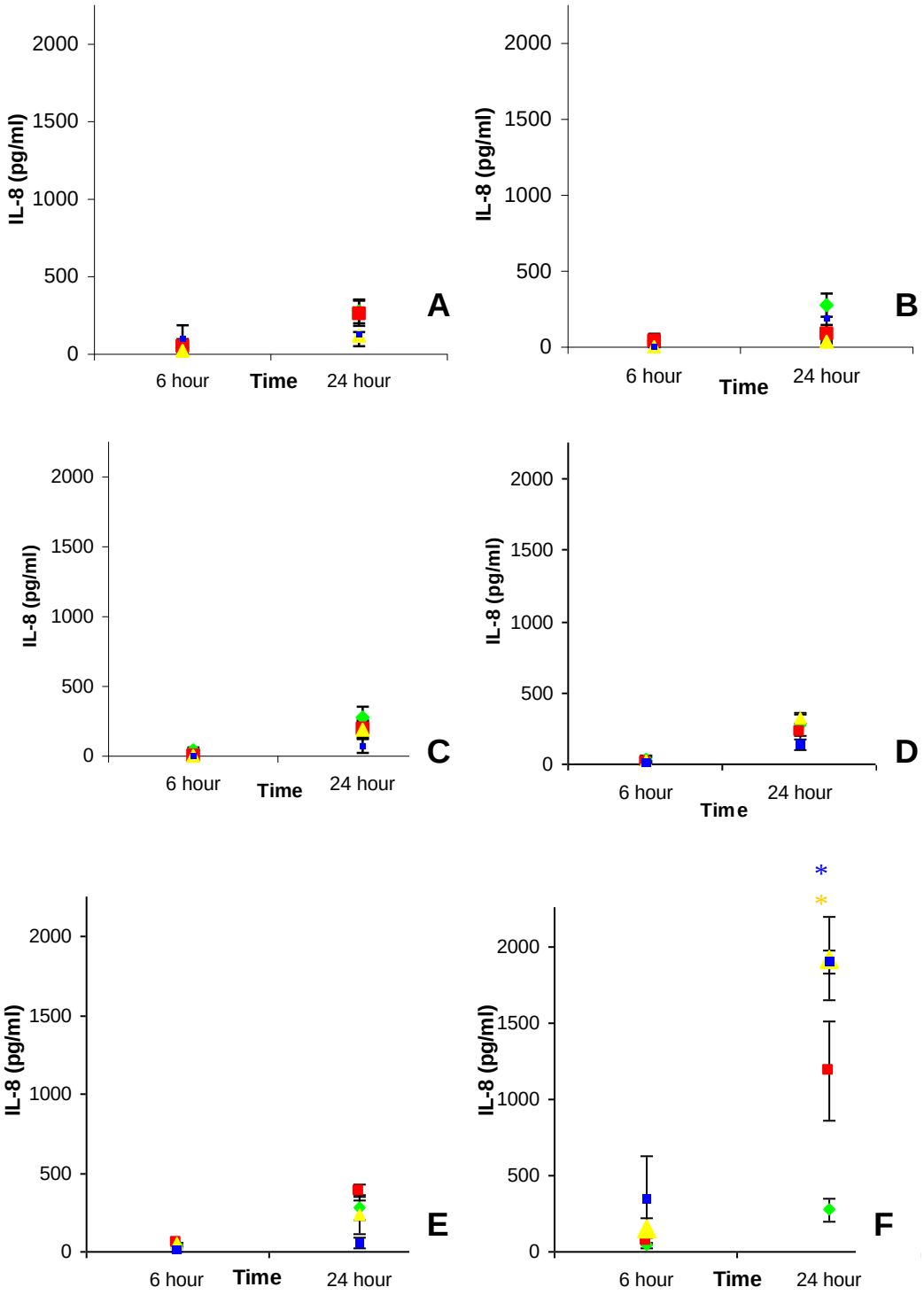
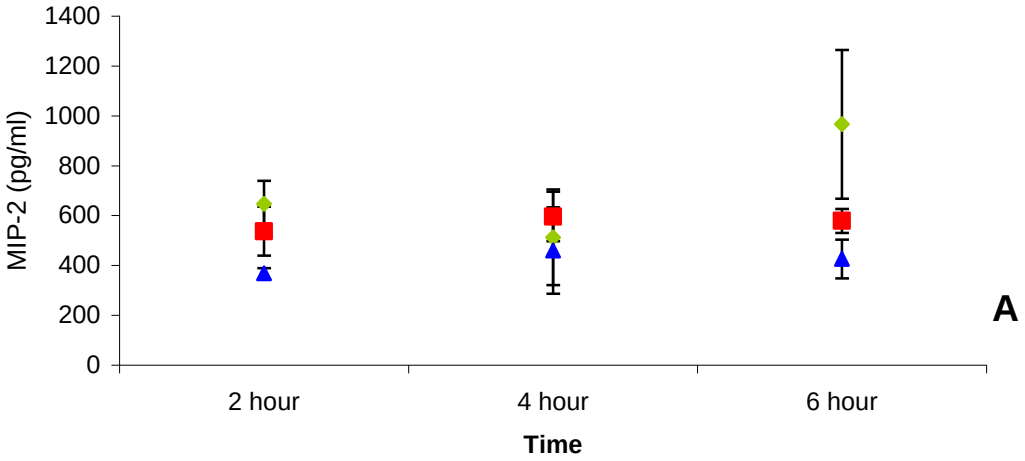


Figure 5.8 IL-8 release from C3A hepatocytes after exposure to the PARTICLE_RISK particle panel. C3A cells were treated with cell medium (control), 0.04, 0.18 or 0.35µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) or QD620 (**F**), for 6 and 24 hours and IL-8 production within the cell supernatants was measured by ELISA. Values represent mean ± SEM (n=3), significance indicated by * = $p<0.05$, when particle treatments are compared to the control group.

5.3.3.2 Evaluating NP induced MIP-2 production by IRHCs

Within the multiplex kit for analysing cytokine production within hepatocyte couplet supernatants, the IL-8 rodent equivalent MIP-2, was not included within the cytokine analysis. However as IL-8 production was increased in cell lines, in response to a number of particle treatments, MIP-2 production was also assessed within the supernatants of particle exposed hepatocyte couplets. It was evident that exposure of hepatocyte couplets to all particles within the PARTICLE_RISK panel elicited no significant increases in MIP-2 release (all p values>0.05), so that MIP-2 release was equivalent to control levels (figure 5.9). However there was a tendency for QD621 exposure (0.04 and 0.08µg/mm^2) to reduce MIP-2 levels to lower than that of the controls at the 6 hour time point (figure 5.9E).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MIP-2 (pg/ml)
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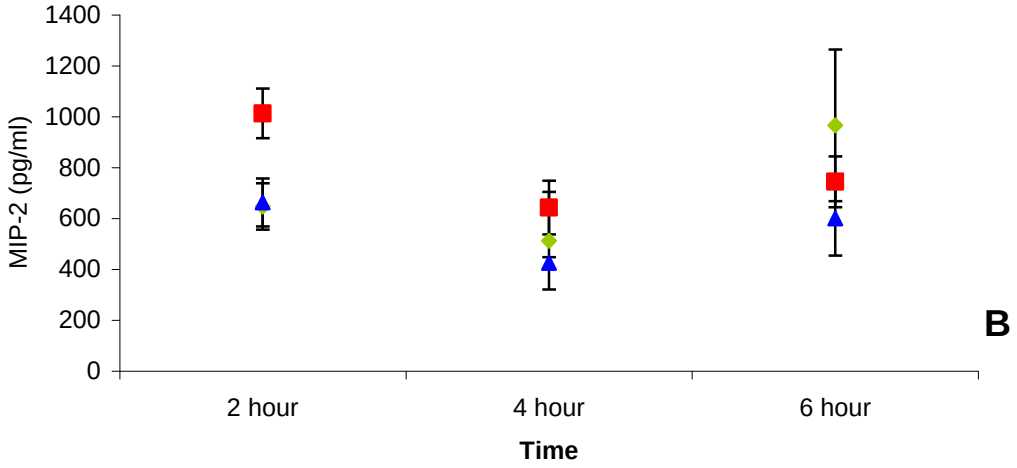
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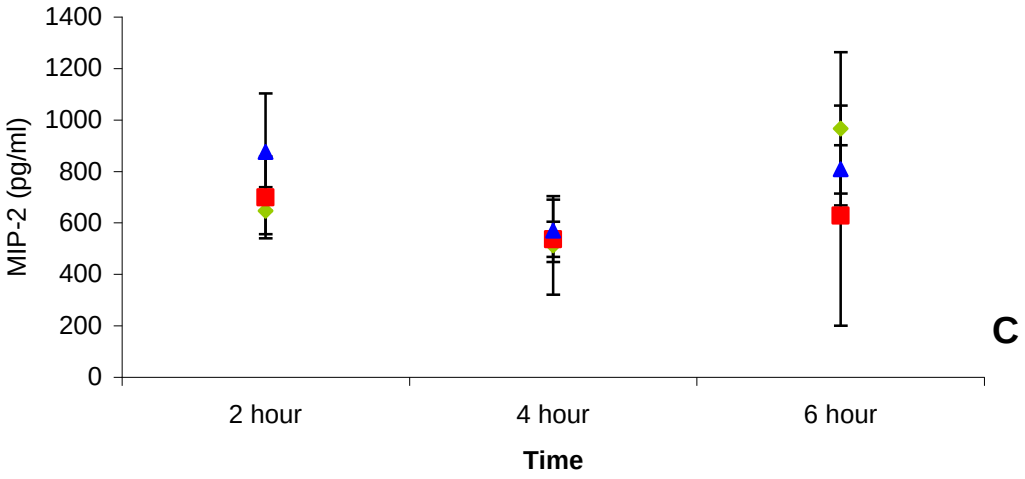
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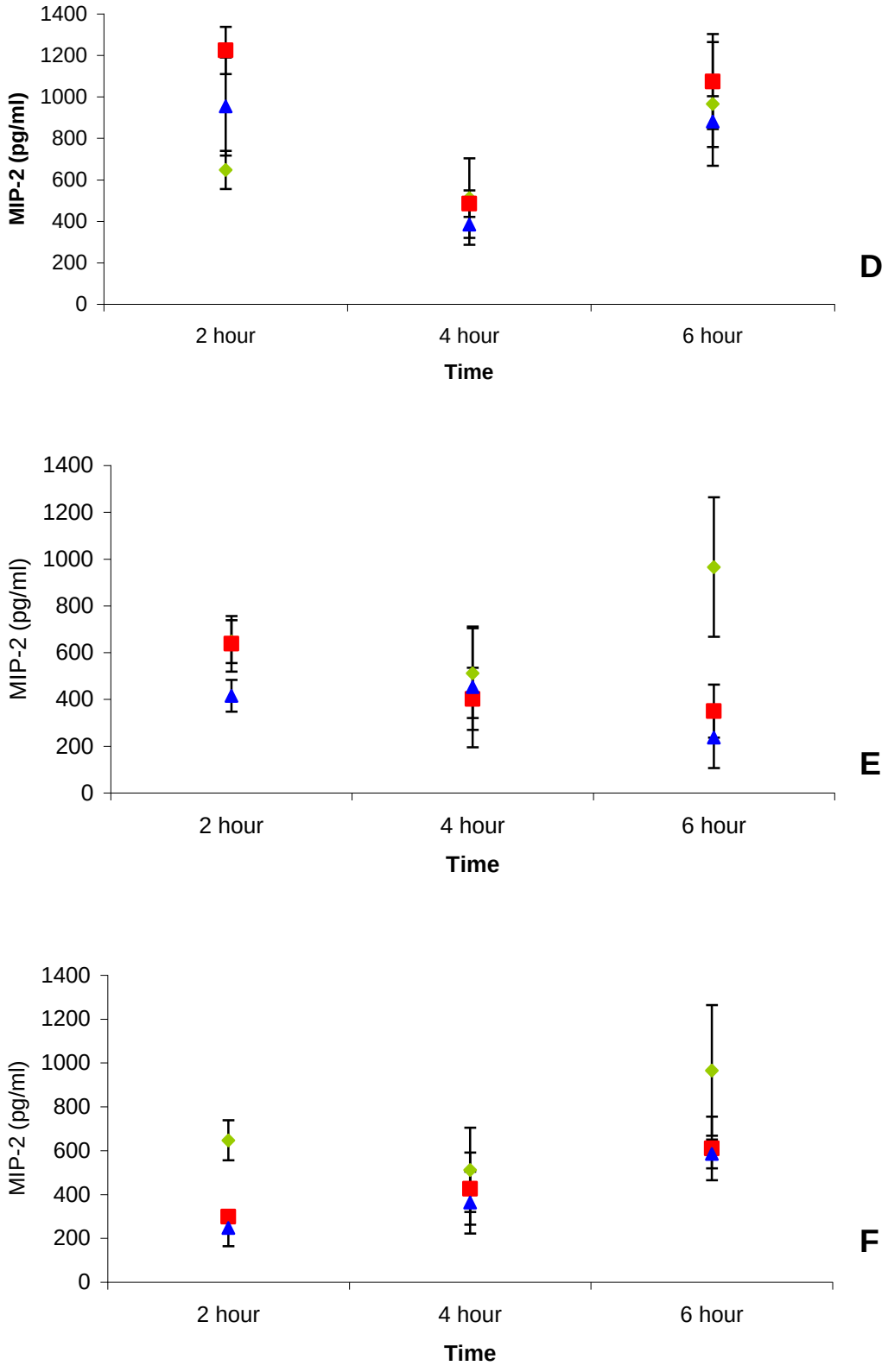


Figure 5.9 PARTICLE_RISK particle induced MIP-2 release from hepatocyte couplets. Isolated rat hepatocyte couplets were treated with cell medium (control), 0.04 or 0.08 µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) or QD620 (**F**), for 2,4, or 6 and MIP-2 production within the cell supernatants determined by ELISA. Values represent mean ± SEM (n=3)

5.3.4 Impact of NPs on bile secretion

It was observed that none of the PARTICLE_RISK particles were able to detrimentally affect bile secretion, when exposed to cells for 30 minutes, at a concentration of 0.04µg/mm^2, so that the percentage of couplets that were able to accumulate CLF (CVA of CLF), was equivalent to that of the control cells (figure 5.10A). It was found that CB, CNT, C_{60}, and QD620 did not have a negative impact on bile secretion when exposed to hepatocyte couplets for 2 hours (figure 5.10B). It was found that ufCB was able to significantly inhibit bile secretion when exposed to hepatocyte couplets (0.04µg/mm^2) for 2 hours ($p=0.033$), and that exposure of couplets to QD621 NPs was able to inhibit bile secretion to the greatest extent subsequent to a 2 hour (0.04µg/mm^2) exposure ($p=0.006$).

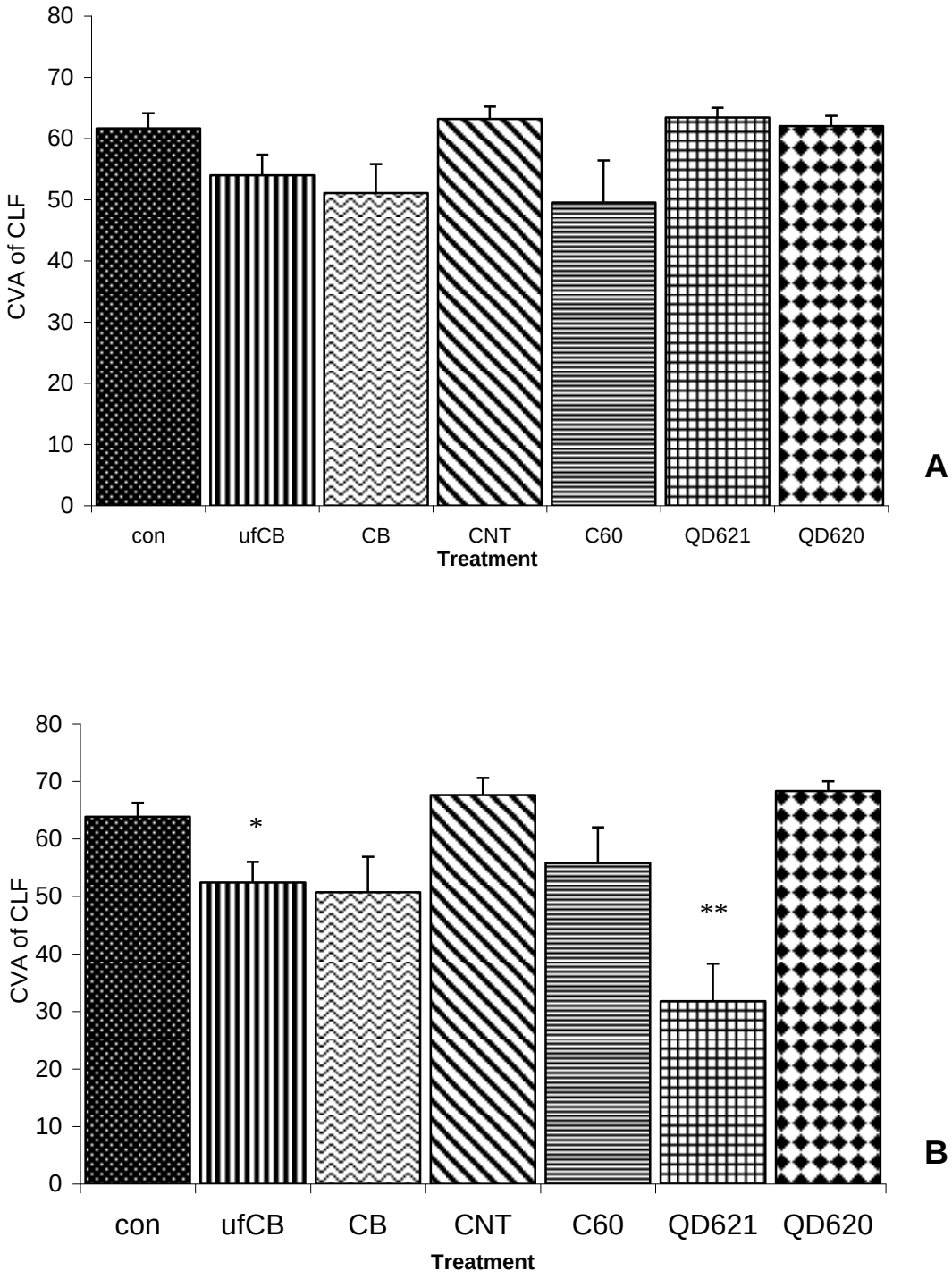


Figure 5.10 Effect of PARTICLE_RISK particles on bile secretion by IRHC. The CVA of CLF (% of couplets able to accumulate CLF within the bile canaliculus) after a 30 minute (**A**) or 2 hour (**B**) exposure time to 0.04µg/mm^{2}of each particle. Values represent mean ± SEM (n=3), significance indicated by * = p<0.05, ** = p<0.01 when particle treatments are compared to the control group.

5.3.5.1 Impact of NP exposure on IRHC morphology using SEM

SEM analysis revealed that control hepatocyte couplets exhibited a morphology which was characterised by round cells that had a smooth, intact plasma membrane (figure 5.11A). It was observed that cells treated with CB had a morphology that was similar to that to control cells (figure 5.11C). It was apparent that cells exposed to ufCB, CNT and C_{60} and QD620 had an increased incidence of bleb formation on the cell surface (figure 5.11), with blebs evident at a higher magnification in figure 5.11H. Exposure of hepatocyte couplets to QD621 caused the greatest extent of cell damage, so that cells had an irregular shape, with evidence of membrane integrity being compromised due to the development of 'holes' within the plasma membrane (figure 5.11F). It was also evident that there were fewer cells present within SEM samples of QD621 exposed cells, so that there were less cells available for imaging.

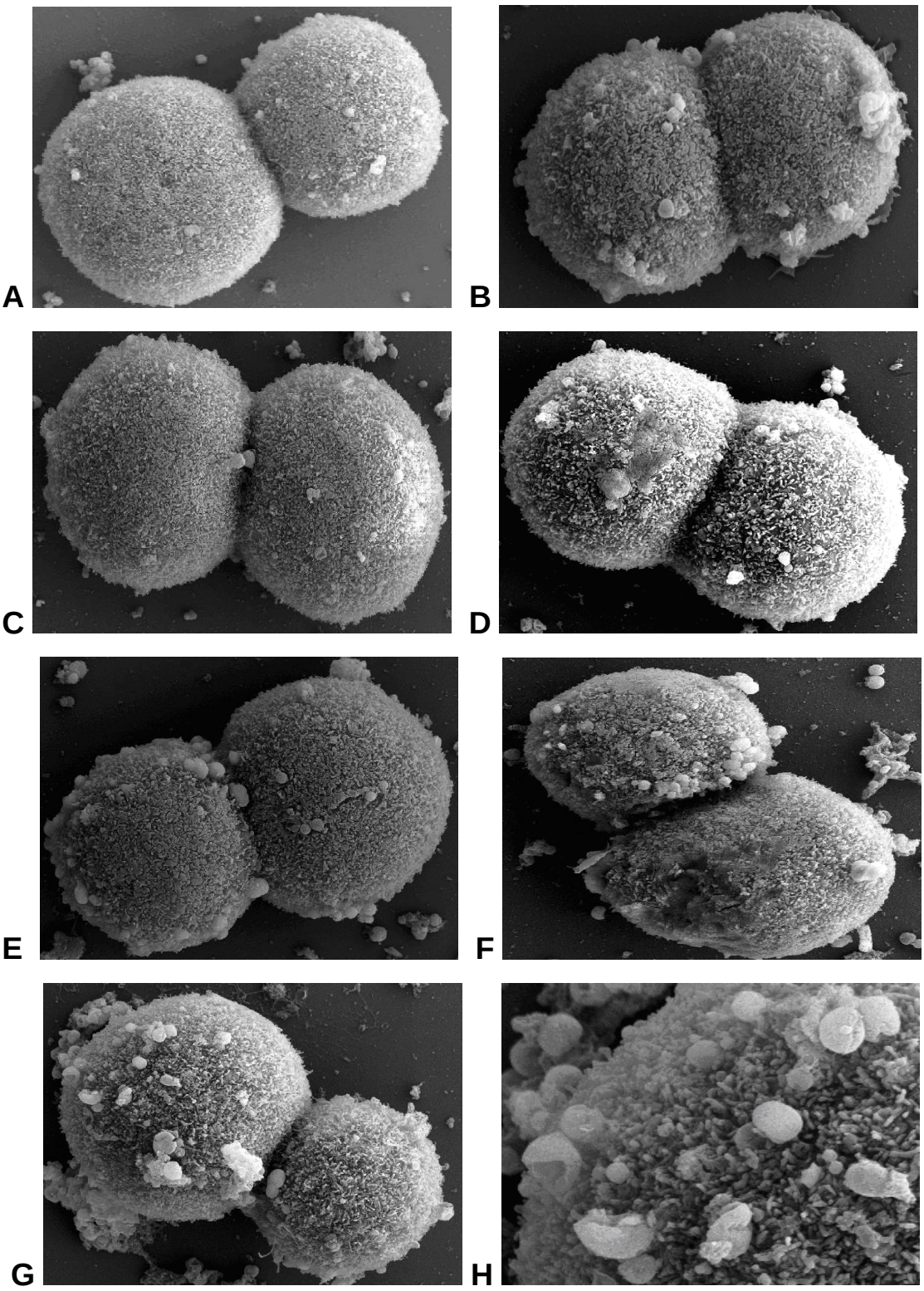


Figure 5.11 SEM analysis of IRHC morphology following exposure to the PARTICLE_RISK panel of particles. Hepatocyte couplets were exposed to L-15 (& 10% FCS) cell culture medium (control **A**), 0.04µg/mm^2 ufCB (**B**), CB (**C**), CNT (**D**), C_{60} (**E**), QD621 (**F**) or QD620 (**G**) for 4 hours. A more detailed image of bleb formation, associated with particle exposure can be visualised in image **H** (representative image taken from QD620 treated cells). Cells were imaged with a Hitachi S-4800 Type II Field Emission Gun-Scanning Electron Microscope.

5.3.5.2 Evaluating IRHC ultrastructure subsequent to NP exposure, using TEM

It was demonstrated that control cells analysed using TEM had a 'normal' cell ultrastructure, so that they had a large, round nucleus, the cytoplasm was filled with mitochondria, and the cells were round in appearance (figure 5.12A). TEM analysis of hepatocyte couplets revealed that the ultrastructure of CB, ufCB, CNT and C_{60} exposed cells was equivalent to that of the control cells (figure 5.12). It was evident that detail of the sub-cellular structure of CNT exposed cells was made difficult by the formation of creases within the TEM samples that arose as a consequence of the processing of cells for TEM analysis, and made it difficult to focus in detail the ultrastructure of cells (figure 5.12D). In QD621 and QD620 exposed cells, sub-cellular organelles such as mitochondria were no longer evident within the cell interior and it appeared that cells were filled with apoptotic bodies (figures 5.12E and F). Furthermore within QD621 exposed cells, the nuclei were no longer evident (figure 5.12E), and in QD620 treated cells, the nuclei were smaller than those contained within control cells (figure 5.12E).

Subsequent to exposure of hepatocyte couplets to the PARTICLE_RISK particle panel there were a number of observations that warranted further investigation using a higher magnification, which also allowed the uptake of particles to be assessed (figure 5.13). It was observed that black, particle-like compartments were found in control cells (figure 5.13A). It was apparent that ufCB was internalised by cells and contained within unknown structures (figure 5.13B). CNTs appeared to be contained within cytoplasmic vesicles within hepatocyte couplets, but that the cells had a normal ultrastructure morphology (figure 5.13D). It was also possible to observe C_{60} within the cell interior, where C_{60} particles were occasionally located within mitochondria or vesicle-like structures (figure 5.13E), however these structures were often similar in appearance to the artefacts or structures observed within control cells (figure 5.13A). QD621 treated cells appeared to contain apoptotic bodies (figure 5.12F) or large cytoplasmic vesicles (figure 5.13F) but under both circumstances the PM appeared to remain intact. Within QD620 exposed cells, apoptotic bodies were evident (figure 5.12G), and the PM and nucleus was intact (figure 5.13G).

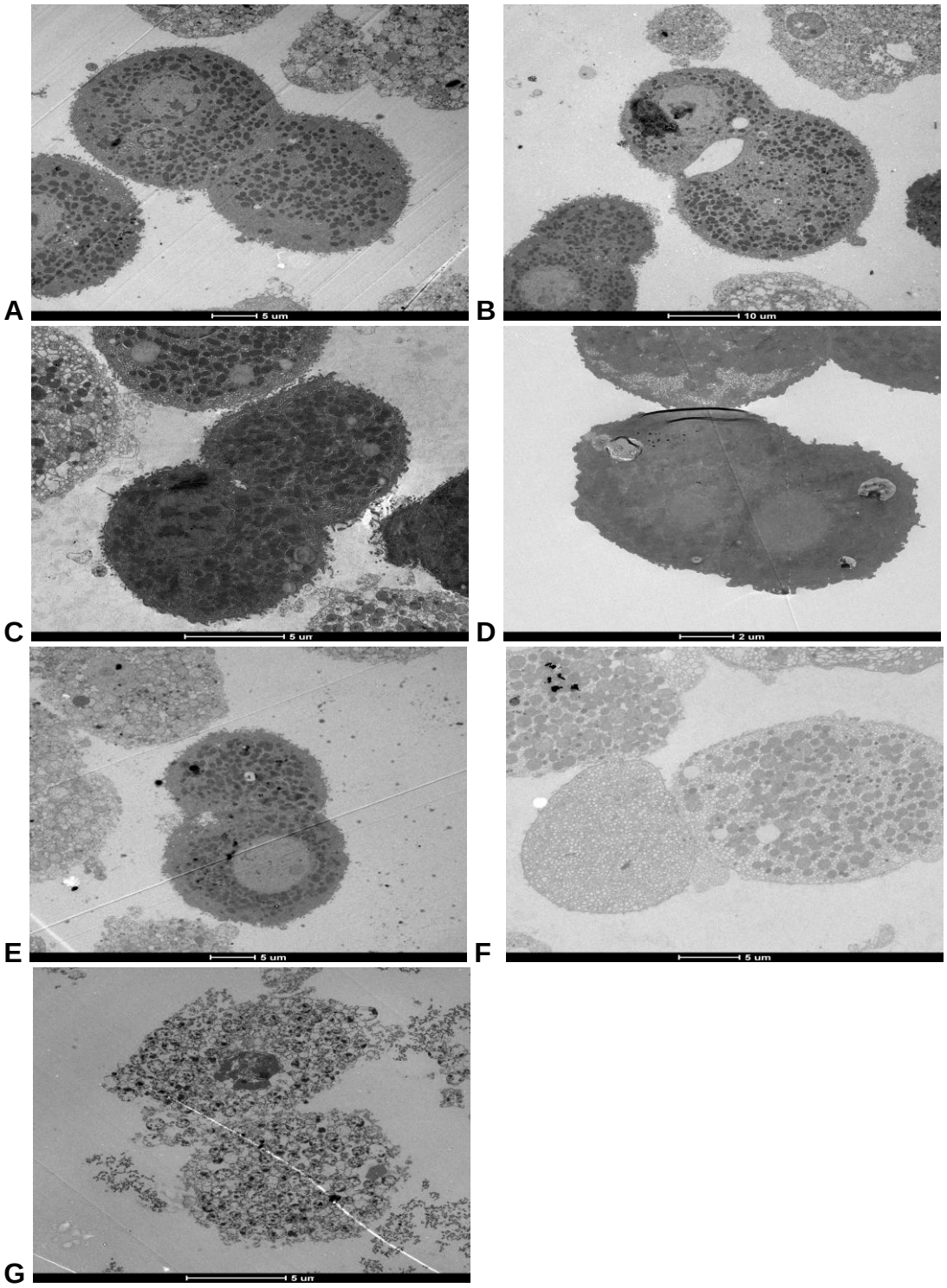


Figure 5.12 TEM analysis of IRHC morphology following exposure to the PARTICLE_RISK particles. Hepatocyte couplets ($2x10^5$ cells/ml) were exposed to L-15 (& 10% FCS) cell culture medium (control **A**), 0.04µg/mm^2 ufCB (**B**), CB (**C**), CNT (**D**), C_{60} (**E**), QD621 (**F**) or QD620 (**G**) for 4 hours and Transmission Electron Microscopy used to reveal the ultrastructure of the cells. Cells were imaged with a Tecnai 12 Transmission Electron Microscope.

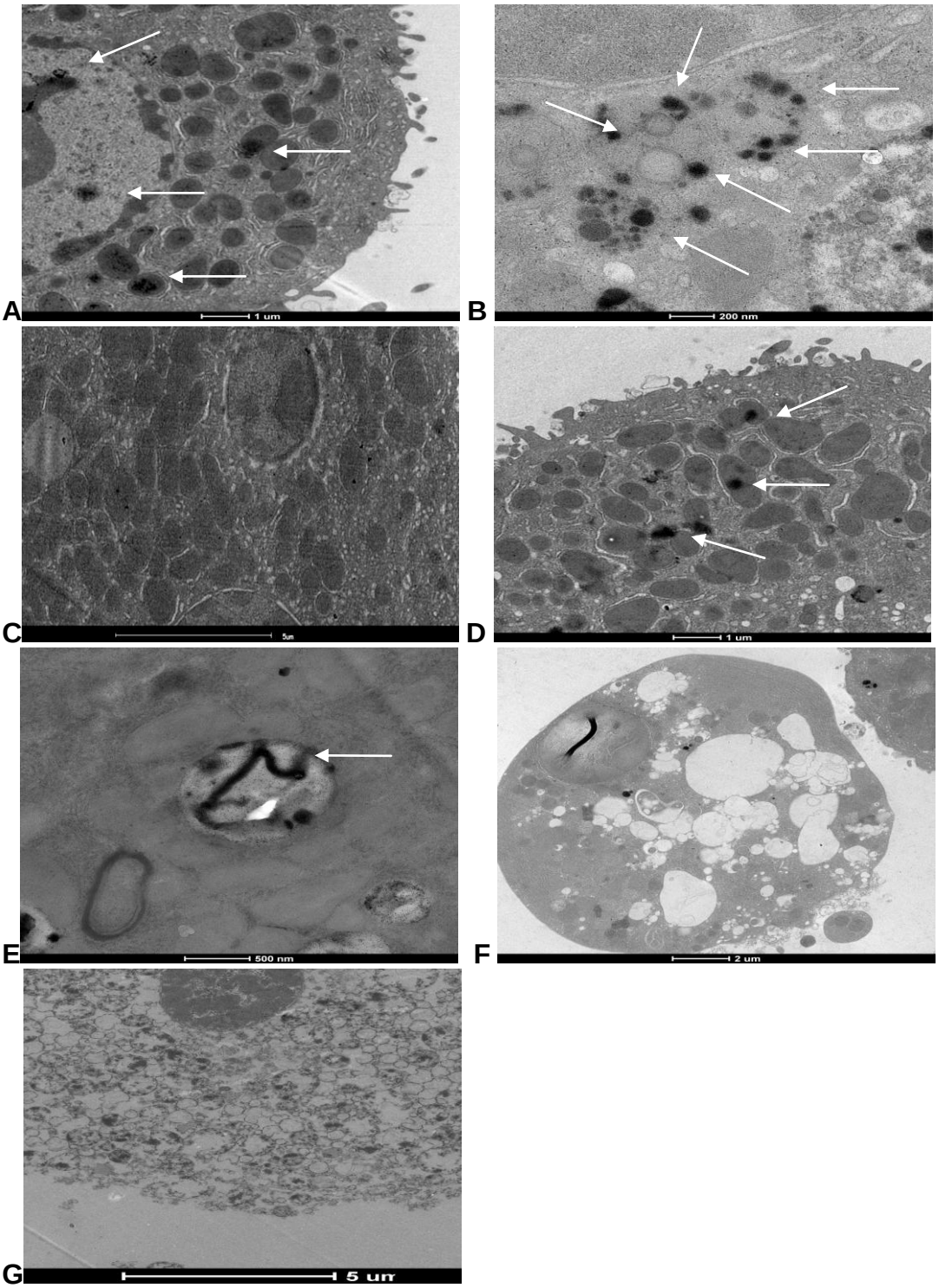


Figure 5.13 TEM analysis of IRHC morphology following exposure to the PARTICLE_RISK particles at high magnification. TEM analysis of particle exposed cells was conducted at higher magnification to gain insight into the fate of internalised particles. Black, particle-like compartments can be observed in control cells (**A**). ufCB appears to be internalised by cells (**B**), with no evidence of CB uptake (**C**). C_{60} particles may be present within the mitochondria of cells (**D**) and CNTs were observed in vesicle-like structures (**E**). Large vacuoles were present within QD621 exposed cells (**E**) and apoptotic bodies were observed in QD620 exposed cells (**F**). White arrows were used to identify internalised particles. Cells were imaged with a Tecnai 12 Transmission Electron Microscope.

5.4 Discussion

The liver has been observed to be a primary site of NP accumulation after exposure, and as hepatocytes are the main liver cell population, and responsible for the majority of liver functions, the ability of particles to impact on hepatocyte function is of great relevance. The purpose of the studies described was therefore to evaluate the response of the hepatocyte C3A cell line to the panel of PARTICLE_RISK particles. This was undertaken to give an indication of any potential hazards associated with particle exposure to the liver, and to compare this to the response of isolated primary rat hepatocyte couplets to gain an indication of the relevancy of the findings, to potentially minimise the use of animals. Furthermore, by comparing the toxicity of different NPs, the toxicity of the different particle types could be ranked and physicochemical factors that might drive toxic responses identified.

Previous reports within the literature have reported that a variety of different hepatotoxins and NPs are capable of inducing cytotoxicity via the selected toxicological endpoints, namely; inflammation, and oxidative stress (see sections 5.1.5 and 5.1.6.3). However different target cells, concentrations of NPs, and exposure times were used, and thus, making comparisons between different NPs, with regards to their toxicity is difficult, but is required to reveal the attributes of NPs that contribute to any observed toxicity. Therefore, to achieve a 'screen-like' test to compare the toxicity of a variety of different particles to hepatocytes, it was relevant to determine if the utilisation of identical particle concentrations and exposure times (in an attempt to standardise the assessment of NP toxicity) made it possible to make generalisations about NP behaviour, or if factors other than their similarity in size can account for different levels or mechanisms of toxicity becoming evident within different particle types. In addition the 'screen-like' nature of the tests conducted allowed comparisons about NP toxicity to be made which is critical due to the vast quantity of available NPs and number of potential targets for toxicity.

5.4.1.1 Determining the impact of NP exposure on oxidative stress development within hepatocytes

The results illustrated that not all particle types investigated were able to induce oxidative stress within all hepatocyte cell types, when GSH depletion was used

as an indicator. It was found that only QD621 was able to decrease GSH levels within the C3A cell line, and tended to lower the GSH content of hepatocyte couplets. The fact that only one particle type within the PARTICLE_RISK panel was able to induce GSH depletion within hepatocytes conflicts with evidence in the literature that indicates that NPs (including ufCB and CNTs) are able to exert toxicity via this mechanism (see later). Therefore it was apparent, that out of all the particles investigated, only QD621 was able to reduce GSH levels within hepatocytes. However, it is likely that the depletion of GSH from cells can be accounted for by the loss of cell viability.

5.4.1.2 Impact of serum presence on oxidative stress

The depletion of GSH from hepatocytes was determined in the presence and absence of serum when considering the toxicity of ufCB and CB. This was carried out due to the fact that serum has antioxidant properties (Stone *et al.,* 1998), which could influence the ability of NPs to induce oxidative stress within hepatocytes. It was established that the absence of serum resulted in a general increase in GSH levels in all treatment groups, which is thought to occur due to the fact that the cells are stressed, and so an increase in GSH occurs as a protective measure. Furthermore it was important that the pattern of response was the same for both particle treatments in the presence and absence or serum. However it has also been demonstrated that serum presence was able to limit the aggregation of NPs (see section 4.4.2.2), which suggests that NPs are exposed to cells at a smaller agglomerate size, and as it is known that size is a primary determinant of particle toxicity, and as serum inclusion is realistic to the *in vivo* exposure scenario, it was deemed appropriate to conduct all experiments in the presence of serum.

5.4.1.3 Determining increases in cellular GSSG as an indicator of oxidative stress

It is customary, when determining the ability of substances to induce oxidative stress (using GSH depletion as an indicator), to express the results as a ratio of GSH:GSSG. This is due to the fact that it is expected that as ROS oxidise GSH to GSSG within cells, GSH levels will decrease and GSSG levels will increase. Accordingly, a decrease in the GSH:GSSG ratio is indicative of oxidative stress. GSSG levels were measured within the experiments (see appendix section A2

for protocol), however the cellular concentration of GSSG was found to be at the lower limit of detection (as cellular levels of GSSG are low, and therefore difficult to detect and quantify). Furthermore the study of cellular GSSG levels was also compounded by the fact the GSSG can be exported from the cell (Stone *et al.*, 1998). Therefore the results were not believed to be suitably accurate and representative of cellular GSSG levels, so that oxidative stress was characterised by expressing GSH concentration per mg of protein in order to standardise the results.

5.4.1.4 Consequences of GSH depletion for normal hepatocyte function

In addition to GSH depletion being an indicator for oxidative stress within cells, it is also important to consider what other roles GSH has within hepatocytes, to determine the consequences of GSH depletion for normal cell function. It is known that GSH participates within Phase II metabolism within hepatocytes, which involves the conjugation of substances to endogenous moieties to facilitate their detoxification and elimination from the body, by making them more 'excretable'. Therefore the ability of substances to decrease GSH levels, will potentially compromise the detoxification of substances via Phase II metabolism, to potentially lead to the accumulation of substances, and their reactive metabolites (produced by Phase I metabolism) within cells, to compromise cell viability. The importance of maintaining GSH levels within cells is demonstrated by knowledge on how acetaminophen induces toxicity within the liver; whereby the depletion of GSH has been proven to be central to the observed toxicity as the reactive metabolite responsible for the toxicity cannot be detoxified (Jaeschke, Knight and Bait 2003). However, it is relevant to consider that there are a number cellular constituents capable of contributing to the Phase II metabolism of substances within hepatocytes (including sulphate, glucuronic acid, acetylation conjugations), that have broad substrate specificity; so that it is often the case that more than one process is able to contribute to the detoxification of substances.

5.4.2 Ability of NPs to impact on hepatocyte viability

When evaluating the ability of particle exposure to compromise the viability of hepatocytes, two assays were utilised; the LDH assay to determine the ability of particles to cause cytotoxicity, and the MTT assay to assess the impact of

particle exposure on cell viability. There is an extensive array of background information available which illustrates the capacity of NPs to compromise cell viability using these assays.

5.4.2.1 Findings from the LDH and MTT assays: are the results comparable?

The MTT and LDH assays, are standard, *in vitro* tests that have been routinely used when assessing viability of cells. It was observed that ufCB, CB, and C_{60} exposure did not cause a reduction in hepatocyte viability when considering the results from the LDH and MTT assays, so that the findings from both assays were in agreement. It was observed that CNTs were able to cause cell death within the MTT assay but not the LDH assay. It was demonstrated that QD620 and QD621 were able to elicit cytotoxicity when considering the results from the MTT and LDH assays, so that again the findings from both assays were comparable. It was observed that QD621 particles were able to induce the greatest cytotoxic response, when compared to the other particles tested. However, QD620 was able to elicit a similar extent of cytotoxicity, albeit at later time points, than that of QD621. It was evident that the cytotoxicity of QDs within the MTT assay was apparent at earlier time points, when compared to the findings obtained from the LDH assay. Consequently, it was apparent that the MTT assay can be regarded as a more sensitive measure when assessing particle induced cell death. This derives from the fact that the MTT assay measures the activity of mitochondria, so that if the metabolic competence of cells is reduced by particle exposure this is expected to precede the plasma membrane damage (associated with cell death) that is necessary to elicit the leakage of LDH from cells. This therefore explains why CNTs did not exhibit toxicity within the LDH assay but were able to decrease MTT activity, and why QDs elicited toxicity at earlier time points than that encountered within the LDH assay.

When assessing the impact of particle exposure on hepatocyte couplet viability it was observed that accurate measurements of LDH activity could not be made due to high background levels. Consequently, only the MTT assay was used to assess the capability of the particle panel to compromise hepatocyte couplet viability. It was found that although the same pattern of toxicity for the particles was observed there were no significant changes in cell viability.

5.4.2.2 NP interference with the MTT and LDH assays

It is acknowledged that NPs are able to interfere with assays utilised to determine their toxicity; whether it be the ability of NPs to contribute to the absorbance of colorimetric assays, the ability of NPs to bind to assay components (including the endpoint being measured, including LDH and cytokines, or to the substances used to detect cytotoxicity, such as the MTT reagent). Accordingly all these factors can contribute to the production of inaccurate, misleading results that make NPs appear more or less toxic than they actually are, thus encouraging and justifying the use of more than one viability assay.

Consequently, it was relevant to ascertain if the PARTICLE_RISK particle panel was able to interfere with the LDH and MTT assays, to confirm that the results obtained are accurate and reflective of the toxic potential of the particles. This was important due to demonstrations within the literature which revealed that CNTs are able to contribute to the production of false positive results within the MTT assay. Worle-Knirsch, Pulskamp and Krug (2006) found that CNTs were able to attach to the MTT formazan product generated by viable cells to prevent its participation within the assay, making it appear that the CNTs detrimentally affected cell viability. Therefore, the ability of the PARTICLE_RISK particle panel to interfere with the MTT assay was of interest, especially for CNTs. The MTT assay was therefore carried out with a number of 'controls'; to determine if the particles were able to bind to the MTT reagent or product, and to determine if NPs contributed to the absorbance readings (at the same wavelength that was used to measure MTT activity) that were generated. All controls were performed in the presence and absence of cells to determine if particles themselves were able to interfere with the assay or if cell-NP interactions were required. It was demonstrated that particles could not interfere with the MTT assay in cell free conditions. It was found that all carbon based particles contribute to MTT absorbance, as a higher absorbance reading was generated than the control (at the wavelength used to detect MTT activity), and so these particles may appear less toxic than they are. However, only the particles internalised by cells, or those attached to the cell surface would contribute to the absorbance reading within the MTT assay, and this was proven as absorbance values were unaffected, when a reading was taken subsequent to

cell exposure to the NPs. The other controls performed demonstrated that QDs and ufCB had the potential to bind to the MTT reagent, and thereby prevent its participation within the assay, potentially making particles appear more toxic than they are. In addition, QDs were able to increase the absorbance reading, when they were added at the same time as the MTT product solubilising agent, propanol. It is therefore suggested that none of the particles within the panel bind to the MTT product, and perhaps are able to encourage the solubilsation of the MTT product. Alternatively the QDs themselves may contribute to the increase in absorbance (as there was a tendency for this within the results, although the finding was not statistically significant). However, overall it was assumed that all particle types did not interfere with the MTT assay so that any findings of cytotoxicity were assumed to be solely particle driven, This arises as a consequence of the fact that the control experiments conducted aimed to determine what interference the NPs had the potential to elicit, but it is unlikely that a high enough concentration of particles would be internalised by cells to achieve the level of interference observed. Belyanskaya *et al.,* (2007) demonstrated that the ability of CNTs to interfere with the MTT assay was influenced by the presence of dispersing agents within particle suspensions. It was observed that CNTs were able to bind to the MTT formazan product and, that CNTs were able to reduce MTT in the absence of cells and enzymes. However they also found that serum was able to reduce the interference of CNTs with the MTT assay, so that perhaps serum was able to coat the CNT surface and reduce its ability to bind to MTT. Therefore as serum was present within the studies conducted using the PARTICLE_RISK panel the findings of toxicity results are thought to be representative of the direct effects of particles on hepatocyte viability using the MTT assay, and not due their interference with the assay.

It was thought that LDH may adsorb onto the surface of particles, thereby preventing its participation within the assay, to provide results that underestimate LDH activity, and therefore misinterpret the capacity of particles to induce cytotoxicity (Stone *et al.*, unpublished observations). It was found that LDH was potentially able to bind to the surface of ufCB particles. However as the MTT assay confirmed that ufCB did not reduce hepatocyte viability the findings from the LDH assay are believed to be reflective of the toxic potential of particles within the panel. Despite this, the potential of biological molecules to

adsorb onto the NP surface reiterates the requirement to utilise more than one test to evaluated NP induced cytotoxicity, and to consider the characteristics of NPs that may provide interference. This is due to the fact that it is known that the adsorption of biological molecules onto the surface of particles is related to particle size, as smaller particles provide a greater surface area (per unit mass) for biological molecules such as LDH to adsorb onto. The critical involvement of particle surface area in the adsorption of molecules onto the particle surface has been illustrated by Brown *et al.,* (2000), who demonstrated that BSA adsorbed onto the surface of ufCB and not CB. The potential for NPs to interfere with the MTT and LDH assays therefore made it relevant to utilise two or more independent cytotoxicity tests to gauge the ability of NP to affect cell viability, and to conduct appropriate controls to prevent against the appearance of inaccurate results.

5.4.3.1 The ability of NPs to impact on IRHC morphology using SEM

The ability of particles to modify hepatocyte couplet morphology was determined using SEM in order to further evaluate the cytotoxic potential of the PARTICLE_RISK particles. It was evident that ufCB, CNT, C_{60}, QD620 and QD621 were able to increase the frequency of bleb formation on the surface of hepatocyte couplets. Cells exposed to CB had a similar morphology to that of control cells, so that this particle type inflicted minimal cell damage. Within the LDH and MTT assays it was found that QD621 were able to induce the greatest cytotoxic response, and this was replicated in the extent of cell damage observed where the damage inflicted by QD621. Cell damage was therefore inflicted by QD621 to such an extent that it would appear that the cell is past the point of recovery, and as a consequence appeared to be associated with a necrotic mediated cell death due to the extent of damage to the plasma membrane which appeared to be irreversible in nature due to the severe loss of membrane integrity in exposed hepatocyte couplets. An observation that further supported the cytotoxic potential of QD621 is that there were fewer cells available to image, suggesting that QD621 exposure killed the cells, which caused them to detach from the coverslip, which made them unavailable for SEM analysis.

Bleb formation can be considered as a universal sign of hepatocyte damage, regardless of its origin (Rosser and Gores 1995), and so it is important to consider their origin. Blebs have been described as 'bubble-like projections that extend from the cell surface', and are assumed to develop due to a breakdown in membrane-cytoskeleton connections, which is thought to arise due to oxidative stress, mitochondrial dysfunction, changes in cytosolic pH, intracellular calcium levels or ATP concentration (Gores, Hermans and Lemasters, 1990, Rosser and Gores, 1995). Multiple processes are therefore thought to contribute to the loss of plasma membrane-cytoskeleton attachments, to culminate in the formation of blebs at the cell surface (Rosser and Gores, 1995).

It is acknowledged that there are three stages within bleb formation; 1) small blebs appear on the cell surface, 2) blebs fuse to form larger terminal blebs (stages 1 and 2 represent reversible events), 3) terminal blebs breakdown and lyse, and are represented by large discontinuities within the plasma membrane structure, so that plasma membrane integrity is compromised, transmembrane ion gradients are lost, and cytoplasmic contents are released, thus representing a state of irreversible damage (Gores, Hermans and Lemasters, 1990, Rosser and Gores, 1995). The 'hole-like' regions evident within QD621 exposed hepatocytes could therefore have developed as a consequence of terminal bleb rupture, so that this is thought to be the eventual outcome of QD621 exposure.

It has been previously demonstrated that alterations in cell morphology can be indicative of toxicity to hepatocyte couplets, for example Roma *et al.,* (1998) illustrated that vasopressin was able to increase bleb formation (determined using SEM analysis) within hepatocyte couplets. Bleb formation on the cell surface of hepatocyte couplets was explained by disruption of the actin cytoskeleton within hepatocyte couplets, whereby the cytoskeleton was redistributed from the peri-canalicular area to the cell surface (Roma *et al.*, 1998). Therefore the finding that bleb formation is apparent in QD621 exposed hepatocyte couplets may be a consequence of cytoskeletal rearrangement which may also explain the impairment CLF accumulation within their bile canaliculi that was observed previously (see section 5.3.4). These functional and morphological impairments have therefore been identified as occurring

concomitantly, and cytoskeletal rearrangements have been suggested to responsible for the cholestatic effects of substances such as vasopressin (Roma *et al.*, 1998).

The reversibility of bleb formation is an important consideration, as evidence of their formation does not equate to the induction of cell death. It has been demonstrated that bleb formation induced by CCl_4 on the cell surface of primary rat hepatocyte couplets was reversible, however this was only in conditions where removal of the toxicant was promoted (Berger, Reynolds and Combes, 1987). Therefore it is plausible that on toxicant removal, plasma membrane structure regresses back to normal, and cell death is avoidable. Furthermore, it is also possible that blebs are able to shed off from the cell surface *in vivo*, which allows the plasma membrane to reseal so that cell integrity is restored to maintain cell viability (Gores, Hermans and Lemasters, 1990).

Therefore it was evident from the results that determining the gross morphology of hepatocyte couplets subsequent to their exposure to particles, revealed that QD621 were again demonstrated to elicit the greatest extent of toxicity, which correlated to the findings from the biochemical endpoints conducted in cell lines and hepatocyte couplets that were used to assess particle toxicity. It is of relevance that only the morphology of hepatocyte couplets was considered subsequent to particle exposure (at only one concentration and time point) due to the cost of SEM sample preparation. There have been other studies that have used cell morphology as an indicator of NP toxicity; for example Arora *et al.,* (2008) used phase contrast microscopy to provide further evidence (in addition to biochemical endpoints) that silver NPs were able to elicit toxicity within HT-1080 (skin carcinoma) and A341 (fibroblast) cell lines, and it was observed that concentrations higher than 6.25µg/ml caused alterations in cell morphology, with a higher proportion of smaller and more rounded cells, and less intercellular contact between remaining cells and they also observed that fewer cells were available to observe due to the high level of cytotoxicity exhibited by the silver NPs. Therefore evaluating the gross morphology of hepatocyte couplets was able to provide further insight into the toxicity of particles, but it is also relevant to consider cell ultrastructure, as alterations to

the appearance of the cell interior can provide information of the mechanism of cell death.

5.4.3.2 Determining the impact NP exposure on the ultrastructure of IRHCs, using TEM analysis

TEM analysis of hepatocyte couplets was conducted to determine if particles were internalised by cells, and to determine if particle exposure caused any changes in cell ultrastructure. This is important as the mechanisms underlying cell death can be uncovered, which is reliant on knowledge on the morphological characteristics of dead cells (outlined in section 5.1.6.1). Grub *et al.,* (2000) revealed that cyclosporin A was able to induce apoptosis within primary rat hepatocytes. This conclusion was reached due to a qualitative assessment of cell morphology using TEM, where there was evidence of cell shrinkage, plasma membrane blebbing, and formation of cytoplasmic apoptotic bodies.

It was observed, using TEM that control hepatocyte couplets had a 'normal' morphology, with a normal distribution and morphology of organelles, mitochondria occupied the majority of the cell interior and the cells had large round nuclei. However it was revealed that the assessment of uptake of carbon based particles was difficult due to the appearance of black dot-like structures within control cells that could potentially be mistaken for particles within exposed cells. The black regions are thought to be artefacts that derive from the staining procedure involved in sample preparation for TEM analysis, or alternatively could represent glycogen stores within cells which are known to have a dark, particle-like appearance within the cell cytoplasm when visualised using TEM (Grub *et al.*, 2000). However, despite this, it was observed that ufCB, CB, CNTs and C_{60} were all evident within the cell interior, (presumably as aggregates). Specifically, CNTs were apparent within defined vesicle structures within cells, which may derive from an endocytic mechanism of uptake or may facilitate CNT removal from the cells. It was also evident that C_{60} was internalised by cells, and found free within the cell cytoplasm, where they were often associated with mitochondria. It was apparent that ufCB was present within defined structures within the cell interior, although there was no evidence of CB uptake by cells.

Within QD621 treated cells it was evident that a large number of cells were dead but a conclusive mechanism of cell death could not be obtained. This was due to the fact that there were cells that had apoptotic or necrotic characteristics within the sample. The necrosis of cells was suggested by the presence of hepatocytes that had large, clear cytoplasmic vacuoles, which is a feature of necrotic cells (Elmore, 2007). These observations corroborate the findings from SEM analysis, where the morphology of QD621 exposed cells suggested that necrosis was primary responsible for QD621 inflicted cell death. On the other hand, there were also cells that had an intact plasma membrane, and that had a cytoplasm that was full of apoptotic bodes, so that organelles, such as mitochondria were not 'free' within the cytoplasm thereby suggesting an apoptotic mechanism of cell death. The fact that apoptotic and necrotic cells co-exist within cells exposed to the same stimulus can be explained by the apoptosis-necrosis continuum, whereby an ongoing apoptotic process can be converted to a necrotic one, as the mechanism of cell death can be influenced by a number of factors including the availability of ATP or caspases (Leist *et al.*, 1997, Zeiss, 2003), so that the two mechanisms of cell death are not necessarily separate, unconnected events. Furthermore the intensity of toxicant exposure (in terms of concentration and/or duration) is also able to impact on the prevalence of either apoptosis or necrosis (Leist *et al.*, 1997), and it is possible that the initiation of apoptosis is an attempt to pre-empt necrosis to protect against damage. Therefore it is not contradictory to observe both apoptotic and necrotic phenotypes in cells exposed to the same stimulus (Zeiss, 2003), and highlights that ascribing a mode of cell death based exclusively on the morphological appearance of cells can provide confusing results, due to the fact that the two processes can occur in response to the same stimulus. Findings of apoptotic mediated cell death following particle exposure corroborates with the findings of Jia *et al.,* (2005) who revealed that SWCNTs and MWCNTs were able to elicit apoptosis (where the nucleus was degenerating, the cell organelles were condensed and vacuoles were present within the cytoplasm). Alternatively it is possible that cells are undergoing cell death via autophagy; whereby the degradation of cytoplasm contents is mediated by a lysosomal mechanism in response to cell damage, and the

presence of large, cytoplasmic vacuoles characterises the process (Levine and Kroemer, 2008).

5.4.4.1 NP induced hepatocyte cytokine production

Toxicity exerted by a number of hepatotoxins (including alcohol, carbon tetrachloride, and acetamphicen) is associated with the increased production of inflammatory mediators, such as TNFα and IL-8 (Dong *et al.*, 1998). Within the liver, cytokine production is usually associated with Kupffer cell activity (due to their immunological origin), however hepatocytes have also been demonstrated to contribute to the initiation and progression of inflammatory responses within the liver (Dong *et al.*, 1998). Consequently, despite the knowledge that Kupffer cells are the primary source of cytokines within the liver, the contribution of hepatocytes must also be considered. This is made even more relevant by the knowledge that hepatocytes are the major liver cell population, so that even if their individual ability to produce cytokines is low, their overall capacity for generation is high.

Multiplex ELISA kits were used to gain an indication of the cytokine profile associated with hepatocyte exposure to the PARTICLE-RISK particles. Sample analysis was completed in singlet, and not duplicate when using multiplex analysis to increase the number of samples that could be analysed within one plate as a consequence of their expense. Consequently, multiplex analysis, was conducted using a 'look and see' approach to determine if particle exposure modified the production of a number of cytokines. This was deemed relevant due to the cascade type nature of inflammatory responses that involve cytokine networks whereby a number of different cytokines contribute to the initiation, perpetuation and termination of inflammatory responses.

Multiplex analysis found that IL-8 production was the only cytokine whose production was elevated by particle exposure, and so enhanced IL-8 production appeared to be integral to the inflammatory response triggered by particles within hepatocytes, and was confirmed using a conventional ELISA kit, where it was demonstrated that only QD620 and QD621 were able to increase IL-8 production by C3A hepatocytes. QD620 was observed to elicit the greatest extent of IL-8 release which was maximal after a 24 hour exposure. A low

concentration of 0.04µg/mm^2 QD621 was able to increase IL-8 release from hepatocytes, however higher concentrations lowered IL-8 concentrations to lower than that of the controls. The ability of QD621 to stimulate IL-8 release is related to the extent of hepatocyte viability, accordingly, as the majority of QD621 exposed cells were dead from the 6 hour time point they were unable to contribute to IL-8 production. Therefore a greater number of QD620 treated cells were available to contribute to the production of IL-8, therefore accounting for the increased production between the 6 and 24 hour time points, despite the fact that the majority of QD620 exposed cells were dead at 24 hours. Consequently, to more fully investigate the impact of QD621 exposure on IL-8 release sub-lethal concentrations of QDs could be used in future experiments, which would aid in revealing the underlying mechanisms of toxicity. The inflammatory and toxic potential of QDs is supported by the findings from partners within the PARTICLE_RISK project (that evaluated the toxicity of the PARTICLE_RISK panel within the lungs), who observed that QDs had the greatest potential to stimulate pro-inflammatory cytokine production (MIP-2 and IL-6), subsequent to i.t. administration of mice, which was maximal at 24 hours (Jacobsen *et al.*, 2008). It was also demonstrated by Jacobsen *et al.*, (2008) that QD621 elicited a stronger inflammatory response than QD620, thus strengthening the notion that if sub-lethal concentrations of QD621 were used, then its true inflammatory potential would have been realised.

MIP-2 levels within cell supernatants were measured within hepatocyte couplet supernatants, subsequent to particle treatments, as this cytokine is the rodent equivalent of IL-8. QD621 exposure had a tendency to reduce MIP-2 levels to lower than that of the controls after a 6 hour exposure of hepatocyte couplets which, as witnessed within the cell lines, correlated with a trend for a reduction in cell viability. MIP-2 production was not enhanced by any of the other particle types, so that the finding that IL-8 production was increased by QD620 in the C3A hepatocytes was not replicated within the hepatocyte couplets. This finding is likely to be a consequence of the NP concentration and exposure time used when assessing the inflammatory response induced by QD620 in hepatocyte couplets (a maximal concentration of 0.08µg/mm^2 and 6 hour exposure), as cytokine release was maximal after a 24 hour exposure, and at higher concentrations, within the C3A cell line.

5.4.4.2 Inflammatory diseases and susceptibility to NP toxicity

It is recognised that there is a susceptibility to the effects of NPs within the lung, so that pre-existing inflammatory disease increases the risk of NP toxicity (Pope and Dockery, 1999), and this has been investigated in animal models (see for example Clarke *et al.*, 1999). Therefore pre-existing inflammatory disease within the liver may lead to a predisposition to NP toxicity, when considering the toxicological outcomes associated with ultrafine particles. For example, alcoholic liver disease develops due to a number of immunological and metabolic processes, and the progression of the disease is thought to be dependent on Kupffer cell activation, lymphocyte infiltration, and TNFα production (Stewart *et al*., 2001). Consequently, the potential for NPs to elicit an inflammatory response within the liver could exacerbate inflammatory based liver diseases.

5.4.5 Impact of NP exposure on bile secretion

It was observed that only QD621 and ufCB were able to inhibit bile secretion, which was indicated by a reduction in the number of couplets that were able to accumulate CLF within their bile canaliculi. It is therefore relevant to consider the mechanisms responsible for the inhibition of bile secretion observed. There was a trend for QD621 to induce cell death within hepatocyte couplets, but this was not the case for ufCB, consequently the reduction in bile secretion cannot be explained solely by a loss of cell viability. This is in agreement with the findings of Stone *et al.,* (1994) who demonstrated that menadione was able to inhibit bile secretion, but cell viability was maintained. Oxidative stress has been demonstrated to contribute to the ability of menadione to reduce the ability of hepatocyte couplets to accumulate CLF within their bile canaliculi (Stone *et al.,* 1994). However, although there was a tendency for a depletion of GSH within hepatocyte couplets exposed to QD621 and ufCB, this mechanism of toxicity cannot be solely responsible for the inhibition in bile secretion observed.

Disruption of the actin cytoskeleton that surrounds the canalicular membrane has been identified as the primary culprit for a decrease in the ability of hepatocyte couplets to accumulate CLF within bile canaliculi (Coleman *et al.*, 1995). This is likely to derive from a decrease in the delivery of carrier proteins to the canalicular membrane, which is necessary for the secretion of bile

components into bile canaliculi (Coleman *et al.*, 1995). Reduced canalicular accumulation of CLF within hepatocyte couplets was observed when exposed to Cyclosporin A, and was accounted for by a decrease in Bsep (transporter) presence at the canalicular membrane, and occurred due to the disruption of the peri-canalicular actin cytoskeleton (Roman *et al.*, 2003). Perez *et al.,* (2006) expanded upon the mechanisms responsible for a loss of canalicular secretory ability within hepatocyte couplets and found that the impairment of canalicular secretion of bile salts elicited by *tert*-butylhydroperoxide (tBOOH) was suggested to occur due to a loss of the internalisation of the Bsep transporter from the canalicular membrane, which was attributed to oxidative stress induced activation of a (calcium dependent) Protein Kinase C mediated signalling cascade to ultimately cause the disorganisation of the actin cytoskeleton. Therefore, the mechanisms that contribute to a decrease in the secretory capacity of hepatocyte couplets are complex, involving a number of sequential events, with an impairment of cytoskeleton function at the root of the toxicity of cholestatic substances, using the hepatocyte couplet model of bile secretion. The ability of QD621 and ufCB to reduce the ability of bile canaliculi to accumulate CLF is therefore likely to involve an impairment of cytoskeletal function, which warrants further investigation.

Several problems were encountered when evaluating the ability of the PARTICLE_RISK particles to impair bile secretion. Firstly, it was not possible to consider the effect of particle exposure on bile secretion within cell lines, despite the fact that they have been demonstrated to form canalicular like structures (see section 4.3.3.4). This was due to the fact that the canalicular like structures were not present with sufficient frequency within the C3A and HepG2 cell lines to provide a realistic indication of the impact of NP exposure on bile secretion, and instead were used only to determine if NPs were eliminated within bile. Furthermore it was apparent that the isolation of hepatocyte couplets from the rat liver produced a mixed population of cell 'units', so that single cells, triplets and quadruplets (and other multiples of cells) were evident. Increasing the couplet population could be achieved through the use of centrifugal elutriation (Wilton *et al.,* 1991), however the equipment required was unavailable.

5.4.6.1 Why were only QDs able to exert toxicity to hepatocytes?

The results demonstrated that hepatocyte toxicity (indicated by oxidative stress development and inflammation) was not induced by the carbon based particles contained within the PARTICLE_RISK panel. This contradicts studies which illustrated that these processes are paramount to NP toxicity (see section 5.1.5). Specifically, only QD621 and QD620 were able to increase the production of IL-8 by C3A cells out of the particle panel investigated. This is contrary to the known tendency of particles, such as ufCB to stimulate IL-8 production within other cell types (see section 5.1.2.3). Consequently, a lack of an inflammatory response within hepatocytes could derive from the fact that NP toxicity may be site specific within the body, and that hepatocytes are less sensitive to the effects of NPs than other cell types. Furthermore it is also possible that cytokines can adsorb onto the surface of particles, including ufCB (Brown *et al.,* 2000). This is suggested from the results, whereby it was apparent that there was a tendency for the carbon based NPs to reduce IL-8 levels to lower than that of the control after a 24 hour exposure, and this warrants further investigation.

It is therefore plausible that some cell types are more sensitive to the toxicity of NPs than others, as it has been demonstrated that NPs are able to induce oxidative stress within cell types derived from the lung (including alveolar macrophages), skin (including keratinocytes), and liver (for example hepatocytes), to name a few (see section 5.1.3.4), thereby implying that there is a common response from cells to NP exposure. Therefore, it is unexpected that the NPs contained within the PARTICLE_RISK panel were unable to elicit oxidative stress, which is supported by the knowledge that other studies utilised much lower NP concentrations than those utilised when evaluating the toxicity of particles to hepatocytes, and found them to elicit oxidative stress. For example, Manna *et al.,* (2005) found SWCNTs to have oxidant properties when exposed to keratinocytes at concentrations ranging from 1-10µg/ml for 12 hours. However it is known that hepatocytes have a high concentration of GSH, in comparison to other cell types, so that they have a greater defence against ROS, and are therefore more resilient to NP toxicity. Therefore it is possible that the reported toxicity of NPs can be regarded as NP and tissue (and cell) specific, which makes it difficult to make generalisations about NP mechanisms

of toxicity. Different cells are therefore likely to respond differently to exposure of the same type of NP (so that NPs are not universally toxic to all cell types within the body), and that the same cells do not respond similarly to different NPs (so that one cell type is not sensitive to the toxicity of all NPs). Furthermore, the toxicity of NPs is also likely to be influenced by the experimental set up, such as NP exposure time and NP concentrations. However despite evidence that NPs, of the same 'type' to those contained within the PARTICLE_RISK panel (such as CNTs), have been illustrated to induce toxicity, these NPs have been produced by different manufacture processes, by different companies or laboratories, have different diameters, lengths, sizes, surface attachments, or contaminants, which will inevitably influence their toxicity. Consequently the toxicological processes that drive the toxicity of ultrafine particles are proposed not to be fundamental to NP toxicity within the liver and therefore a unifying hypothesis which explains the toxicity of NPs at all potential targets within the body is unlikely, so that NP toxicity has to be exposed on a NP by NP, experimental and/or site specific basis

There are a number of studies that are in agreement with the findings, for example Pulskamp, Diabate and Krug, (2007) found that CNTs (multi and single walled) were unable to cause cytotoxicity or induce an inflammatory reaction (measured by NO, TNFα and IL-8 production) in the NR8383 alveolar macrophages or A549 epithelial cells. However, they were able to increase ROS levels, and decrease mitochondrial membrane potential so perhaps the cells initiated protective measures to overcome these potentially lethal observations. Therefore within the results it was observed that only the QDs were able to elicit toxicity that was consistent in nature, and that ufCB, CB, CNTs, C_{60} were, in the main, unable to impact on hepatocyte function or viability.

5.4.6.2 What is responsible for QD toxicity?

QDs were consistently observed to induce toxicity within hepatocytes, with positively charged QDs (QD621) eliciting a greater extent of toxicity than their negatively charged counterparts (QD620). The ability of QDs to induce toxicity within hepatocytes is likely to derive from their charge, instability, composition or small size.

5.4.6.2a The contribution of QD instability to their toxicity

It is relevant to consider the impact of QD stability, to their toxicity. Specifically, cadmium contained within QDs is usually enclosed within the core of the QD structure. However it has been postulated that cadmium can be released, due to the unstable nature of QDs. This is particularly important within the liver, due to the knowledge that cadmium is a known liver toxicant (Dong *et al.*, 1998).

Derfus, Chan and Bhatia, (2004) demonstrated that the toxicity of QDs was reliant on their unstable nature, whereby the liberation of free cadmium ions from the QD structure was integral to their toxicity. Primary rat hepatocytes were exposed to CdSe QDs, and it was found that under certain circumstances they were cytotoxic. It was observed that if the surface of NPs was oxidised (through exposure to UV light), cadmium was released from the QD and that this was responsible for the observed cytotoxicity. This may be relevant to *in vivo* conditions, where QDs have been illustrated to cause oxidative stress within cells (observed by Lovric *et al.,* 2005b), so that the oxidative environment will make it more likely that QDs exert toxicological outcomes due to the release of cadmium. Derfus, Chan and Bhatia, (2004) also reported that the addition of a ZnS shell to the QD structure was able to virtually eliminate the toxicity associated with QD exposure, illustrating that the shell is an important component in protecting against the toxicity of QDs, due to the stability it provides to the QD structure. The addition of a shell to the QD structure therefore affords a certain degree of protection against QD mediated toxicity, and provides further evidence to confirm the finding that cadmium ion release represents an important aspect of QD toxicity. It also illustrates that knowledge on the characteristics of NPs that contribute to toxicity can aid in the design of NPs, to avoid the incorporation of physicochemical attributes that render them toxic.

The instability of QDs was exemplified by the appearance of the QD suspensions supplied by the manufacturers (see figure 5.14), where it was apparent that the QD suspensions decompose with time, which occurs in an unpredictable manner, as some vials decomposed more quickly than others.

Figure 5.14 QD suspensions. The stock vials of QD621 can be observed to decompose and become unstable. The vials on the left hand side of the picture are regarded as stable QD suspensions, and the vials on the right hand side unstable, which can be observed due to the colour change of stock solutions.

5.4.6.2b The importance of charge to QD toxicity

The results suggested that the charge of NPs may be fundamental to their toxicity. This assumption was based on the knowledge that positively charged QDs (QD621) were consistently able to induce cytotoxicity within hepatocytes to a greater extent than their negatively charged counterparts (QD620). The importance of NP charge in influencing the extent of toxicity was observed by Lockman *et al.,* (2004) who demonstrated that positively charged NPs were more toxic to the blood brain barrier, however Ryman-Rasmussen *et al.,* (2007) observed that the toxicity of negatively charged QDs was greater than that of their positively charged equivalents. Furthermore Hoshino *et al.,* (2004a) demonstrated that the cytotoxicity of CdSe QDs was related to the surface molecules that were attached to the QD surface. The QDs were all identical in composition, but had different surface coatings which provided the QDs with a particular charge; namely cysteamine (positively charged), carboxylic acid and thioglycerol (both negatively charged). It was found that the QDs that contained the carboxylic attachment were the most toxic, and the positively charged and thioglceryol surface (relatively less negatively charged than the carboxylic acid QDs) modified QDs did not induce cytotoxicity in the WTK1 (human lymphoblast) cell line. It is therefore apparent that the type of surface attachment, and therefore charge of the QD was able to influence QD toxicity. This observation was made possible due to the fact that the QDs utilised contained a ZnS shell within their structure that provided a more stabile QD, so that cadmium release was minimal and toxicity was based solely on the QD surface. The findings from this study therefore indicated that the toxicity of QDs

may be charge dependent, where negatively charged QDs were more toxic than their positively charged counterparts. However these findings are contrary to those observed within QD621 and QD620 toxicity, thereby highlighting the difficulty in assessing QD, and NP toxicity, as diverse QD structures may elicit different levels of toxicity. However, other studies have confirmed the finding that positively charged particles are more toxic than negatively charged ones of identical composition. Yacobi *et al.,* (2007) demonstrated that positively charged CdSe/ZnS QDs were more toxic to rat alveolar type 1 epithelial cells than their negatively charged counterparts. Again, the fact that the QDs used contained a ZnS shell provided a more stable QD, structure to allow toxicological assessment to be based solely on charge.

Consequently, as the PARTICLE_RISK QDs do not have a shell to protect against the release of cadmium, it is likely that both charge and instability act concomitantly to account for the observed toxicity. This hypothesis is supported by the findings of Cho *et al.,* (2007) who investigated the importance of cadmium release to the toxicity of QDs, by using a number of different QDs that varied with respect to charge, composition and surface attachments, to determine if QD instability, or the composition of the core and shell of the QD was important to their toxicity. It was observed that the release of cadmium from CdTe QDs occurred to a much greater extent than that observed from CdSe/ZnS QDs, therefore insinuating that shell may confer some protection from a cytotoxic response. This was proven, as CdTe QDs induced cytotoxicity within MCF-7 cells but not CdSe/ZnS QDs, however it was observed that the release of cadmium from the CdTe QDs was not wholly responsible for the cytotoxicity as there was no dose dependent correlation between cell viability and free cadmium, which is contrary to the findings of Derfus, Chan and Bhatia, (2004). Therefore to more fully comprehend the importance of charge to NP toxicity within the liver, the ability of polystyrene beads of positive or negative charge to induce toxicity within hepatocytes could be compared. This would allow an evaluation of NP toxicity to be focussed solely on charge.

5.4.6.2c QD characteristics responsible for their toxicity

Other QD properties, than charge and instability could contribute to QD toxicity. Lovric *et al.,* (2005b) observed that positively charged CdTe QDs (no shell)

were cytotoxic to PC12 (pheochromocytoma cells) and N9 (microglia) cells, and that the extent of toxicity elicited was dependent on the size of the QDs. It was evident that smaller QDs produced a more pronounced toxicity which was hypothesised to result from their greater level of uptake (when compared to larger QDs of equivalent composition), which derived from their smaller size.

Therefore the properties of the PARTICLE_RISK QDs that are most influential in dictating the observed toxicity need to be delineated, and of particular relevance is the contribution of QD charge, and/or instability. Therefore cadmium release from QDs is believed to be an important component of the cytotoxicity elicited by QDs, however as the two different QDs tested did not produce an equivalent level of cytotoxicity it is conceivable that other particle characteristics influence, and contribute to the toxicity of QDs, including the most likely contributor of particle charge. Therefore QD621 could induce a greater extent of toxicity as they are more unstable than QD620, and therefore leach more cadmium. However it is also possible that the positive charge of QD621 contributes to their toxicity, so that any positively charged QD621 that remain 'intact' are more toxic than the negatively charged QD621, which could account for their greater toxicity. Therefore perhaps both QD instability and charge act concurrently to account for the greater toxicity of QD621. Understanding what aspects of the QD structure are primarily responsible for any observed toxicity will therefore make harnessing their exploitation safely more attainable.

5.4.7 Why do IRHCs appear to be less responsive to NP toxicity than cell lines?

The pattern of response observed within cell lines and primary hepatocyte couplets was similar when considering the effects of the PARTICLE_RISK particle panel on hepatocyte viability, and GSH depletion. However within hepatocyte couplets the findings did not reach significance. This is likely to be a consequence of the fact that lower concentrations of particles, and shorter incubation times were used when assessing the toxicity of the particles to the hepatocyte couplets. As a result, a greater number of replicative experiments,, using hepatocyte couplets may have provided results that replicated the extent of toxicity observed when hepatocyte cell lines were exposed to the

PARTICLE_RISK particles. The lower concentration of NPs used was justified by the assumption that primary cells are ordinarily more sensitive to toxicant exposure. In addition, when assessing the impact of NP exposure on bile secretion, a particle concentration of 0.04µg/mm^{2}was used due to the limited supply of some of the particles, which was compounded by the high volumes required for assessing the impact of particles on bile secretion, due to the size of the imaging apparatus. Furthermore, the use of shorter exposure times was warranted due to the limited number of experiments that can be completed in one day when using primary cells. In addition, an exposure time of 24 hours was not completed due to the fact that bacterial contamination was often evident [which occurs as part of the cell isolation due to the close proximity of the liver to the gut (and its associated bacterial content)], which would be expected to interfere with the results as their incubation time increased.

A lack of significant findings may also derive from the fact that the primary hepatocytes isolated contain different 'populations' (specifically hepatocytes that originate from peri-portal, or peri-venous locations) of hepatocytes which would make the response observed more variable due to differences in sensitivities of the different hepatocyte types (see section 2.3.3). The importance of separating peri-venous and peri-portal hepatocytes is highlighted by the finding that different hepatocyte couplet populations differ in their susceptibility to toxicants. Specifically peri-venous couplets are more resistant to the toxicity of menadione, as peri-portal couplets were less able to maintain canalicular secretory activity (Wilton *et al.* 1993). Centrifugal elutriation can be used to separate peri-venous and peri-portal hepatocyte couplets, but this was equipment was unavailable (Wilton et al, 1993). However, this is likely that particle concentration and exposure time is most influential in particle toxicity, as it is not known if C3A cells are derived from peri-venous or peri-portal hepatocytes.

5.4.8 Justification of particle concentrations used when assessing PARTICLE_RISK toxicity to hepatocytes

When assessing the toxicity of the PARTICLE_RISK particle panel to the C3A hepatocyte cell lines, studies were performed using increasing concentrations of particles, namely; low (0.04µg/mm^2) medium (0.18µg/mm^2) and high

(0.35µg/mm^2). These concentrations were used as it was intended that the toxicity of particles would be assessed using a 'screen' type approach which would to allow for comparisons and generalisations about particle behaviour to be made. Limiting the concentrations of particles used, instead of carrying out full dose response characterisation, was necessary due to the large number of particles that required toxicological assessment, and due to the limited supply of particles. It was thought that the highest particle concentration used, although not expected to be physiologically relevant would allow toxicity to become apparent, to reveal what particle exposure to hepatocytes has the potential to elicit (specifically to highlight the hazard associated with exposure). A concentration of 0.18µg/mm^2 was used due to the fact that this concentration of particles had been previously used within the lab group when assessing the toxicity of particles within the lung, so that comparisons could be made to previous demonstrations of NP toxicity (Stone *et al.*, 1998). The time points of 2,4,6 and 24 hours, can be justified by the fact that the acute toxicity of particles was of particular interest.

5.4.9 Conclusion

The impact of particle exposure on hepatocytes has been determined, and it was observed that the only particles within the PARTICLE_RISK particle panel, that were consistently capable of inducing cytotoxicity within hepatocytes, were the QDs. The mechanisms underlying the development of cytotoxicity were investigated; specifically the initiation of an inflammatory response and oxidative stress, and by evaluating the gross and sub-cellular morphology of cells. By ascertaining which particles within the panel were capable of eliciting toxicity to hepatocytes, NP characteristics that were thought to be most influential in driving the observed toxicity were identified, to give an indication of what NP properties to avoid when designing NPs to avoid the appearance of toxicity. It is therefore suggested that the toxicity of NPs in general and QDs in particular is reliant on their instability, composition, size and charge. The *in vitro* investigation of NP toxicity to hepatocytes is limited by the fact that *in vivo* the combined behaviour of a number of cells types and tissues. It is now necessary to determine if interactions between the different cell populations within the liver will contribute to, or prevent against toxicity associated with NP exposure.

Chapter Six

Evaluating the importance of macrophage- hepatocyte interactions to NP toxicity within the liver

6.1 Importance of considering cellular interactions within the liver when evaluating NP toxicity

The liver is an example of an organ where multiple cell types must co-operate to sustain normal liver function. As a result, cell-cell interactions are an important consideration when evaluating the impact of NP exposure on liver function. Of particular relevance, is the contribution of Kupffer cells to NP mediated toxicity to hepatocytes within the liver. Consequently Kupffer cells present within the lining of blood vessels may enable the removal of NPs from the circulation to limit the contact of NPs with the underlying hepatocytes. As a result, exposure of Kupffer cells to NPs may stimulate the generation of signals to change their function, so that hepatocytes are prepared for NP exposure, or paradoxically may initiate a (inflammatory) response that is able to initiate or potentiate NP toxicity to hepatocytes and thereby affect their function or viability.

6.1.2.1 The contradictory roles exhibited by Kupffer cells within the liver

When a number of cell types co-exist within a tissue, the behaviour of one cell type will inevitably be affected by the activity of surrounding cells; this is accomplished by direct cell contact or via the release of extra-cellularly acting mediators. It is often the case that experiments *in vitro* investigating the toxicity of substances to the liver concentrate on the target cell of interest. However, the relevancy of this is debatable, as cells *in vivo* do not exist in isolation, and are dependent upon communication with surrounding cells, which may contribute to, or attenuate the toxicity of substances. Kupffer cells are considered to act as a primary, first line of defence against foreign material present within the circulation, so that they are strategically situated to enable them to encounter, recognise, internalise and destroy foreign material, and therefore act as a protective barrier to hepatocytes, to prevent potentially toxic substances coming into direct contact with the underlying hepatocytes (Muriel *et al.* 2001). This is exemplified by the finding that polystyrene NPs (50nm) have been observed to accumulate in the liver, which is accounted for by their predominant localisation within Kupffer cells (Ogawara *et al.*, 1999a). However, conversely, on activation Kupffer cells release mediators that may be detrimental to hepatocyte function so their response to toxicants can be regarded as a double-edged sword (Roberts *et al.,* 2007).

The Kupffer cell therefore appears to have contradictory roles; one as a mediator of toxicity and one as a protector from toxicant exposure (Roberts *et al.,* 2007). The 'threshold' hypothesis, also has to be taken into consideration, whereby a limited release of inflammatory mediators from Kupffer cells is protective but their prolonged or excessive release elicits a damaging response (Roberts *et al.,* 2007). This is exemplified within the toxicity of LPS, which is delivered to the liver via the hepatic portal vein from the GIT, and its recognition by Kupffer cells stimulates their activation, which is associated with a moderate release of inflammatory mediators (such as TNFα). It has therefore been suggested that there is a background level of Kupffer cell stimulation, so that they are permanently in a semi-activated state from continued exposure to LPS (Sahu, 2007). However, excessive alcohol consumption, for example, allows more LPS to pass into the circulation from the GIT, and thereby causes an increased stimulation of Kupffer cells, which in turn stimulates the increased release of mediators (such as TNFα) that have damaging effects on hepatocytes (Sahu, 2007). The importance of the 'priming' Kupffer cells has also been observed within the toxicity of lead; whereby pre-treatment with LPS increased TNFα production by Kupffer cells in response to lead administration (Milosevic and Maier, 2000). Therefore, as NPs have been demonstrated to be inflammogenic (see section 5.1.2.3) their propensity to initiate a similar response to that exhibited by the increased exposure of primed Kupffer cells to LPS, is plausible, and of concern.

6.1.2.2 Consequences of Kupffer cell stimulation

The actions of Kupffer cells are thought to be central to the mediation of hepatotoxicity; it is therefore important to consider what particular events are most influential in driving the observed toxicity. The extra-cellularly acting mediators released from activated Kupffer cells that could elicit an inflammatory response, or detrimentally impact on hepatocyte function include cytokines (including TNFα and IL-1), ROS, lipid metabolites (such as prostaglandins and leukotrienes), nitric oxide, hydrolytic enzymes (including proteases), and growth factors (Roberts *et al.,* 2007, Milosevic and Maier, 2000). It has therefore been established that hepatocyte function can be regulated in a paracrine manner to have beneficial or detrimental consequences (figure 6.1). This is exemplified by the finding that Kupffer cells have been demonstrated to induce hepatocyte

proliferation to facilitate repair subsequent to toxicant exposure, but paradoxically this proliferative response can also lead to the development of neoplasia, so that Kupffer cell activity can potentially contribute to hepatic carcinogenesis (Milosvic and Maier, 2000, Roberts *et al.,* 2007). Furthermore communication between different liver cell populations has the ability to detrimentally affect normal hepatocyte function, as exemplified by the finding that decreased CYP2B1 activity in hepatocytes was correlated with increased TNFα release from Kupffer cells in a Kupffer cell/hepatocyte co-culture in response to LPS (Milosevic, Schawalder and Maier, 1999), highlighting that normal hepatocyte function can be negatively affected by Kupffer cell activity. Consequently the Kupffer cell has paradoxical roles within the liver, insinuating that there may be a delicate balance in their ability to provide protection for surrounding cells or inflict damage (Roberts *et al.*, 2007). Cellular communication is therefore an important aspect of liver function and plays a central role in the hepatic response to toxicants (figure 6.1).

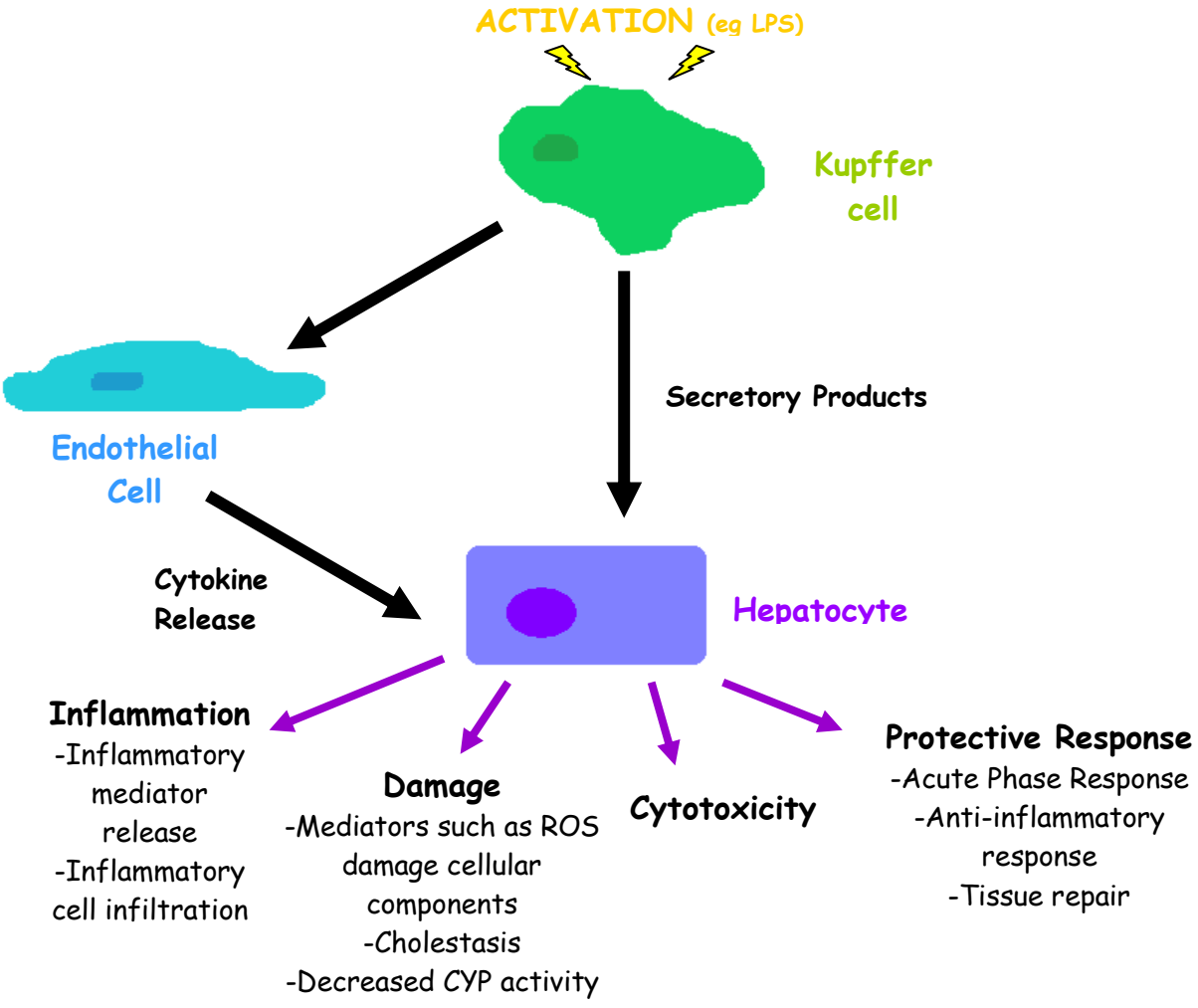


Figure 6.1 Inter-cellular communication within the liver. Kupffer cell activity and its potential outcomes for hepatocyte function are outlined (adapted from Tilg, Kaser and Moschen, 2006).

6.1.2.3 Contribution of Kupffer cells to the toxicity of known hepatotoxins

The toxicity of a variety of well-known hepatotoxins is thought to require the involvement of Kupffer cells. Laskin *et al.,* (1995) confirmed the importance of the contribution of Kupffer cells to acetaminophen toxicity, as rats pre-treated with substances that suppressed Kupffer cell activity, reduced the observed toxicity. Mediators, including ROS, hydrolytic enzymes, TNFα, IL-1 and IL-6, released from activated Kupffer cells were therefore implicated in the toxicity of acetaminophen, due to their cytotoxic action and ability to disrupt normal hepatocyte function (Laskin *et al.,* 1995). However it is noteworthy that the primary mechanism of toxicity is known to be mediated through the production of a reactive metabolite which binds to hepatocyte macromolecules to disrupt their normal function (Sahu, 2007).

Toxicity associated with carbon tetrachloride (CCl_4) exposure arises as a consequence of its metabolism to a reactive molecule which induces hepatocyte cytotoxicity via lipid peroxidation (Sahu, 2007). However it has been suggested that the damage inflicted on hepatocytes also involves the activation of Kupffer cells whose secretory products are able to contribute to hepatocyte damage (Muriel *et al.*, 2001). The pivotal role played by Kupffer cells was confirmed by their depletion, which attenuated CCl_4 mediated toxicity (Muriel *et al.*, 2001). This is therefore another example where Kupffer cells have been implicated in contributing to the toxicity of known hepatotoxins, despite not being the primary cause.

The enhanced production of ROS, cytolytic proteases and inflammatory cytokines from Kupffer cells is thought to be integral to the pathogenesis of alcohol induced liver toxicity (Bautista, 2000). Therefore the ability of Kupffer cells to produce and secrete a wide spectrum of biological mediators, which have the ability to inflict damage, incriminates Kupffer cell activity within alcohol toxicity. The involvement of increased LPS mediated stimulation of Kupffer cells (and subsequent release of TNFα) has been illustrated as being central to alcohol toxicity, which is confirmed by the demonstration that alcohol toxicity can be diminished by preventing Kupffer cell activity (Yin *et al.*, 2001). The involvement of TNFα is further implicated as antibodies against TNFα (to inhibit its function) are able to attenuate the observed toxicity (Iimuro *et al.,* 1997).

Hepatocyte cytotoxicity associated with alcohol exposure has consequently been suggested to occur as a consequence of increased TNFα generation which stimulates apoptosis within hepatocytes (Albano, 2006). Oxidative stress development within hepatocytes is also thought to be central to alcohol toxicity, and arises as a consequence of alcohol metabolism by CYP2E1 within hepatocytes, and indirectly due to the release of ROS from Kupffer cells and neutrophils (Albano, 2006). Therefore the toxicity of alcohol is multifactoral, involving complex interactions between a variety of cell types, including Kupffer cells, neutrophils, and hepatocytes, and a variety of effectors of damage, such as cytokines and ROS.

6.1.2.4 Indirect mechanisms of Kupffer cell mediated hepatotoxicity

In addition to the release of substances from Kupffer cells influencing hepatocyte function, it is also recognised that Kupffer cells that come into direct contact with hepatocytes are able to influence their activity. This is possible due to extensions from Kupffer cells that extend into the space of Disse through endothelial cell fenestrations (Sahu, 2007). The importance of hepatocyte-Kupffer cell contact in influencing the outcome of LPS induced inflammatory responses was investigated by Hoebe *et al.,* (2001) and it was found that cytokine production (TNFα and IL-6) was greater when the cell types were in direct contact. This is an important observation as the experimental set up encompasses the reality that Kupffer cells and hepatocytes can be in direct contact within the liver *in vivo*.

It is not just the action of released factors from Kupffer cells that can affect hepatocyte function, but other indirect mechanisms of toxicity are also evident. The activation of endothelial cells by mediators released from Kupffer cells has been demonstrated to elicit a pro-coagulant state within the liver, leading to the activation of platelets, which compromises blood flow, to have detrimental consequences for hepatocytes due to the decreased supply of oxygen and nutrients (Roberts *et al.*, 2007), and this has been observed by Khandoga *et al.*, (2004) in response to ufCB. It is therefore important to recognise that the liver is an organ composed from a number of cell types that exert a variety of functions, and that many processes contribute to the toxicity observed within hepatocytes, thereby highlighting the complexity of hepatotoxicity.

6.1.3 Investigating cell interactions within the liver

Revealing the processes underlying the communication between different liver cell populations enables the contribution of various biological mediators released from cells to be exposed. Isolated cells allow the response of target cells to potential toxicants to be investigated, however they are thought to be unsuitable for predicting the response of tissues to toxicant exposure, as they have the obvious drawback that they lack the contribution of neighbouring cells. To overcome this, co-cultures composed of two liver cell populations can be used to compare the response that is elicited when separate cell responses are considered. Alternatively conditioned medium (CM), can be collected (from the exposure of one liver cell type to a potential toxicant) and then exposed to another cell liver population to determine how the response differs from that elicited within the individual cells.

The importance of *in vitro* models, which allow the contribution of the different liver cell populations to hepatotoxins, can be gained from the following examples. Milosevic and Maier, (2000) investigated the response of primary rat hepatocytes and Kupffer cells to lead individually, and in combination (termed a co-culture). The findings revealed that Kupffer cells were able to increase the sensitivity of hepatocytes to lead exposure, as hepatocyte cytotoxicity was witnessed only in co-culture conditions. Mawet *et al.,* (1996) investigated the contribution of cell communication to alcohol mediated neutrophil infiltration to the liver. This was achieved through the use of Kupffer cell CM, which was obtained through the exposure of primary rat Kupffer cells to LPS. It was observed that IL-8 production by hepatocytes was enhanced when they were exposed to CM. By using antibodies directed against TNFα, IL-1ß and LPS (to inhibit their function), the substance contained within the CM that was responsible for the enhanced production of IL-8 by hepatocytes was elucidated, and found to be IL-1ß. Therefore it was concluded that IL-8 production by hepatocytes could be regulated by cytokines released from Kupffer cells, which is demonstrative of cytokine networking (Standiford *et al.,* 1990), and is an important aspect of cell interactions within the liver.

6.1.4 Importance of cell interactions to NP toxicity

Revealing the contribution of the different liver cell populations is also worth considering when evaluating the potential toxicity of NPs within the liver, due to the knowledge that more than one cell type contributes to NP toxicity within the lung. It has been demonstrated that L-2 type 2 epithelial cells are able to release cytokines in response to NP treatment (ufCB and TiO_2), which stimulates the chemotaxis of macrophages, so that they are recruited to sites of particle deposition and inflammation (Barlow *et al.,* 2005), thus demonstrating that epithelial cells within the lung were able to influence the activity of alveolar macrophages in an attempt to reduce the extent of damage elicited by particles.

Furthermore Jimenez *et al.,* (2002) illustrated the involvement of an inflammatory cascade in response to particle exposure, whereby macrophages were able to regulate the release of IL-8 from epithelial cells on exposure to PM_{10}, despite the fact that it was found that no increases in IL-8 production were encountered when epithelial cells were exposed directly to PM_{10}. As CM obtained from PM_{10} exposed macrophages contained elevated levels of TNFα it was suggested that PM_{10} stimulated the production of TNFα from macrophages that enhanced IL-8 production from epithelial cells. These findings were corroborated by Ishii *et al.,* (2004) who found that mediators (TNFα and IL-1ß) released from PM_{10} stimulated alveolar macrophages affected the transcriptional regulation of inflammatory mediator genes in epithelial cells. This was supported through the utilisation of antibodies directed against TNFα and IL-1ß within CM, which was able to prevent the inflammatory response evoked within epithelial cells. Cytokines acting in a paracrine manner are therefore an important component of inflammatory responses within tissues, and are implemented, in order to enable the efficient removal of potentially damaging substances.

The studies discussed address the importance of communication between different cell types to particle toxicity, but were primarily conducted within cells derived from the lung, so that the relevance of the findings to the toxicity of NPs within the liver requires consideration. Consequently, it has been observed that the toxicity of NPs involves a cascade of events, whereby the participation of a variety of cells types and cytokines is integral to the inflammatory response *in*

vivo, to encourage the recruitment of cells to the inflammatory site and permit the efficient removal of the inflammogenic stimulus. Furthermore it is not only inflammatory cells that contribute to inflammatory responses, but other resident tissue cells, and their contribution to NP toxicity within other target organs.

6.1.5 Aim

The aim of these experiments was to evaluate the ability of macrophages to influence NP mediated toxicity to hepatocytes. This was achieved through the exposure of macrophages to the PARTICLE_RISK particle panel to obtain conditioned medium (CM) that was exposed to hepatocytes to gain an indication of the importance of cell interactions to NP toxicity within the liver.

6.2 Materials and Methods

6.2.1Materials

RPMI 1640 cell culture medium, and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (Poole, UK). All other materials and equipment were obtained from sources previously stated.

6.2.2 Standardisation of NP exposures

As described for the exposure of hepatocytes (see section 5.2.2.1), the exposure of macrophages to particles was standardised, so that particle concentrations were expressed as µg/mm^2. This enabled the exposure of the different liver cell populations to the PARTICLE_RISK panel to remain consistent. This is further justified by the requirement for the exposure of macrophages to particles to be up-scaled, so that the experiments were performed in 6 well plates, at a volume of 3ml, due to the volume of particle supernatants that were required. Accordingly, macrophages were exposed to particle concentrations of 13, 58 or 113µg/ml in 6 well plates (diameter of 35mm, and surface area of 961.6mm^2), equivalent to 0.04, 0.18 and 0.35 µg/mm^2 respectively, and corresponded to those used previously when hepatocytes were treated.

6.2.3 Obtaining macrophage Conditioned Medium: THP-1 cell subculture, and exposure of differentiated THP-1 cells to the PARTICLE_RISK particle panel

The macrophage CM was generated by Dr Lesley Young (Napier University), using the following protocol. THP-1 cells are a human monocyte leukaemia cell line obtained from the European Collection of Cell Cultures (Salisbury, UK). THP-1 cells are a suspension cell line and are maintained in RPMI 1640 cell culture medium that was supplemented with 10% FCS, 2mM L-Glutamine and 100U/ml Pen/Strep (termed complete THP-1 cell culture medium). THP-1 cells were differentiated so that they acquired a macrophage-like phenotype prior to particle exposure, which was achieved using PMA. PMA was stored in aliquots at -20°C at a concentration of 1mg/ml and diluted in complete cell culture medium to 5ng/ml when required (prepared fresh on the day of the experiment). Cells were plated at a concentration of 3x10^5 cells/ml in 6 well culture dishes (at a volume of 3ml per well) in complete THP-1 cell culture medium that contained

PMA (5ng/ml) and incubated for 48 hours, at 37^{o}C, 5% CO_2, during which time the cells became adherent. The cells were then washed with PBS and exposed to the PARTICLE_RISK particle panel at concentrations of 13µg/ml, 58µg/ml and 113µg/ml (in C3A complete cell culture medium), or appropriate control, for 24 hours (at a volume of 3ml). The supernatants (termed conditioned medium) were collected and centrifuged at 2320g, for 10 minutes in 15ml falcon tubes, to remove the particles. The supernatants were then aliquoted and frozen at -80^{o}C until required.

6.2.4 The impact of particle exposure on THP-1 cell viability, and cytokine release

The LDH activity of CM was assessed as described in section 5.2.5.2. IL-8 levels within CM were determined by ELISA as previously described (see section 5.2.8.3). TNFα content of the CM was determined using a Human TNFα ELISA kit purchased from BioSource (Wheatly, UK) using the protocol outlined in section 5.2.8.3.

6.2.5 Treatment of C3A cells with CM

C3A cells were plated at density of 3x10^{5} cell/ml in 24 well plates (at a volume of 1ml/well) and incubated overnight at 37^{o}C, 5% CO_2. The cells were then exposed to CM for 2,4,6, and 24 hours. The impact of CM exposure on C3A hepatocyte GSH depletion (see section 5.2.3.1), cell viability [using the LDH assay (see section 5.2.5.2)], cytokine analysis using a Human 17-plex kit (see section 5.2.8.2) and IL-8 cytokine release using a BioSource kit (see section 5.2.8.3) were conducted as previously described.

6.2.6 Significance analysis

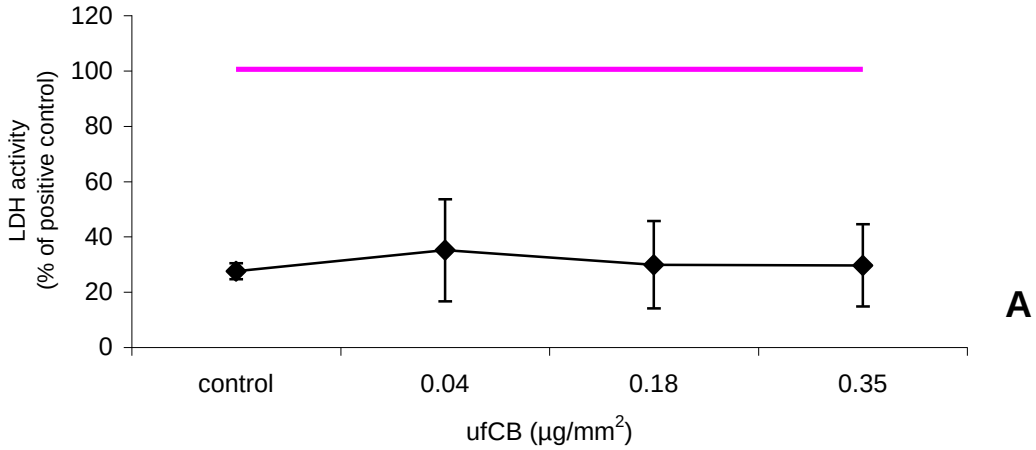
One way ANOVA analysis was used to assess the significance of results, whereby particle exposures were compared to the control treatments at each time point. Results were considered statistically significant if $p<0.05$.

6.3 Results

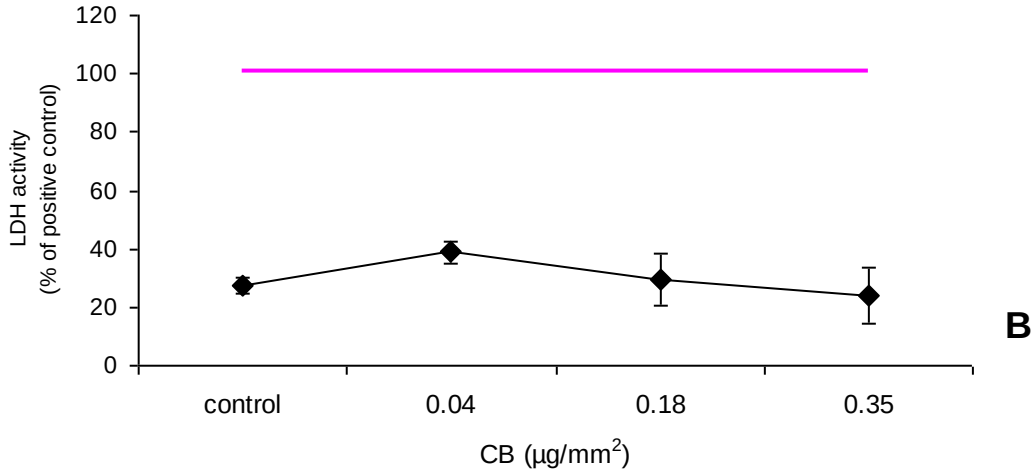
6.3.1 Impact of NP exposure on THP-1 macrophage viability

No significant changes in THP-1 macrophage cell viability, relative to the control (using LDH release as an indicator), were observed when exposed to ufCB, CB, and CNT for 24 hours, at all concentrations investigated (figure 6.2).

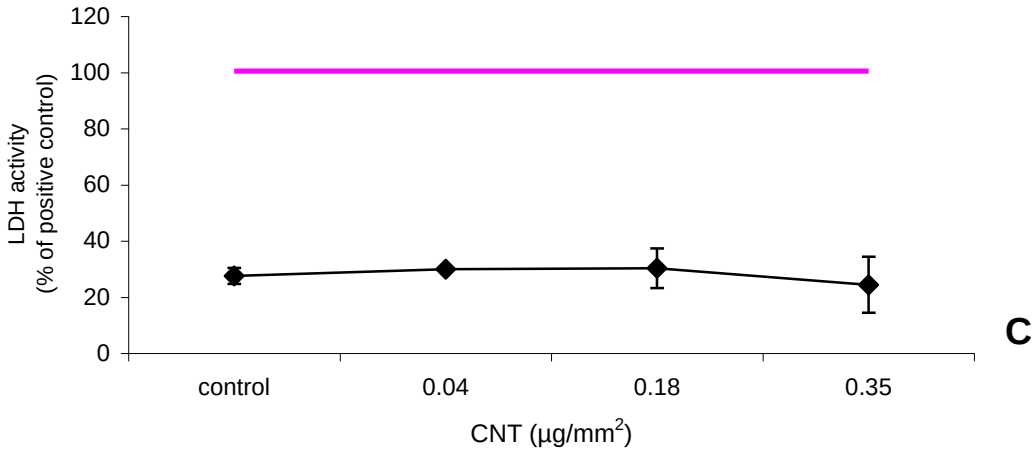
Exposure of macrophages to QD621, QD620 and C_{60} induced cell death (figure 6.2). Macrophages exposed to QD621, induced significant increases in LDH activity within cell supernatants at concentrations of 0.04µg/mm^2 (p=0.0305), 0.18µg/mm^2 (p=0.0074) and 0.35µg/mm^2 (p=0.03) after a 24 hour exposure. It was evident that exposure to QD620 (p=0.0436) and C_{60} (p=0.050) were cytotoxic when exposed to macrophages at a concentration of 0.35µg/mm^2, for 24 hours.


120
100
80
60
40
20
0
LDH activity
(% of positive control)
control
0.04
0.18
0.35
ufCB (µg/mm2)

A


120
100
80
60
40
20
0
LDH activity
(% of positive control)
control
0.04
0.18
0.35
CB (µg/mm2)

B


120
100
80
60
40
20
0
LDH activity
(% of positive control)
control
0.04
0.18
0.35
CNT (µg/mm2)

C

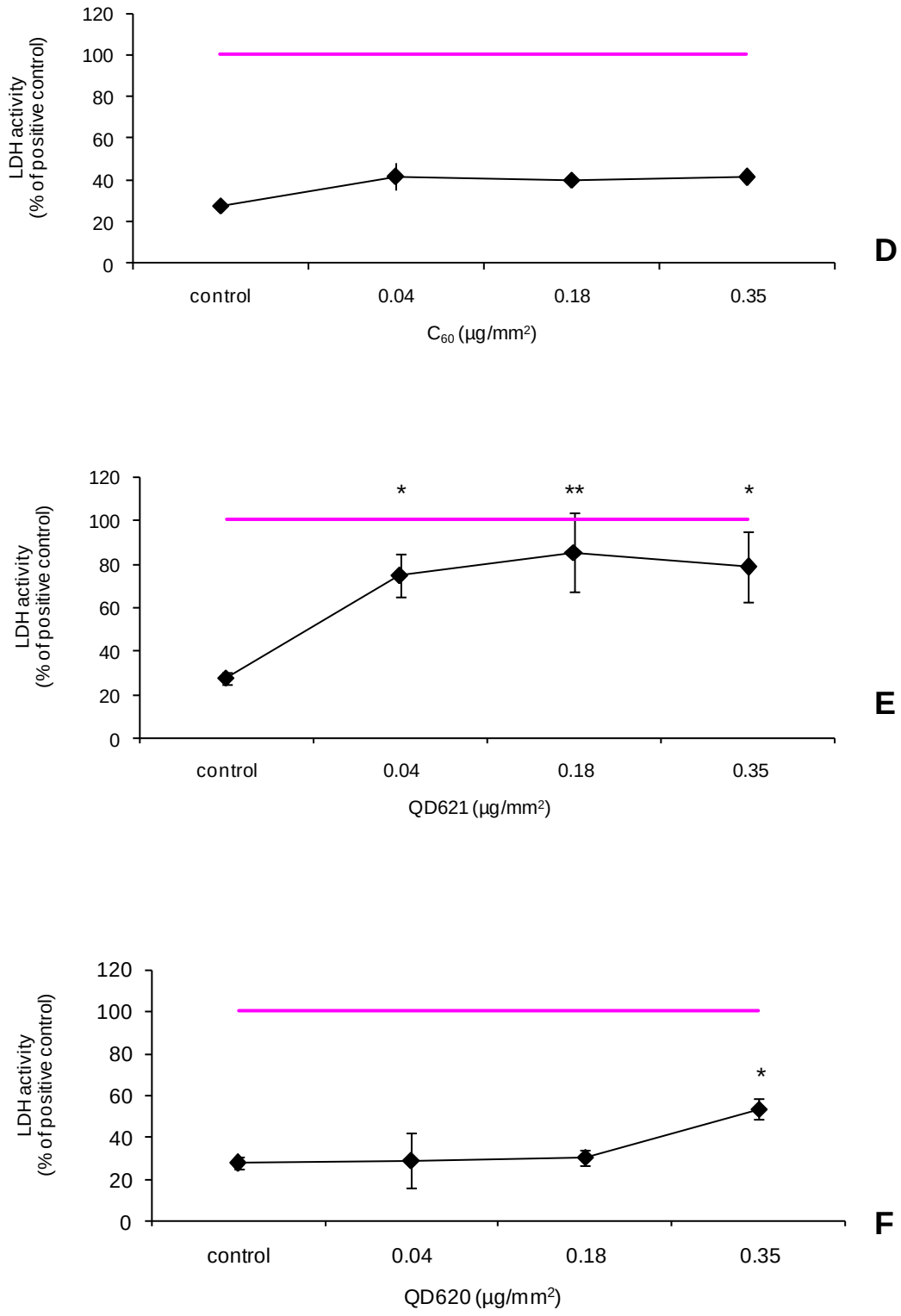
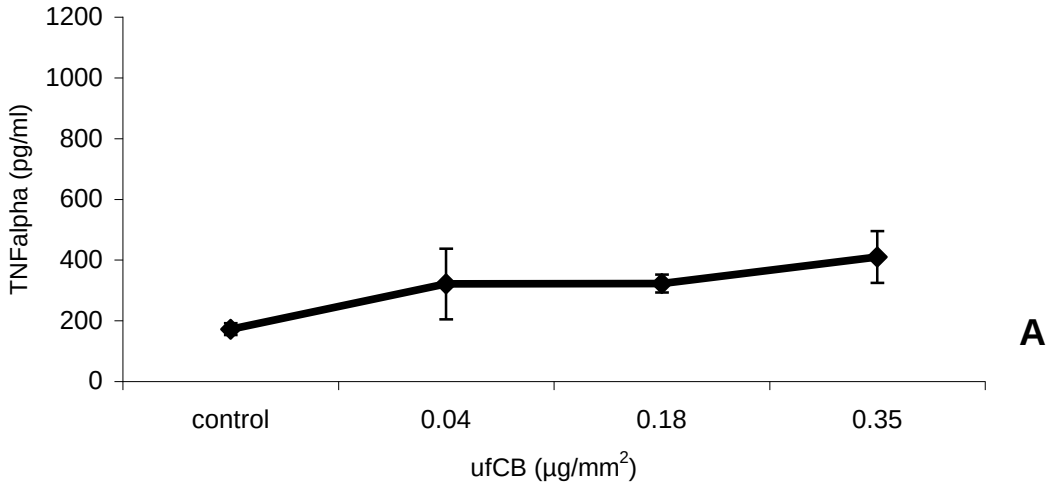


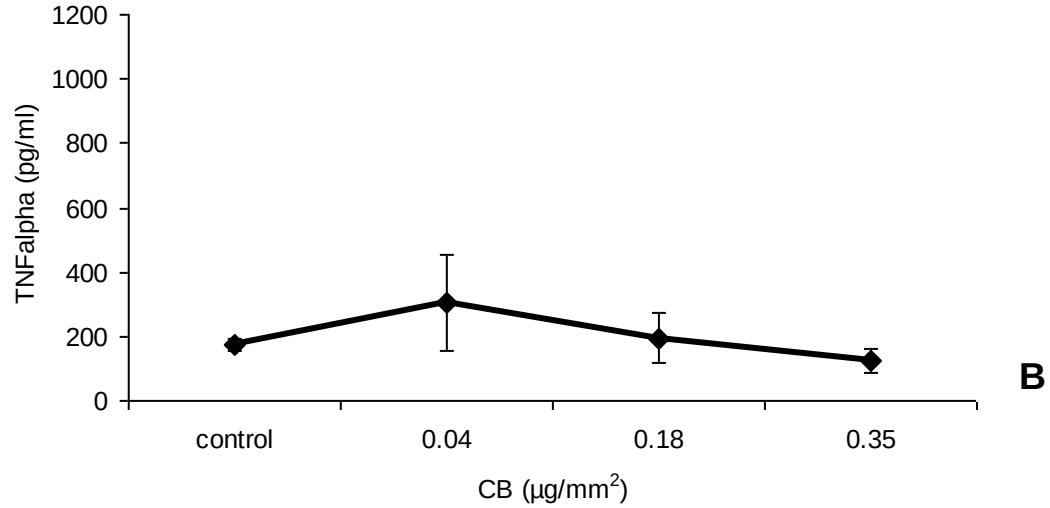
Figure 6.2 THP-1 cell LDH release after exposure to the PARTICLE_RISK panel of particles. Differentiated THP-1 cells were exposed to cell medium (control), 0.04, 0.08, or 0.35µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**), or Triton X-100 (positive control) for 24 hours and LDH activity in the cell supernatant measured. Results are expressed as a % of the positive control and values represent mean ± SEM (n=3), significance indicated by * = $p<0.05$, ** = $p<0.01$, when particle treatments were compared to the control group.

6.3.2.1 Effect of NP exposure on TNFα release from THP-1 macrophages

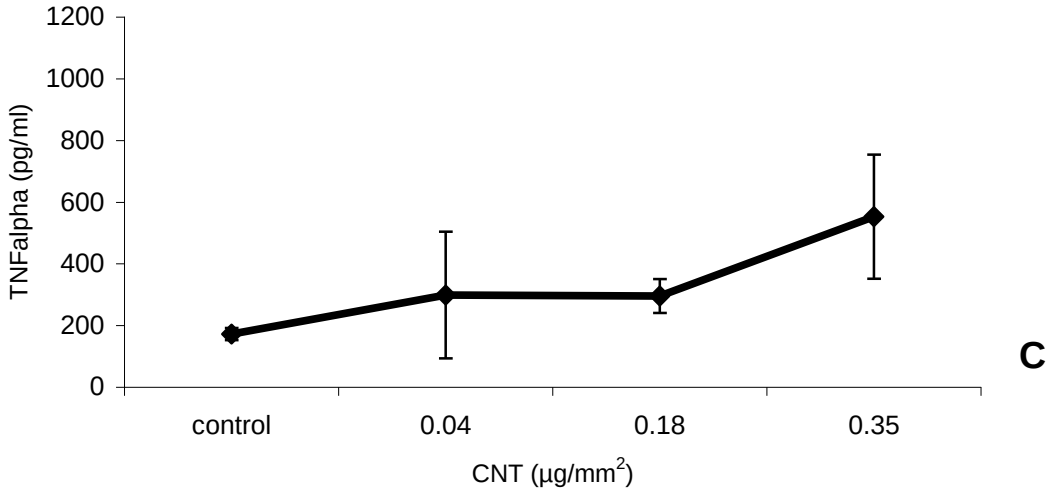
No significant increases in TNFα production were observed when THP-1 macrophages were exposed to ufCB, CB, CNT or C_{60} for 24 hours, when compared to the control group (figure 6.3). However there was a tendency for ufCB to elicit a dose dependent increase in TNFα production by THP-1 cells (figure 6.3A). It was observed that a significant increase in TNFα release was stimulated by QD621 after a 24 hour exposure of THP-1 cells, at a concentration of 0.04µg/mm^2 (p=0.0312, figure 6.3E). Higher concentrations of QD621 tended to induce an increase in TNFα levels, for example a concentration of 0.35µg/mm^2 QD621 increased TNFα levels from 172±20pg/ml in the control group to 616±111 (figure 6.3E), but this effect was lesser than the lower concentration of QD621. The greatest increase in TNFα release from THP-1 cells was evident when exposed to QD620 for 24 hours at concentrations of 0.18µg/mm^2 (p=0.006) and 0.35µg/mm^2 (p=0.0021), which can be observed in figure 6.3F.


1200
1000
800
600
400
200
0
TNFalpha (pg/ml)
control
0.04
0.18
0.35
ufCB (µg/mm2)

A


1200
1000
800
600
400
200
0
TNFalpha (pg/ml)
control
0.04
0.18
0.35
CB (µg/mm2)

B


1200
1000
800
600
400
200
0
TNFalpha (pg/ml)
control
0.04
0.18
0.35
CNT (µg/mm2)

C

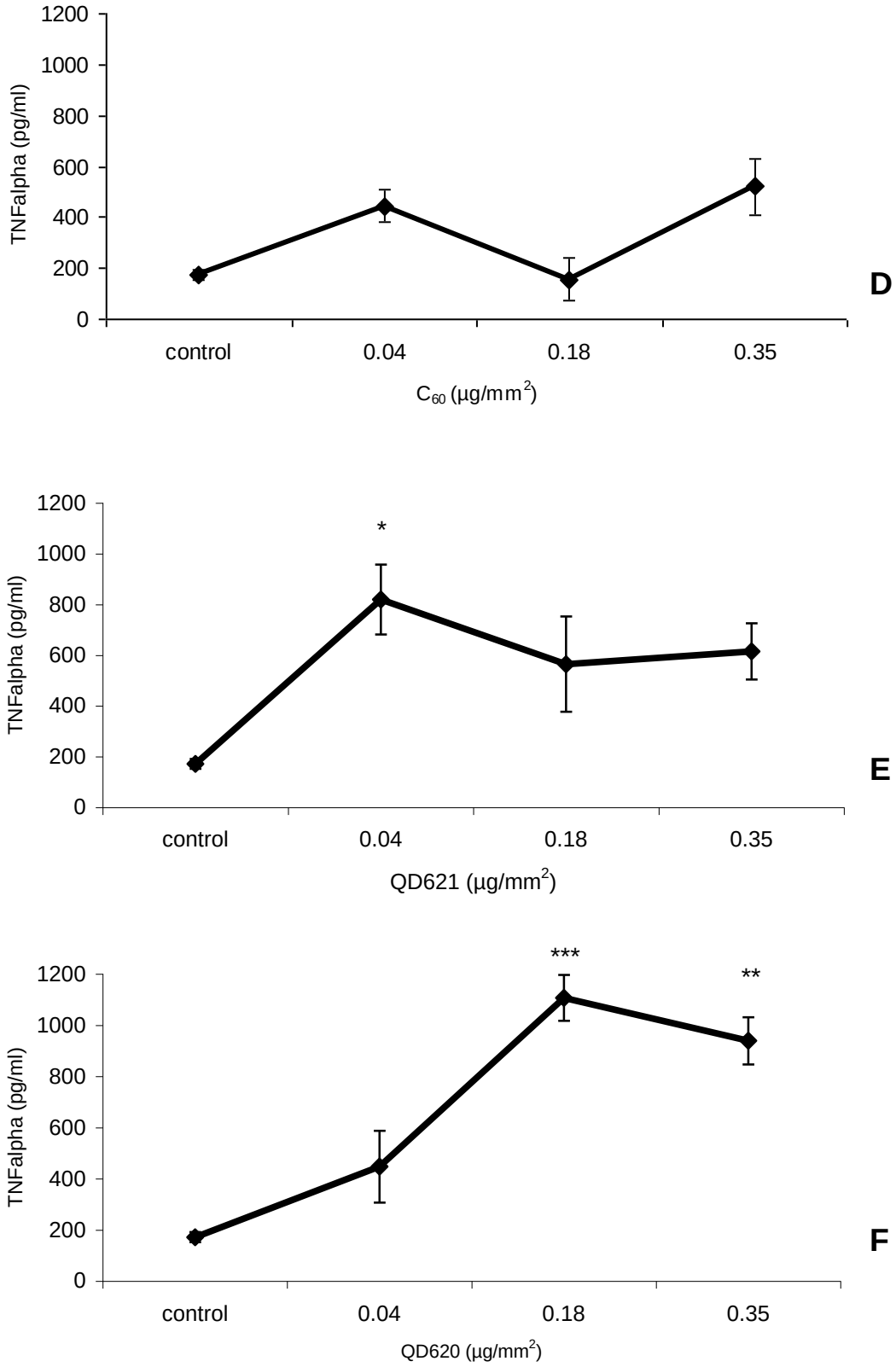
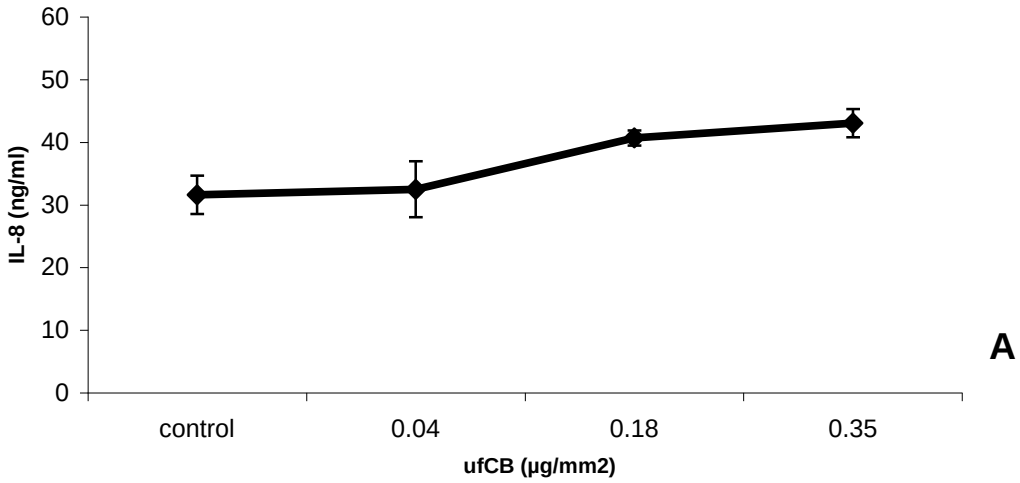


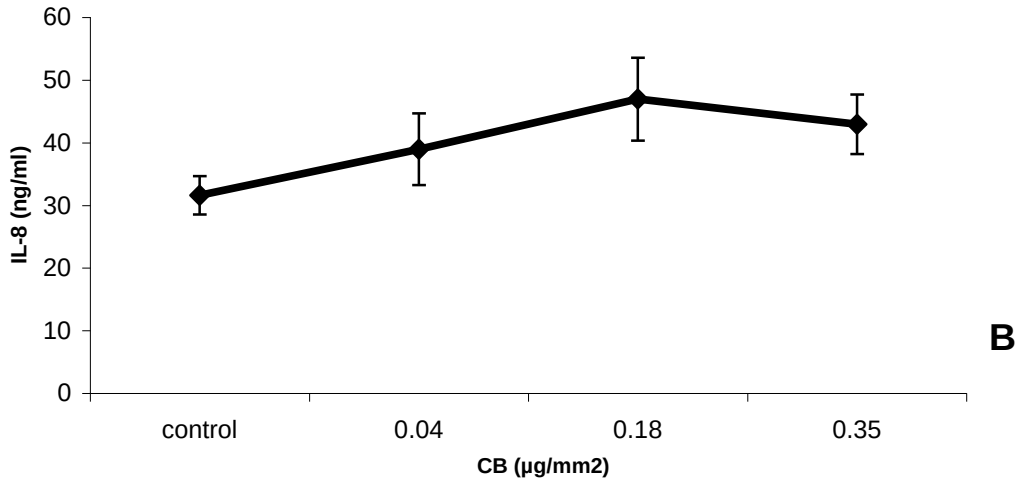
Figure 6.3 TNFα release from THP-1 cells following exposure to the PARTICLE_RISK particle panel. Differentiated THP-1 cells were treated with cell medium (control), 0.04, 0.18 or 0.35µg/mm^2 ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) or QD620 (**F**), for 24 hours and TNFα production within cell supernatants determined by ELISA. Values represent mean ± SEM (n=3), significance indicated by * = p<0.05, ** = p<0.01 *** = p<0.001 when particle treatments were compared to the control group.

6.3.2.2 NP induced IL-8 release from THP-1 macrophages

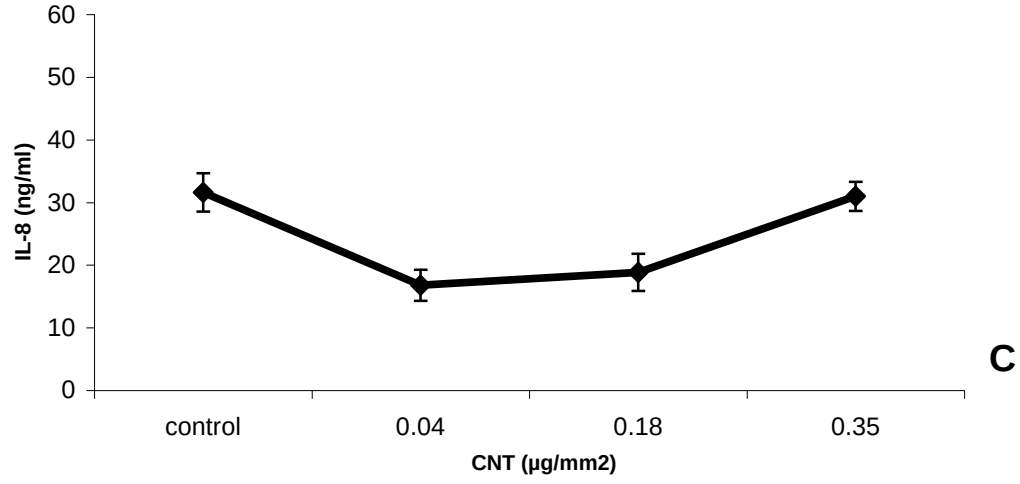
No increases in IL-8 production were observed when macrophages were exposed to ufCB, CB, CNT, C_{60} and QD621 (figure 6.4). There was a tendency for ufCB to increase IL-8 levels in a dose dependent manner but this was not significant (figure 6.4A). There was also a tendency for QD621 to increase IL-8 production at a low concentration, as IL-8 levels increased from 31608±3041pg/ml in the control group to 45128±4108pg/ml in cells exposed to 0.04µg/mm^2 QD621 (figure 6.4E). At higher concentrations (0.18 and 0.35µg/mm^2) QD621 tended to reduce IL-8 levels to lower than that of the control group (figure 6.4E). A significant increase in IL-8 release was apparent when THP-1 cells were exposed to QD620 for 24 hours at concentrations of 0.18µg/mm^2 (p=0.0204) and 0.35µg/mm^2 (p=0.0212).


60
50
40
30
20
10
0
IL-8 (ng/ml)
control
0.04
0.18
0.35
ufCB (µg/mm2)

A


60
50
40
30
20
10
0
IL-8 (ng/ml)
control
0.04
0.18
0.35
CB (µg/mm2)

B


60
50
40
30
20
10
0
IL-8 (ng/ml)
control
0.04
0.18
0.35
CNT (µg/mm2)

C

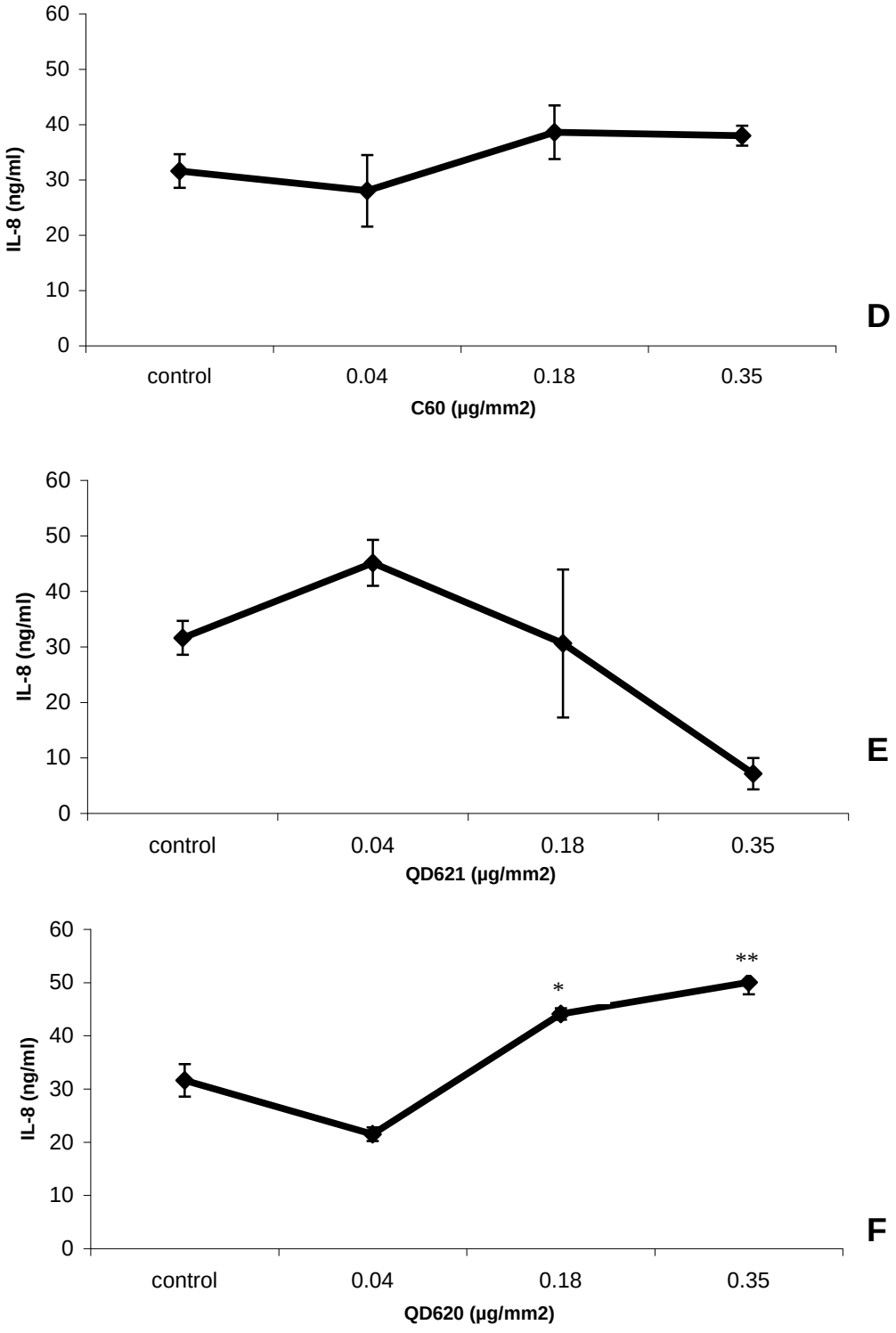
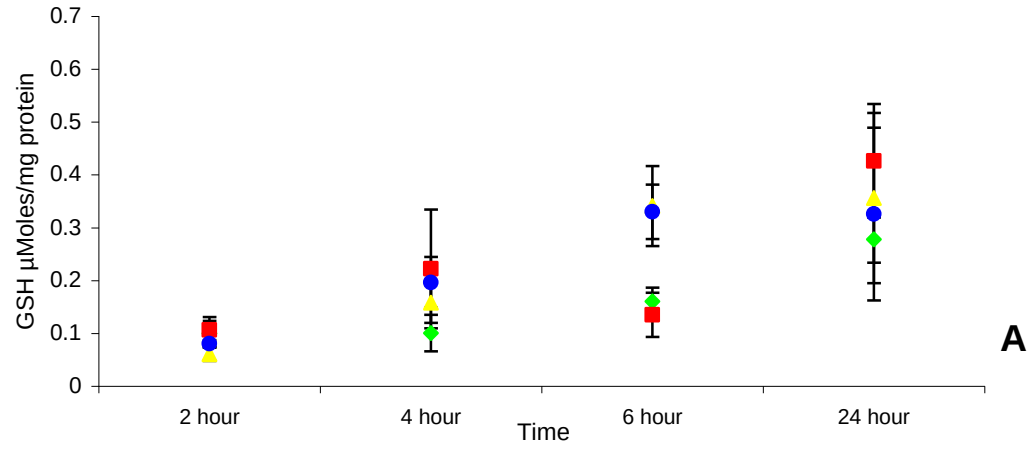


Figure 6.4 IL-8 release from THP-1 cells following exposure to the PARTICLE_RISK panel of particles. Differentiated THP-1 cells were treated with cell medium (control), 0.04, 0.18 or 0.35µg/mm^{2}ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) or QD620 (**F**), for 24 hours and IL-8 release within the cell supernatants determined by ELISA. Values represent mean ± SEM (n=3), significance indicated by * = $p<0.05$, ** = $p<0.01$ when particle treatments are compared to the control group.

6.3.3 Ability of CM to induce oxidative stress in C3A hepatocytes

When exposed to ufCB, CB, CNT, C_{60} and QD620 CM, no changes in GSH content within C3A cells were observed at any of the time points investigated (figure 6.5). It was apparent that there was a dose and time dependent depletion of GSH on exposure of C3A hepatocytes to QD621 CM. After a 4 hour exposure to QD621 CM (0.35µg/mm^2) there was a definite tendency for GSH depletion, as GSH levels decreased from 0.101±0.04µMoles GSH/mg protein in the control group to 0.016±0.008 µMoles GSH/mg protein, however this finding was not significant (figure 6.5E). After a 6 hour exposure to 0.35µg/mm^{2}QD621 CM, a significant depletion of GSH was evident (p=0.0316), within hepatocytes, with a tendency for a dose dependent depletion of GSH with the other treatments (figure 6.5E). At 24hours, exposure to QD621 CM induced a significant decrease in cellular GSH with 0.18µg/mm^2 (p=0.0016) and 0.35µg/mm^2 (p=0.0016) QD621 CM treatments, with a tendency for a depletion in GSH content evident at a concentration of 0.04µg/mm^2 (figure 6.5E). There was a tendency for QD620 CM exposure to increase GSH levels within hepatocytes after a 4 hour exposure, for example exposure of hepatocytes to QD620 CM at a concentration of 0.35µg/mm^2 induced an increase in GSH levels from 0.1±0.03µMoles GSH/mg protein in the control group to 0.28±0.03µMoles GSH/mg protein in C3A cells exposed to 0.35µg/mm^2 QD620 CM, although this was not statistically significant (figure 6.5F).


0.7
0.6
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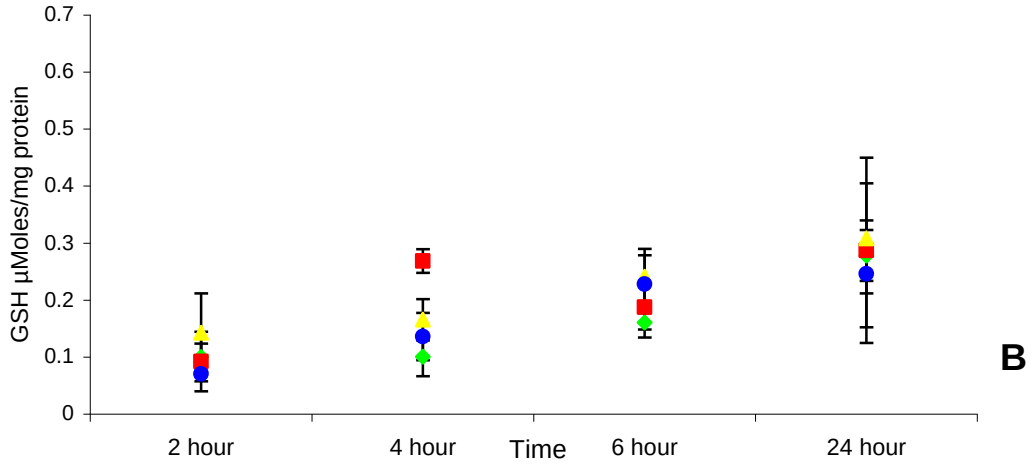
0
GSH µMoles/mg protein
2 hour
4 hour
6 hour
24 hour
Time

A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GSH µMoles/mg protein
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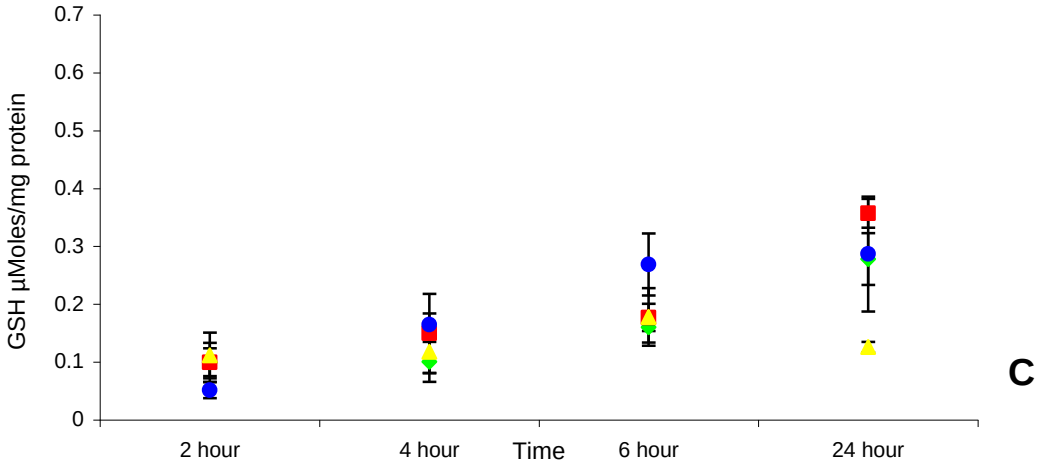
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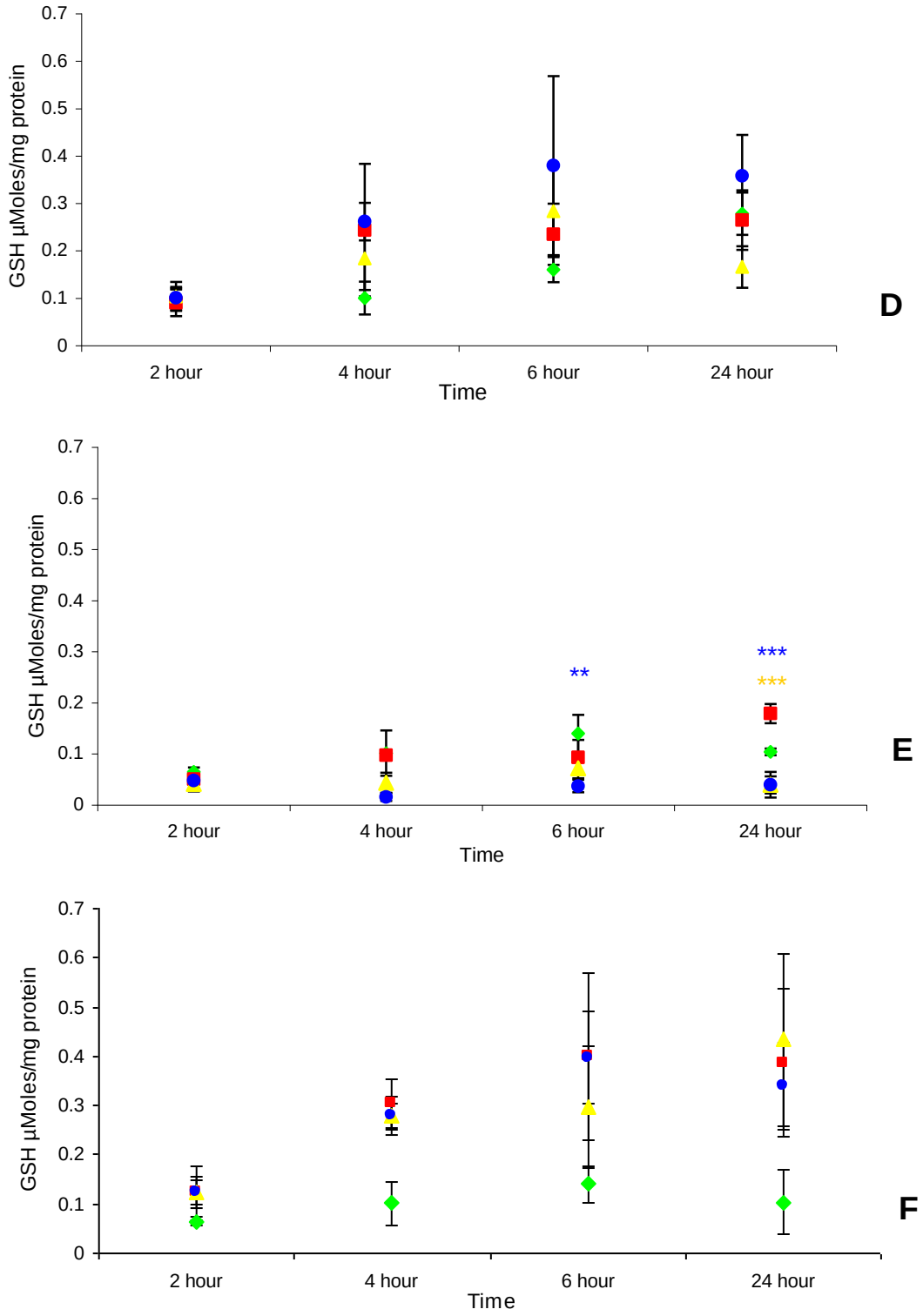
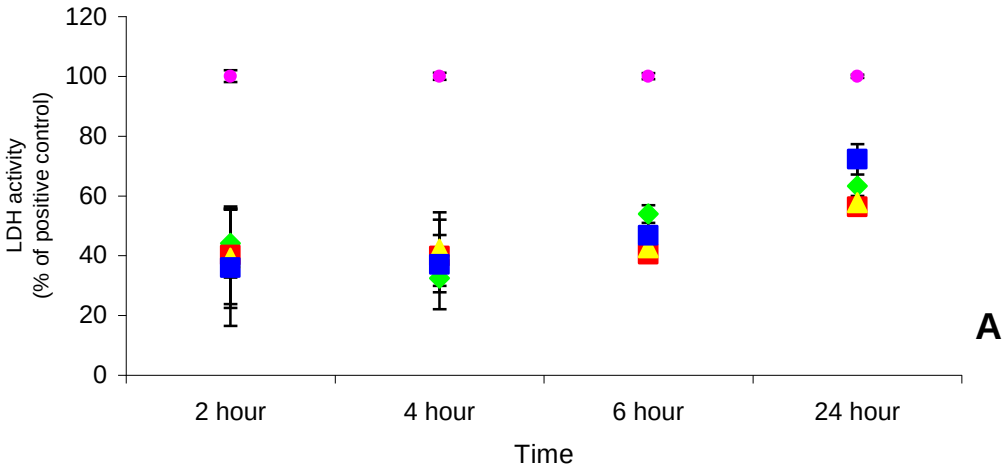


Figure 6.5 C3A hepatocyte GSH content after exposure to CM. THP-1 cells were exposed to the PARTICLE_RISK particle panel (at concentrations of 0.04, 0.18 or 0.35µg/mm^2), or control (complete C3A cell culture medium) for 24 hours and the supernatants, termed CM, collected and exposed to C3A hepatocytes for 2,4,6 or 24 hours. GSH concentrations within C3A cells exposed to ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**) and ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**) CM were determined. Values represent mean ± SEM (n=3), significance indicated by ** = p<0.01 *** = p<0.001 when particle treatments are compared to the control group.

6.3.4 Impact of CM on hepatocyte viability: assessed using the LDH assay

Exposure to ufCB, CB, CNT, C_{60} and QD620 CM, elicited no significant changes in LDH release from C3A cells at any of the time points investigated, although exposure to QD620 CM tended to increase cell death within C3A cells at 24 hours (figure 6.6).

It was observed that LDH release from C3A cells increased in a dose and time dependent manner, when exposed to QD621 CM (figure 6.6E). At 6 hours, a significant release of LDH (compared to the control treatment) from C3A cells was induced by QD621 CM at particle concentrations of 0.18µg/mm^2 ($p=0.0005$) and 0.35µg/mm^2 ($p=0.0003$), with the extent of cell death being comparable to that of the positive control. At 24 hours all QD621 CM treatments of 0.04µg/mm^2 ($p=0.0015$), 0.18µg/mm^2 ($p=0.0008$), and 0.35µg/mm^2 ($p=0.0012$) elicited a release of LDH from C3A cells that was equivalent to that of the positive control.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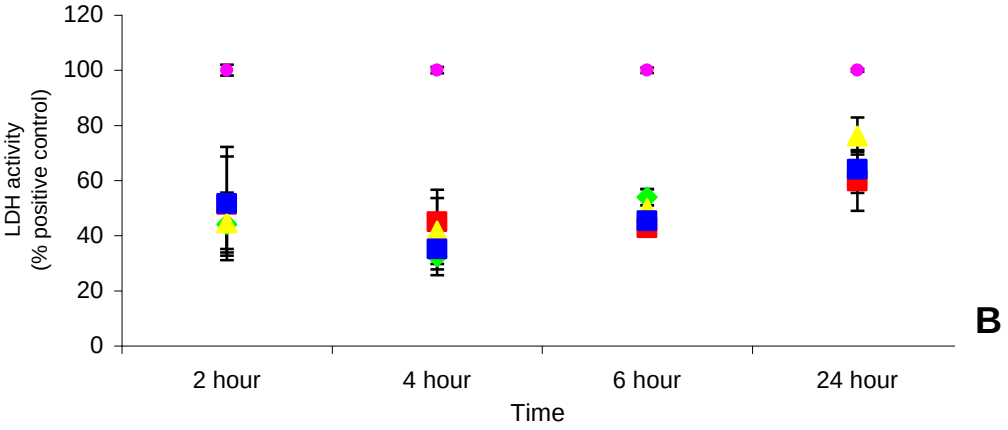
100
80
60
40
20
0
LDH activity
(% of positive control)
2 hour
4 hour
6 hour
24 hour
Time
A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LDH activity
(% positive control)
2 hour
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24 hour
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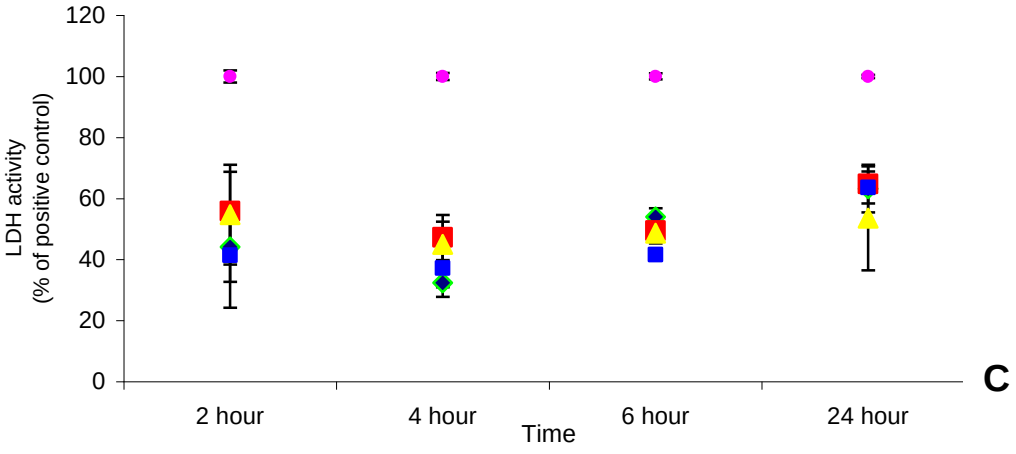
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LDH activity
(% of positive control)
2 hour
4 hour
6 hour
24 hour
Time
C

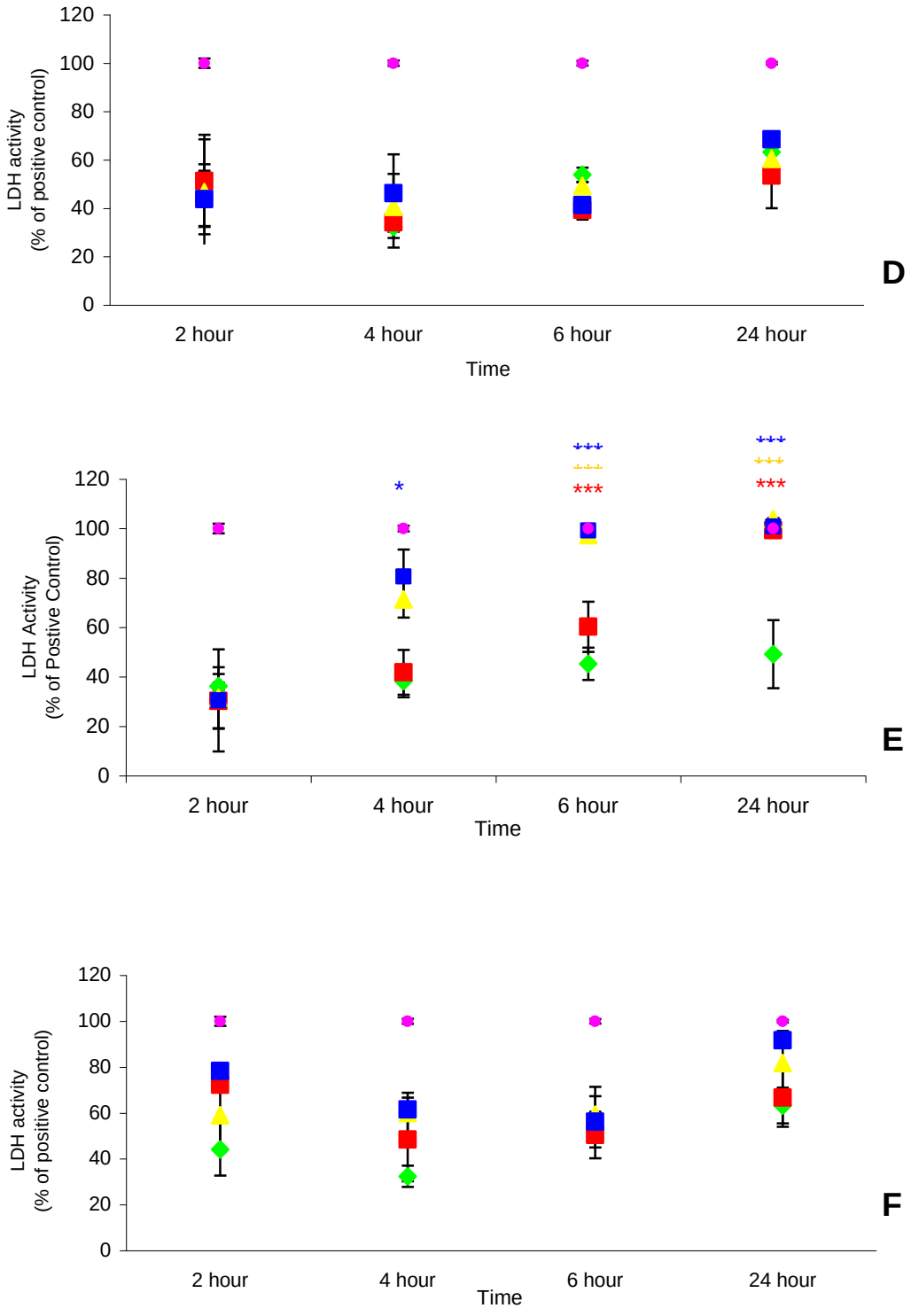
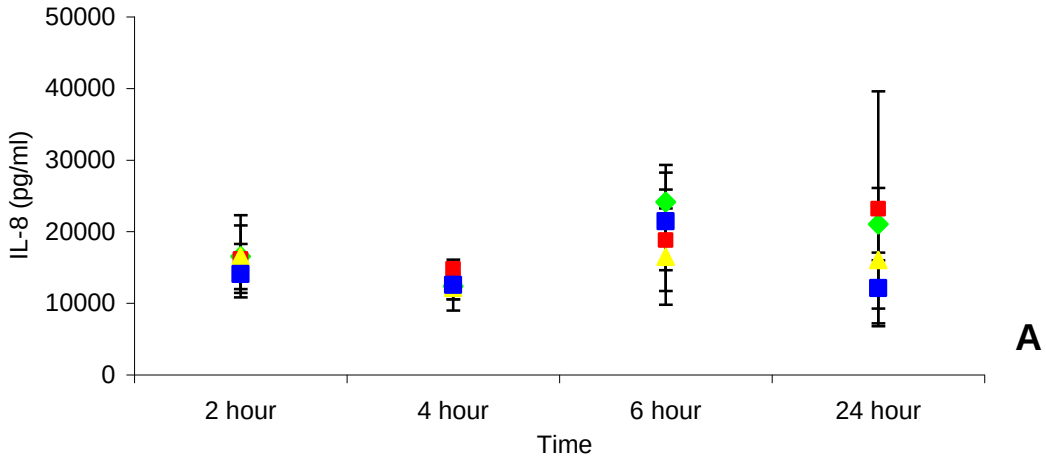


Figure 6.6 CM stimulated LDH release from C3A cells. THP-1 cells were exposed to the PARTICLE_RISK particle panel (at concentrations of 0.04, 0.18 or 0.35 µg/mm^2), equivalent control (complete C3A cell culture medium) or Triton-X100 positive control for 24 hours and the supernatants, termed CM, were collected and exposed to C3A hepatocytes for 2,4,6 or 24 hours. LDH activity within the C3A supernatants of cells exposed to control CM and ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**) and ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**) CM were determined. Results are expressed as a % of the positive triton control. Values represent mean ± SEM (n=3), significance indicated by * = p<0.05, ** = p<0.01 *** = p<0.001 when particle treatments are compared to the control group.

6.3.5 Impact of CM exposure on IL-8 release from C3A hepatocytes

When exposed to ufCB, CB, CNT, C_{60}, QD621, and QD620 CM, there were no significant changes in IL-8 release from C3A cells at any of the time points investigated (figure 6.7). It was observed that there was a tendency for QD621 CM treated cells to reduce IL-8 levels to lower than that of the controls which was especially evident at higher concentrations (0.18 and 0.35µg/mm^2) at all time points (figure 6.7E). There was also a tendency for QD620 CM treated cells to increase IL-8 levels, but this was not significant (figure 6.7F).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IL-8 (pg/ml)
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Time
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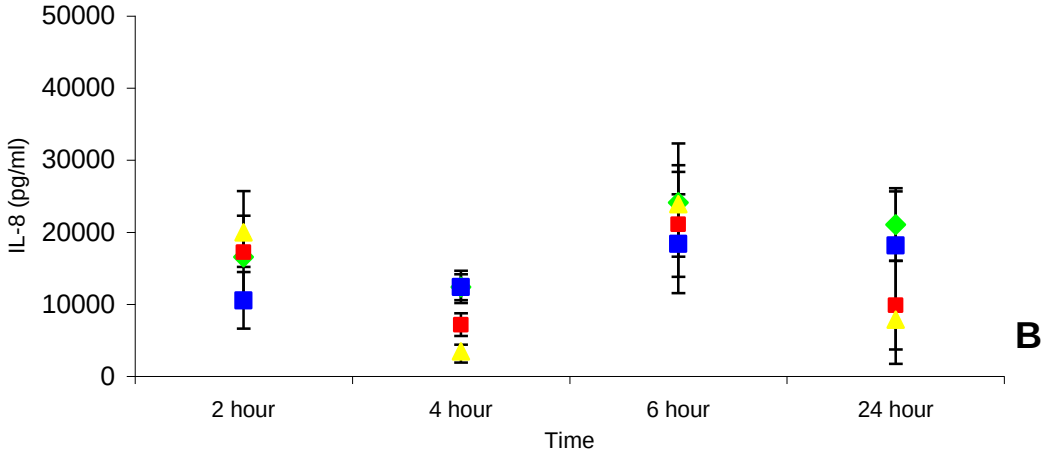
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Time
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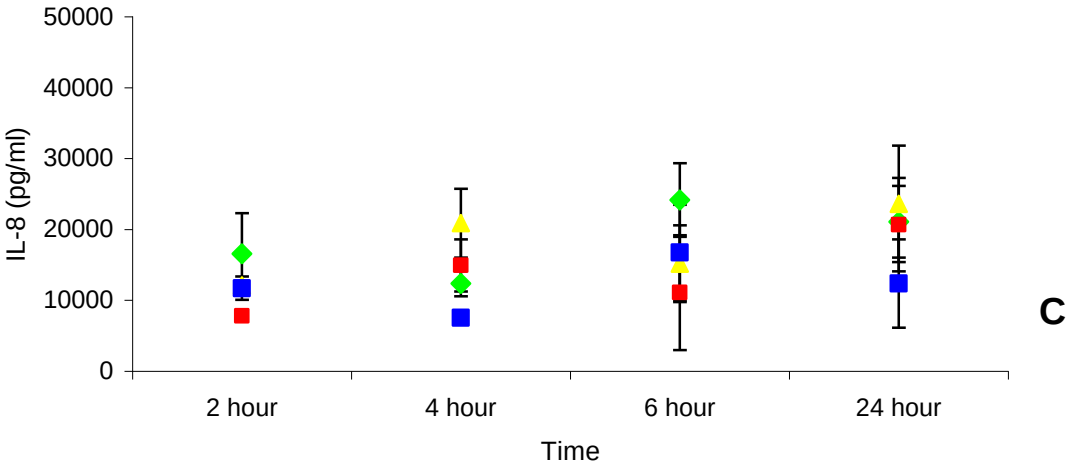
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IL-8 (pg/ml)
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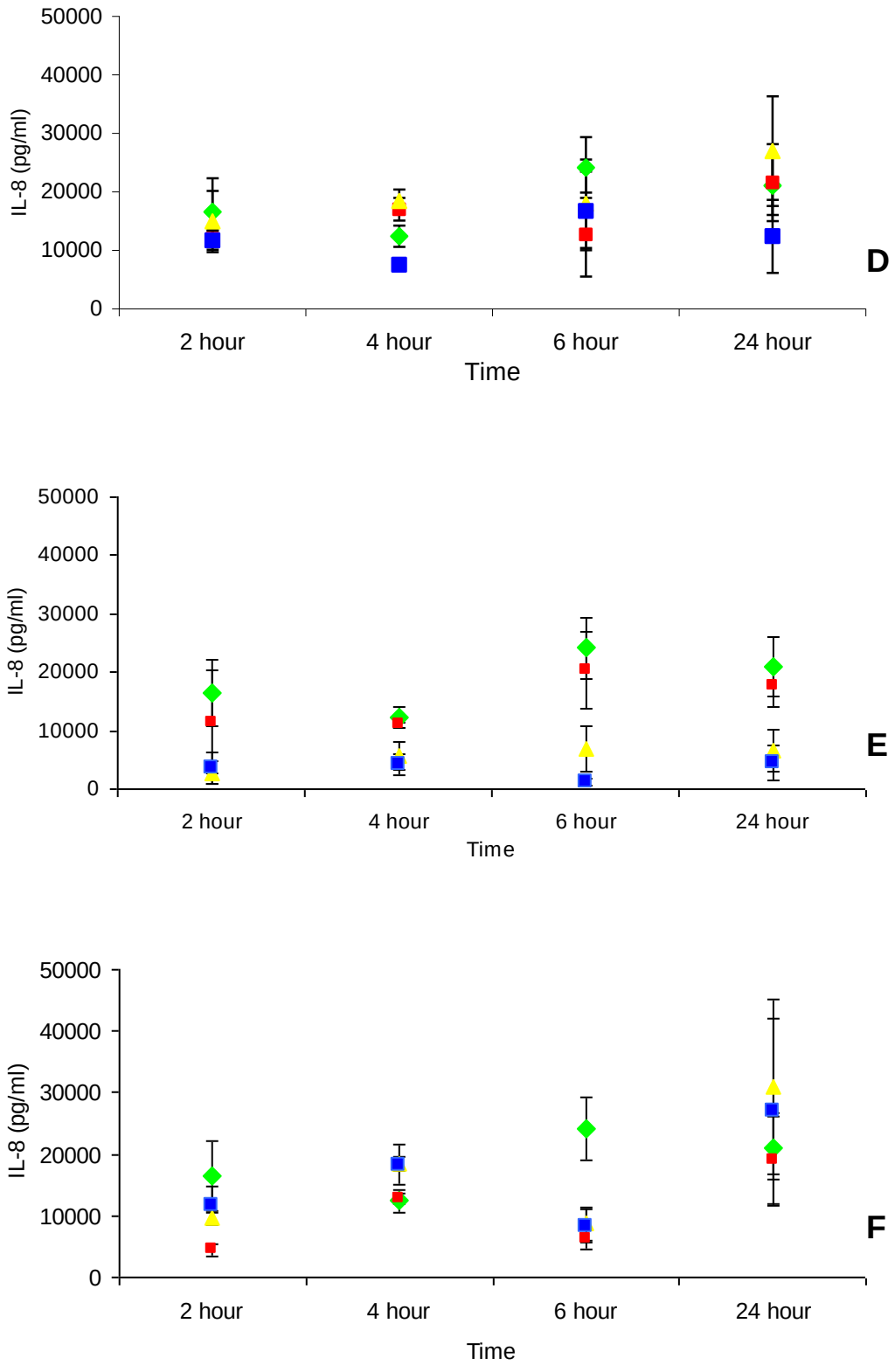

Figure 6.7 CM induced IL-8 release from C3A hepatocytes THP-1 cells were exposed to the PARTICLE_RISK particle panel (at concentrations of 0.04, 0.18 or 0.35 μg/mm^2), or equivalent control (complete C3A cell culture medium) for 24 hours, and the supernatants, termed CM exposed to C3A cells for 2,4,6 or 24 hours. IL-8 levels within C3A cell supernatants of cells exposed to ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**) and ufCB (**A**), CB (**B**), CNT (**C**), C_{60} (**D**), QD621 (**E**) QD620 (**F**) CM were determined by ELISA. Values represent mean ± SEM (n=3).

6.4 Discussion

Within the liver several cell types are evident and thus cell-cell interactions are an important consideration when evaluating the impact of NP exposure on normal liver function. Accordingly the influence of macrophage-hepatocyte interactions to the toxicity of the PARTICLE_RISK particle panel within the liver was investigated so that the ability of macrophages to attenuate or potentiate particle toxicity to hepatocytes was determined.

6.4.1 Relevance of the model used

It is acknowledged that the synthesis and release of biological factors by Kupffer cells is able to exert profound effects on neighbouring cells. It was therefore of interest to determine if macrophages were able to impact on the toxicity of NPs to hepatocytes within the liver. The experimental set up allowed the ability of macrophage secretory products to influence hepatocyte function to be investigated to determine the importance of cellular interactions within the liver. The response of the individual cells to particles was ascertained, in addition to evaluating the impact of CM on hepatocyte function, so that the ability of macrophages to potentiate particle toxicity to hepatocytes could be explored. The experimental set up was therefore deemed an appropriate model to investigate cell interactions within the liver as the responses of individual cells, and CM exposed hepatocytes could be compared. With regards to the CM protocol used, it would have been preferable to obtain a human Kupffer cell line to assess the importance of macrophage–hepatocyte interactions to NP toxicity, in order to strengthen the relevance of the study, however this was limited by their availability. Kupffer cells were therefore substituted with the THP-1 monocytic cell line that could be differentiated to allow cells to adopt a macrophage phenotype. As Kupffer cells within the liver are derived from circulating monocytes (Bilzer *et al.,* 2006), these cells are believed to be a relevant alternative.

6.4.2 The impact of NP exposure on macrophage function

6.4.2.1 NP induced macrophage cytotoxicity

Cytotoxicity was observed when macrophages were exposed to QD621, QD620 and $C_{60,}$ with the other particles present within the PARTILCE_RISK panel unable to compromise macrophage viability. The ability of QD621 to induce cell

death was observed to the greatest extent, and evident at all concentrations investigated. This finding is therefore in keeping with the toxicity of the PARTICLE_RISK panel to hepatocytes. *In vivo* the ability of particles to induce cytotoxicity within macrophages would inevitably affect the function of neighbouring cells (particularly hepatocytes, due to their abundance), as factors released from dying cells have the ability to initiate an inflammatory reaction or cell death within surrounding cells (Gores, Herman and Lesmaster, 1990). Furthermore the fact that macrophage viability was reduced could make the other liver cell populations more susceptible to circulating toxins that would otherwise be effectively cleared from macrophages, so that the ability of NPs to reduce macrophage viability is able to compromise their role within host defence. However, it is worthy of consideration that just because THP-1 cells were sensitive to the toxicity of certain NPs, this may not be evident within Kupffer cells, which can be illustrated by the different responses exhibited by hepatocyte cell lines and primary cells.

6.4.2.2 The inflammatory response associated with exposure of macrophages to NPs

The ability of particles to induce an inflammatory response within the liver was determined, as the release of inflammatory mediators from macrophages has the potential to initiate an inflammatory cascade that involves the participation of hepatocytes to ultimately have detrimental consequences for their normal function. The release of TNFα from macrophages exposed to the PARTICLE_RISK particle panel was considered, as the release of this inflammatory mediator has been implicated in the hepatotoxicity of a number substances including alcohol (Stewart *et al*., 2001), and has been central to the inflammatory response induced as a consequence of NP exposure to the lung (Driscoll *et al.*, 1997). Furthermore TNFα is a multi-functional cytokine, and is capable of contributing to a number of key events within inflammatory responses, such as increased adhesion molecule expression, and the stimulation of cytokine and chemokine production, so that elevated production is integral to the initiation, progression, and maintenance of inflammatory responses. IL-8 is a chemokine that is responsible for the attraction of neutrophils to sites of inflammation, and its release has been demonstrated to

characterise the response of NP exposure *in vivo* (Li *et al.*, 1999) and *in vitro* (Brown *et al.*, 2001).

Exposure to ufCB, CB, CNT, and C_{60} did not induce an increase in the release of TNFα or IL-8 from differentiated THP-1 cells. This is contrary to previous findings within the literature; as differentiated human monocytes have been demonstrated to be susceptible to the inflammatory effects of NPs, such as ufCB (Brown *et al.*, 2004). Furthermore it was unexpected that exposure of macrophages to the PARTICLE_RISK particle panel did not elicit an increase in IL-8 levels, as this cytokine has been documented to characterise pulmonary inflammatory responses to particles *in vivo* and *in vitro* (see section 5.1.2.3), often at a similar concentration to those used within the studies described, with macrophages postulated to be a primary source of this cytokine. Only QDs were capable of eliciting an increase in cytokine production by macrophages. It was found that a low concentration (0.04μg/mm^2) of QD621 was able to induce a significant increase in TNFα production by macrophages, and a trend for an increase in IL-8 production was identified. However higher concentrations (0.18 and 0.35μg/mm^2) of QD621 were observed to increase TNFα levels (but to a lesser extent than a concentration of 0.04μg/mm^2), and to lower IL-8 levels to less than that of the control group. The reduction in cytokine production at higher concentrations of QD621 can be explained by the knowledge that the majority of THP-1 cells were non-viable and therefore unable to contribute to IL-8 and TNFα production. Accordingly, QD621 are thought to be capable of stimulating macrophages, at sub-lethal concentrations. The most profound particle induced release of cytokines from macrophages was observed on exposure to QD620. However it is noteworthy that a decrease in cell viability was used to explain the lack of cytokine production in treated cells (at high concentrations). It is therefore contradictory to observe the greatest increase in cytokine levels within macrophages exposed to 0.35μg/mm^2 QD620 when a significant level of cytotoxicity was detected. However it is known that QD621 has a greater toxic potential in comparison to QD620. This is exemplified in the finding that all concentrations of QD621 were able to elicit cytotoxicity within macrophages, but only the highest concentration of QD620 was able to cause significant levels of cell death. Therefore it was likely that a higher proportion of QD620 treated THP-1 cells were viable at earlier time points, and were

therefore capable of contributing to cytokine production despite a loss of viability comparable to that of QD621 exposed cells at 24 hours. Therefore, as witnessed for the hepatocytes (see section 5.4.4.1) QD621 are thought to have a greater inflammatory potential than QD620, if sub-lethal concentrations are used.

As cytokine release was associated with macrophage exposure to QDs it is relevant to discuss the implications of their increased production. The increased production of TNFα is known to be an early event within liver inflammatory responses (Wullaert *et al.*, 2007) and one of its many roles is to trigger the recruitment of inflammatory cells. IL-8 is a neutrophil chemoattractant, and its release *in vivo* is likely to promote the infiltration of neutrophils to the liver. Therefore in addition to resident cell populations contributing to the toxicity of particles, inflammatory cells such as neutrophils that are recruited to the liver have the ability to contribute to liver toxicity. However the potential of QDs to induce an inflammatory response that culminates in the infiltration of neutrophils to the liver is of concern, as neutrophils are capable of eliciting hepatocyte damage due to the release of damaging factors such as proteases, inflammatory mediators and ROS (Jaeschke *et al.*, 2002). Furthermore, it has been demonstrated that particle mediated neutrophil infiltration and activation results in the release of ROS which can influence the generation of carcinogenicity due to the ability of ROS to regulate cell proliferation and induce DNA damage (Knaapen *et al.*, 1999). Therefore the infiltration of neutrophils, as a consequence of increased pro-inflammatory cytokine production within the liver requires further consideration which would necessitate the completion of *in vivo* studies.

Excessive exposure of hepatocytes to TNFα is known to be capable of inducing cell death, and thus TNFα has been implicated in the pathogenesis of ischemia reperfusion injury (where liver damage occurs following the restoration of the oxygen supply) and within chronic inflammatory conditions such as alcoholic liver disease (Wullaert *et al.*, 2007). Conversely, TNFα can stimulate hepatocyte proliferation, which is beneficial as the replacement of damaged cells is promoted. However if compensatory hepatocyte regeneration is too high, cancer development can occur (Wullaert *et al.*, 2007). Pathologies within

the liver have therefore been observed to result from a disruption in the balance of excessive cell death and proliferation, in which TNFα plays a fundamental role in regulating (Wullaert *et al.*, 2007). Furthermore Kupffer cells have been demonstrated to be the primary source of TNFα, for example lead induced TNFα production within hepatocyte-Kupffer cell co-cultures were equivalent within Kupffer cell and co-culture conditions, so that Kupffer cells were identified as the main source of this cytokine (Milosevic and Maier, 2000).

6.4.3 Importance of interactions between hepatocytes and macrophages to NP toxicity within the liver

6.4.3.1 The impact of CM exposure on hepatocyte GSH levels

It was established that the only particle CM capable of inducing a depletion of GSH within hepatocytes was exposure to QD621 CM. This was consistent with findings from experiments that were conducted in hepatocytes exposed to particles suspended in cell culture medium, where only QD621 was capable of depleting GSH within hepatocytes. However, QD621 CM exposure was able to deplete GSH levels after a 6 hour exposure, which occurred earlier than previously encountered. This suggests that exposure of macrophages to QD621 results in the release of factors, or soluble cadmium, that act to encourage the development of oxidative stress within hepatocytes. However, as previously apparent, it is likely that the depletion of GSH mediated by QD621 arises as a consequence of a loss of cell viability. Thus, QD621 CM was able to induce cytotoxicity at earlier time points (4 hours onwards), which accounts for the depletion of GSH evident within a shorter exposure time, than observed previously when hepatocytes were directly exposed to NPs.

6.4.3.2 CM mediated hepatocyte cytotoxicity

It was demonstrated that exposure to ufCB, CB, CNT, C_{60} or QD620 CM did not impact on hepatocyte viability. It was observed that only QD621 CM was able to cause an increase in hepatocyte cell death. Therefore the pattern of response was observed to be consistent with the findings observed when hepatocytes and macrophages were exposed to particle suspensions directly, whereby QD621 was the most potent particle within the PARTICLE_RISK panel capable of inducing toxicity.

It was apparent that QD621 CM exposure elicited a reduction in cell viability that was evident at earlier time points than that encountered previously when hepatocytes were exposed to particles directly, so that secretory products released from macrophages are thought to potentiate QD621 toxicity. Conversely, the enhanced cytotoxicity of QD621 CM on hepatocytes when compared to QD621 exposure alone may also be a consequence of the fact that soluble components (such as cadmium) are released from QD621 into the CM and are responsible for the observed toxicity Therefore, although the release of macrophage derived factors (such as TNFα) was of particular relevance within the CM studies, soluble factors released from NPs in general, and QDs in particular are also a potential source of toxicity.

Alternatively, LDH already contained within QD621 CM, prior to the exposure of hepatocytes may contribute to the increased LDH release witnessed in QD621 CM treated hepatocytes. This is feasible due to the fact that QD621 was observed to elicit cytotoxicity within macrophages, and therefore LDH is known to be contained within the CM. Therefore to determine if LDH present within CM contributed to the observed toxicity, the LDH activity within CM was subtracted from that evident within supernatants derived from hepatocytes exposed to CM, and was determined in all treatment groups, at the 2 hour time point. It was found that hepatocytes exposed to control and particle CM, except for QD621, contributed to the LDH activity measured within the hepatocyte supernatants, indicating that LDH activity within the hepatocyte supernatants derived from hepatocytes. However it was revealed hepatocytes exposed to QD621 CM, do not contribute further to LDH release due to the high background level of LDH present within CM. The lack of hepatocyte contribution to LDH activity may arise due to the fact that components within QD621 CM bind to LDH to prevent its detection at early time points so that the LDH activity measured is lower than expected, or that macrophage derived LDH is responsible for the apparent toxicity observed.

6.4.4 CM induced release of IL-8 from C3A hepatocytes

Multiplex analysis revealed that, as witnessed in hepatocytes exposed to particles, only IL-8 levels were increased within hepatocyte supernatants exposed to CM. It was observed that the levels of IL-8 released from C3A

hepatocytes exposed to CM were much higher (>10000pg/ml) than those encountered when hepatocytes were directly exposed to particles where IL-8 production was lower (<400pg/ml) in all treatment groups. However it is assumed that the majority of IL-8 contained within hepatocyte supernatants derives from CM, as the levels of IL-8 were lower than those derived from macrophages. Consequently, it was observed that only limited increases in IL-8 were apparent on exposure of hepatocytes to CM, and it is therefore suggested that the majority of IL-8 present within CM exposed hepatocyte supernatants derives from that produced by macrophages. Consequently, despite the fact that hepatocytes have been illustrated to secrete IL-8 in response to particle exposure (see section 5.4.4.1), the macrophage population tested within this *in vitro* system is thought to be the primary source of this cytokine. However this is thought to be a realistic finding as within the liver, macrophages will be responsible for responding first to inflammogenic stimuli, and due to their immunological nature would be expected to contribute greatest to inflammatory responses.

6.4.5 Protective effect of macrophages to QD620 mediated toxicity to hepatocytes

QD620 CM treatments elicited no cytotoxicity in the hepatocytes, which is in contrast to the findings produced when particles were exposed directly to hepatocytes. Therefore perhaps macrophages initiate a protective response through the release of, unknown factors, or that macrophages or centrifugation steps remove the toxic components associated with QD620 CM exposure, so that they do not have the opportunity to elicit toxicity within hepatocytes. It was also found that exposure to QD620 CM resulted in an increase in GSH cell content at 4 hours, so that perhaps macrophages, on exposure to QD620 prepare hepatocytes for exposure to a potential toxicant, through an upregulation of GSH to ultimately protect hepatocytes from cell death. Furthermore TNFα is known to participate in the development of inflammatory responses which can elicit damage, however TNFα is also capable of initiating protective responses within the liver. This is exemplified by the knowledge that TNFα can initiate an acute phase response to protect and warn of danger (Dong *et al.* 1998) and can initiate liver regeneration and hepatocyte proliferation (Tigl, *et al*., 2006). Therefore it is also possible that TNFα released from

macrophages is able to promote hepatocyte proliferation to counteract and compensate for and toxicity mediated by QD620. Consequently, as TNFα levels were highest in QD620 exposed macrophages, and QD620 viability was not reduced as a consequence of QD620 (as was expected) it is suggested that TNFα may be one of the mediators released from macrophages that is able to afford protection to hepatocytes from QD620 toxicity, or promote hepatocyte regeneration that is lost due to QD620 toxicity.

6.4.6 Contribution of NPs to CM effects

The particles contained within CM were eliminated via centrifugation (as evidenced by the production of a particle pellet), however CM may be contaminated with a small concentration of particles. Therefore it is possible that particles remained within the CM that could be directly responsible for the increased toxicity of CM exposed hepatocytes when compared to particle treatments alone, as CM induced hepatocyte toxicity preceeded that of particle mediated toxicity. However, it is relevant to assume that the enhanced toxicity of CM is associated with the release of mediators and factors from macrophages, or due to the release of cadmium into the CM, as particle concentrations within CM would be minimal. Furthermore it was a possibility, when evaluating cytokine release and LDH activity that the CM contained these components, and that their increased presence within hepatocyte supernatants was responsible for the enhanced toxicity of CM as discussed previously.

6.4.7 Conclusion

The response of the liver to particles is thought to involve the participation of a number of cell types, and evaluating the responses of individual cells in isolation does not provide a realistic model of NP toxicity to the liver. By considering the arrangement of liver cells *in vivo,* a more accurate reflection, and prediction of NP behaviour *in vivo* can therefore be obtained. Consequently, as macrophages present within the sinusoidal lining are likely to encounter NPs first, they may protect against or enhance the toxicity of NPs to underlying hepatocytes. The results obtained highlight how different cell types can respond similarly to NPs, as it was apparent that hepatocytes and macrophages were most susceptible to the toxicity of QD621, and that QD621 toxicity to hepatocytes was potentiated by the stimulation of macrophages. In addition it is

relevant to consider that the response of cells will be dictated by the role of the cell in question; for example macrophages have a defensive immunological role and are therefore equipped with a number of substances that are able to eliminate and detoxify substances, which explains why macrophages are primarily accountable for the inflammatory response initiated by QDs. Therefore, the response of a tissue to NPs *in vivo* is inevitably going to involve the participation of multiple cell types, and it was revealed that communication between cell types may enhance (in the case of QD621), or protect (as for QD620) against the toxicity of particles. In addition it is necessary to emphasise that although not all particle types were capable of inducing toxicity within the liver, they may be toxic to another organ.

Chapter Seven

General Discussion

7.1 How the toxicity of NPs to the liver was investigated

The aim of the studies outlined within the thesis was to investigate the toxicity of the PARTICLE_RISK particle panel to the liver. The ability of NPs to be internalised by hepatocytes, and negatively impact on hepatocyte function was investigated with greatest interest, due to their abundance within the liver and importance to the maintenance of normal liver function. The impact of NP exposure on macrophage function was also evaluated, due to their role within host defence. Finally, the importance of cellular interactions to NP toxicity within the liver was investigated through the exposure of hepatocytes to macrophage CM, due to the fact that when multiple cell types are evident within an organ such as the liver, neighbouring cells are able to elicit profound effects on one another, by protecting against or potentiating the toxicity of substances.

7.2 Evaluating the uptake and intracellular fate of fluorescent polystyrene particles within hepatocytes *in vitro*

The uptake of carboxylated fluorescent polystyrene particles by HepG2 and C3A cell lines and hepatocyte couplets was found to be in the main, comparable, so that particle uptake was size, time and serum dependent. The uptake of 200nm polystyrene particles was minimal, with the majority of particles evident at the cell surface. However, it would be ideal to confirm, and quantify the level of uptake using, for example, flow cytometry. Polystyrene NPs (20nm) were internalised to a greater extent by hepatocytes, with little or no evidence of accumulation within early endosomes or lysosomes, but some limited NP accumulation was identified within the mitochondria of cell lines but not primary hepatocyte couplets, however this requires verification. There was some suggestion of excretion of NPs into bile canaliculi; this was not extensive, but may provide a route of elimination of NPs from the body. It is noteworthy that fluorescent polystyrene NPs were used as an experimental tool, prior to the supply of the PARTICLE_RISK particles, to investigate the uptake and fate of NPs within hepatocytes, so that their use provided generalisations about the potential size and time dependence of NP uptake by hepatocytes to be made. However it is acknowledged that not all NPs behave similarly and studying the uptake and so the intracellular fate of the PARTICLE_RISK panel was also determined to a limited extent, through the use of TEM.

7.3 Comparing the toxicity of the PARTICLE_RISK particle panel

An *in vitro* screen-like approach was used to evaluate the toxicity of the PARTICLE_RISK particles, in order to identify the particles within the panel that had the greatest potential to elicit toxicity within the liver. This was accomplished using particle concentrations deemed to be low, medium or high in magnitude (based on previously published work conducted within lung epithelial cells and macrophages, Stone *et al.*, 1998) within an acute exposure setting (ranging from 2 to 24 hours). This allowed a comparison in particle toxicity to be made, and was of benefit when a large number of particles required toxicological assessment.

7.3.1 The impact of NP exposure on hepatocyte function

The toxic potential of each particle type within the PARTICLE_RISK panel to hepatocytes was determined, to allow a comparison of their toxicity to be made, and is outlined in table 7.1. It was apparent that QDs were consistently able to negatively impact on hepatocyte function, which was evident within all aspects of hepatocyte function investigated, so that these NPs were highlighted to have the greatest toxic potential within the panel. It was observed that QD621 were most toxic, with CB exhibiting a lack of toxicity to hepatocytes, and that CNTs were the most toxic carbon based particle.

Table 7.1 The negative impact of the PARTICLE_RISK particle panel on hepatocyte function. The detrimental effect of particle exposure on GSH depletion, cell viability (using the MTT and LDH assays), bile secretion, IL-8 release and gross and sub-cellular morphology is indicated by; - for no effect, + for an effect, ++ a moderate effect and +++ for a relatively large effect.

Endpoint / Particle	GSH Depletion	Cytotoxicity (LDH)	Cytotoxicity (MTT)	Bile Secretion	IL-8 Release	Gross Morphology (SEM)	Sub-cellular Morphology (TEM)
ufCB	-	-	-	+	-	+	-
CB	-	-	-	-	-	-	-
CNT	-	-	+	-	-	+	+
C_{60}	-	-	-	-	-	++	+
QD621	+++	+++	+++	+++	++	+++	+++
QD620	-	++	++	-	+++	++	++

7.3.2 The impact of NP exposure on macrophage function

The response of macrophages to the PARTICLE_RISK particles was also considered, and the toxicity induced by the particles to macrophages is outlined in table 7.2. It was again evident that QDs induced the greatest extent of toxicity (witnessed by their ability to induce cytotoxicity and an inflammatory response) in comparison to the other particle types investigated, where it was noteworthy that CNTs and CB were unable to have a negative impact on macrophage function or induce an inflammatory response.

Table 7.2 The detrimental impact of the PARTICLE_RISK particle panel on macrophages. The detrimental effect of particle exposure on cell viability (using the LDH assay), IL-8 and TNFα release is indicated by - for no effect, + for an effect, ++ a moderate effect and +++ for a great effect.

Endpoint / Particle	Macrophage Cytotoxicity	Macrophage TNFα release	Macrophage IL-8 release
ufCB	-	+	+
CB	-	-	-
CNT	-	-	-
C60	+	-	-
QD621	+++	++	++
QD620	+	+++	+++

7.3.3 The ability of conditioned medium to impact on hepatocyte function

The importance of macrophage-hepatocyte interactions was investigated to determine if factors released from macrophages were able to influence hepatocyte function, and the findings are summarised in table 7.3. It was found that QD621 CM produced the greatest toxic response within hepatocytes, with toxicity evident at earlier time points than the toxicity encountered within hepatocytes exposed to particles. Conversely QD620 CM was able to protect against the toxicity evident within hepatocytes exposed to particles, and was believed to derive from an upregulation in GSH, and increased hepatocyte proliferation induced by TNFα. All other carbon particles within the panel induced no, or minimal toxicity.

Table 7.3 The detrimental impact of CM on hepatocyte function. The ability of CM to negatively influence intracellular GSH concentration, hepatocyte viability (using the LDH assay), and IL-8 release, is indicated by -, for no effect, + for an effect, ++ a moderate effect and +++ for a great effect.

Particle / Endpoint	GSH depletion	Hepatocyte Cytotoxicity	Hepatocyte IL-8 release
ufCB CM	-	-	+
CB CM	-	-	-
CNT CM	-	-	-
C60 CM	-	-	-
QD621 CM	+++	+++	+
QD620 CM	-	+	+

7.4 Do cell lines and primary isolated rat hepatocyte couplets behave similarly?

Primary cells are not able to grow indefinitely in culture, due to the fact that after a number of population doublings cells enter a state of replicative senescence, where they are no longer able to divide, which impacts on their function. Therefore, in order to assess the consequences of toxicant exposure on primary cells, cells must be isolated each time they are required. Cell lines are cells that have been immortalised so that they acquire an extended replicative capacity. An important remit of the assessment of NP toxicity to the liver was to determine if cell lines were capable of mimicking the response of primary cell exposure, in order to ensure that a relatively realistic prediction of NP behaviour was obtained *in vitro*. This was conducted in order to assess the possible usefulness or limitations of the human HepG2 and C3A (HepG2 sub-clone) hepatocyte cell lines, when compared to primary rat hepatocytes. The C3A cell line was chosen determine the impact of NP exposure on hepatocyte function due to their ease of culture in comparison to HepG2 cells, as C3A cells grow in a monolayer whereas HepG2 cells had a tendency to grow on top of each other in ‘clumps’. In general the response of the different cell types was consistent. However it would appear that the hepatocyte couplets and macrophages were

more sensitive to the toxicity of C_{60}. Furthermore although there was a trend for QDs to induce toxicity within hepatocyte couplets there was a lack of significance, in comparison to the findings derived from studies conducted within the cell lines, however this is thought to be overcome through more repetitions to reduce the variability within the results, or through the use of higher concentrations of NPs and longer exposure times. In addition, it is also necessary to consider if species differences were responsible for impacting on the toxicity of NPs, as the cell lines were of human origin, and the primary cell isolated from rats.

It was concluded that cell lines are a good but, not perfect model for assessing and predicting the toxicity of NPs. Therefore the use of *in vitro* toxicity screening to investigate particle toxicity allows a quick and inexpensive evaluation of NP toxicity within the liver, with the use of primary cells assisting in determining the relevance of the findings from cell lines. However addressing the true predictive capabilities of these *in vitro* models would require *in vivo* studies. This is made especially relevant due to the knowledge that comparative studies that assess the toxicity of NPs *in vivo* and *in vitro* have demonstrated a lack of correlation, with NPs exhibiting toxicity *in vitro* that is not realised *in vivo* (Sayes *et al.*, 2007). However, it is of relevance that collaborators within the PARTICLE_RISK project have conducted *in vivo* studies and observed that NPs are able to elicit toxicity within the liver. Jacobsen *et al.* (2008) observed that QDs were able to elicit hepatic necrosis and Kreyling *et al.* (unpublished observations) observed that gold NPs were eliminated, in a size dependent manner within bile after i.v. exposure.

7.5 Attributes of NPs responsible for toxicity

It was observed that QDs were capable of inducing the greatest extent of toxicity within the particle panel, which is thought to be most influenced by their charge and/or instability and small size. It is therefore suggested that cadmium is released from the QD structure and is responsible for the observed toxicity, and that positively charged NPs are more toxic than their negative counterparts. Consequently, it is suggested that QD621 are more toxic than QD620 due to the fact that their structure is more unstable, and/or that positively charged NPs are more toxic than negatively charged NPs.

In addition, the findings suggest that there may be an element of particle size related toxicity within the liver; so that the greatest toxic potential of QDs could be related to their small size. However, C_{60} particles did not elicit a similar extent of toxicity to QDs despite being smaller. However, it is known that it is difficult to disperse C_{60} as it is poorly soluble in aqueous environments so that it tends to form large aggregates (Ju-Nam and Lead 2008). Therefore exposure of C_{60} (within cell medium) probably occurs primarily in its aggregated form, which would account for its limited toxicity. The phenomenon that particle toxicity may be size dependent was also backed up by the finding that CB, the largest particle within the particle panel, did not induce toxicity in any cell type, suggesting that CB was the least toxic particle investigated. Thus under these circumstances, particle toxicity to the liver appeared to fit the generalisation that smaller particles are more toxic than their larger counterparts. However, within the literature, the common perception that NP forms of materials are more toxic than their fine counterparts was based on a limited number of studies that compared the toxicity of TiO_2, CB and polystyrene particles, and the wide ranging NP types available means that other particle characteristics may be more influential in modifying NP toxicity. The composition of particles also appears to impact on their toxicity, as the cadmium leached from QDs was proposed to be responsible for their toxicity. However it was also observed that carbon based particles elicit similar extents of toxicity, and that differences in carbon NP toxicity is thought to be affected by the structure of the particle in question, and the cell type being investigated. However the toxicity of carbon based particles to the liver appears to be driven by a number of factors such as the cell type, cell origin (i.e. primary vs cell line) and endpoint being measured. The observation that particle charge, instability, size, aggregation, composition and structure are able to influence particle toxicity highlights the complexity of evaluating NP toxicity.

7.6 Future Work

It would have been of interest to investigate some of the toxicological findings of the particle panel in more detail, to provide further verification of their importance.

When determining the uptake and fate of fluorescent polystyrene particles a number of findings warranted further investigation. Firstly, revealing the mechanism of uptake is of interest, as the lack of localisation of NPs within endosomes suggested that endocytosis was not involved within the internalisation of these particles, however other endocytic pathways follow different intracellular pathways. Therefore, evaluating the uptake of polystyrene particles at 4^{o}C would allow the contribution of endocytosis to be revealed and provide greater insight into the mechanisms underlying particle uptake. Evaluating the elimination of NPs within bile, and accumulation within mitochondria was determined using fluorescent probes, with the intracellular fate of internalised fluorescent NPs being based on the assumption that the co-localisation of NPs and the sub-cellular structure of interest occurred due to their location within the same region of the cell using confocal microscopy. Therefore the use of software that permits the analysis of microscopic images in more detail through the generation of 3D projections that allow the localisation of NPs within sub-cellular compartments to be investigated in more detail could be used to confirm the findings. However these programmes require z stacks to be performed which is impeded by the use of fluorescent probes, such as MitoTracker and CLF that bleach easily. Consequently the utilisation of probes that had a more limited tendency to bleach would be of benefit to confirm the findings. Furthermore it is perhaps more realistic to consider the use of *in vivo* models, to measure liver responses, such as cytochrome P450 expression, and bile duct cannulation that could be used to determine if NPs are eliminated within bile. It was also anticipated that TEM of hepatocyte couplet sub-cellular morphology, subsequent to particle exposure, would provide more detailed analysis of the localisation of NPs within mitochondria to be analysed but this was hindered by the similarity in appearance of NPs to cellular components.

Evaluating the impact of particle exposure on hepatocyte function revealed a number of findings that could be expanded upon. Firstly, when determining the toxicity of QDs it was found that the particle concentrations used within the toxicity screen were often lethal as the exposure time increased, especially within the QD621 treatment groups. The use of sub-lethal concentrations of QDs would therefore allow the mechanisms underlying their toxicity to be more easily revealed. Furthermore, it would have been useful to receive more

detailed characterisation information from the PARTICLE_RISK partners, to gain insight into the properties of NPs that were primarily responsible for any observed toxicity. This would include quantification of cadmium leaching from QDs, over time, which would have allowed experiments to be conducted that exposed cells to equivalent concentrations of cadmium, and evaluated the toxicity as a comparison. In addition, it would have been more relevant if particle characterisation was completed with particles dispersed in cell culture medium and not MilliQ water, but this was out of my control.

In addition, determining the ability of NPs to elicit oxidative stress within hepatocytes was accomplished by investigating the depletion of cellular GSH, however it would have also been relevant to determine if NPs were capable of increasing ROS levels, which could be accomplished, for example, through the utilisation of the DCFH probe to truly determine the ability of NPs to induce ROS production within liver cells. In addition, it was revealed that several particles within the PARTICLE_RISK panel were capable of compromising cell viability, and it is of interest to determine the mechanism by which this occurs; specifically via necrosis or apoptosis. Determining the predominant mechanism of cell death could be accomplished by using a number of techniques, including the Annexin V Propridum iodide assay (simultaneously detects necrotic and apoptotic cells), or detection of caspase activation (which occurs during apoptosis). Determining the ability of NPs to impact on cytochrome P450 activity proved to be unsuccessful within C3A and HepG2 cells, due to the inconsistent nature of findings produced from the test protocol used, which is thought to derive from the low level of CYP activity (see appendix section A3). Therefore perhaps measuring CYP450 mRNA (using polymerase chain reaction) or protein levels (using Western blots) may aid in determining the impact of NP exposure on hepatocyte metabolism, or through the use of primary cells. This was not possible due to the nature of the PARTICLE_RISK project which required a number of objectives were evaluated within a limited time period.

The relevance of using hepatocyte *in vitro* models was confirmed through the use of primary hepatocytes, however it would have also been beneficial to isolate primary Kupffer cells, to carry out the CM studies using primary cells.

However due to time constraints which impeded learning their isolation from the rat liver, and lack of availability of cell lines, a monocytic cell line was differentiated and used to represent Kupffer cells within the experiments. Within the studies that evaluated the importance of cellular interactions within the liver to particle toxicity it would have been of interest to evaluate IL-1β release from macrophages as this cytokine has been illustrated as being central to inflammatory responses within the liver, however as multiplex analysis did not reveal an increased production in this cytokine further analysis into its production using conventional ELISA was deemed unnecessary.

7.7 Conclusions

The pattern of response elicited by particles in all exposure scenarios was in the main, consistent, so that the toxicity of the particles can be ranked in the following order: QD621>QD620>CNT=ufCB=C_{60}>CB. The toxicity screen approach utilised therefore allowed the toxicity of a battery of particles to be assessed, and the results highlighted that different cell types respond similarly to particle exposure, as the pattern of toxicity was paralleled within the responses of hepatocytes and macrophages. The findings therefore support the utilisation of a toxicity screen approach when a comparison of particle toxicity is required, whereby if particles are available in infinite quantities further investigations can be undertaken to reveal mechanisms of toxicity by carrying out full dose response investigations. Therefore, it would have been of interest to complete full dose response experiments to enable LD_{50} and ED_{50} values for each particle to be estimated, which was not possible due to the limited availability of particles. Furthermore although the screen like nature of the assessment of NP toxicity to the liver was important to allow comparisons between the toxicity of the PARTICLE_RISK particle panel to be made it is acknowledged that particle toxicity, when determined using *in vitro* tests, is dependent on a number of factors, including NP characteristics (such as composition, size, charge, shape, stability, aggregation), sample preparation (the dispersal of the NPs) and the cell culture conditions (cell confluency, cell type) and experimental set up (such as exposure time and particle concentrations). The use of predictive *in vitro* models is therefore of relevance as the development of an *in vitro* screen to assess NP toxicity is necessary due to the large number of NPs that require toxicological assessment, and to limit

the demand for *in vivo* studies. The potential adverse effects elicited by NPs was gained from the toxicity of ambient particles, however the toxicity of NPs to the liver does not appear to follow the same toxicological paradigm, so that perhaps toxicity is tissue and NP specific.

7.9 Relevance to man

The potential of NPs to translocate to the liver is a realistic prospect, and has been demonstrated in humans (Nemmar *et al.*, 2002,) however it is important that this has been contradicted by other studies (Mills *et al.*, 2005). Therefore although there are discrepancies within the findings the possibility of NP localisation within the liver necessitates that the threat of NP exposure to normal liver function must be considered and investigated. Furthermore the knowledge that NP toxicity cannot be extrapolated from bulk forms of the same material, indicates that NPs have to be thought of as separate entities and as such require toxicological assessment. The high cost, and ethical implications of *in vivo* studies, and large number of NPs that require toxicological assessment necessitates that appropriate *in vitro* approaches are developed in order to yield inexpensive and accurate predictions of NP toxicity *in vivo*. The toxicity of particles to the liver was therefore assessed using *in vitro* models, and it is therefore necessary to discuss the relevance of this, specifically in relation to the applicability to the findings *in vivo*. The use of primary cells was important to determine if their response was mirrored within cell lines, which is important as primary cells are assumed to be more representative of the response of cells *in vivo*.

In the main, the investigations concluded that the response of cell lines and primary cells to particles were in agreement, with the predictive capability of the findings *in vivo* requiring further investigation. In addition, considering the implications of cellular interactions to the toxicity of particles within the liver, using an *in vitro* model, was able to more appropriately replicate the *in vivo* situation. This was achieved by taking into consideration the arrangement of cells within the liver, so that macrophages are exposed first to circulating NPs and the subsequent release of factors from stimulated macrophages has the ability to interfere within hepatocyte function.

It is now relevant to outline the implications of the toxicological findings observed. It has been observed by partners within the PARTICLE_RISK project that the instillation of QDs caused hepatic necrosis within mice (Jacobsen *et al.*, 2008). Consequently the pulmonary exposure of mice was able to exert toxicity within the liver, highlighting that QD toxicity to the liver warranted further exploration. It was observed that only the QDs were capable of initiating an inflammatory response *in vitro*, which if replicated *in vivo* is likely to stimulate the infiltration of neutrophils into the liver. Furthermore the ability of particles to induce cytotoxicity has obvious consequences for normal liver function; whereby the loss of macrophage viability has the potential to interfere with host defence and increase the likelihood of circulating toxins accessing the underlying hepatocytes, and a decrease in hepatocyte viability may equate to a loss of liver specific functions such as cytochrome P450 activity or bile secretion. The findings obtained therefore provide hazard data for the PARTICLE_RISK particles that can be used within risk assessments, conducted by partners within the project, to determine the health implications of particle exposure. Furthermore knowledge of the extent of NP exposure is imperative as this will impact on their ability to elicit toxicity on exposure, as risk is dictated by the hazard associated with particle exposure and the extent of exposure.

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Appendix

A1. Differential centrifugation

Differential centrifugation was used to separate the rat liver into five distinct sub-cellular fractions, using increasing speeds of centrifugation. The purity of the liver fractions was then determined by assessing the activity of marker enzymes (specific to particular sub-cellular organelles) within the different liver fractions; namely acid phosphatase (lysosomes), alkaline phosphatase (plasma membrane), succinate dehydrogenase (mitochondria) and NADPH cytochrome c reductase (endoplasmic reticulum) using protocols adapted from Harris, Graham and Rickwood (2006). Once the differential centrifugation procedure was established rats were exposed to fluorescent polystyrene NPs via i.v. and i.t. exposure in an attempt to reveal if polystyrene NPs were able to accumulate within the liver, and if they did what their sub-cellular localisation was.

Protocol for differential centrifugation of the rat liver

The differential centrifugation protocol was adapted from Chen *et al.* (1999). Specifically, the rat liver was removed, weighed and coarsely minced with scissors in ice cold homogenisation buffer (20mM Hepes buffer, containing 0.25M sucrose, 1mM EDTA, pH 7.4), at a volume of 7.45ml/g of liver. The liver was then homogenised using a motor driven Teflon Potter-Elvehjem homogeniser, with 4 up and down strokes.

The homogenate was centrifuged at a low speed to remove unbroken cells and debris (50 x g, 7 minutes). The supernatant was subsequently separated into nuclear, mitochondrial, lysosomal, microsomal and cytosolic fractions, using the protocol outlined in table A1. The organelle pellets were resuspended in homogenisation buffer (6ml) and the purity of each fraction evaluated by

Table A1. Differential Centrifugation protocol. The liver was homogenated then subjected to sequentially higher speeds of centrifugation using an ultracentrifuge.

Liver Fraction	Centrifugation Speed (g)	Time (minutes)
Nuclear	1000	10
Mitochondrial	9000	10
Lysosomal	30000	30
Microsomal	100000	120
Cytosolic	Last supernatant	

Creation of a standard curve to detect fluorescent polystyrene particles within different liver fractions

To determine if fluorescent polystyrene 20nm NPs could be detected (subsequent to exposure *in vivo*) within the different liver fractions a 'standard curve' was created for each of the sub-cellular fractions. This was achieved by adding an increasing particle concentration (20-320µg/ml, or until the maximal fluorescent intensity was reached) to each liver fraction (2ml in a quartz cuvette), and the associated fluorescence was detected with a fluorimeter (10nm slit width), which can be observed in figure A1. Therefore the fluorescent intensity associated with particular particle concentrations was evaluated, so that if the fluorescent intensity is known from samples obtained from treated animals, the concentration of particles present could be quantified, due to the compilation of regression equations for each liver fraction. It was observed that the ability to detect particles was variable within the tissue homogenate and different liver fractions (figure A1).

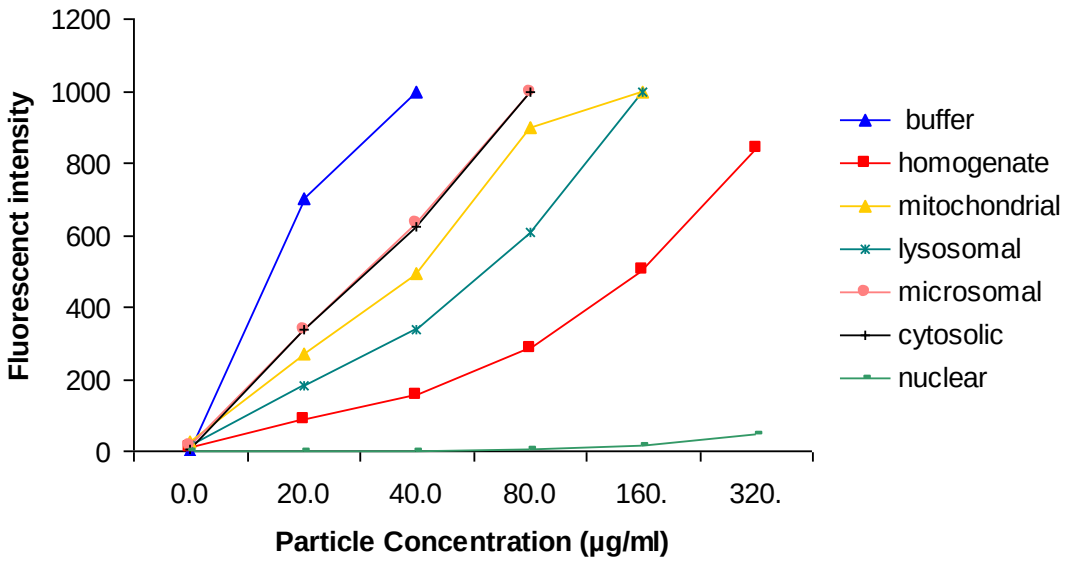

Figure A1. Fluorescent polystyrene particle standard curve. An increasing fluorescent particle (20nm) concentration was added to each liver fraction (2ml) and fluorescent intensity detected within a fluorimeter, and 10nm slit width.

Determining the sub-cellular location of fluorescent polystyrene particles within the rat liver after intratracheal and intravenous administration

Wistar rats (male, >3 months old) were anaesthetised with halothane (via inhalation), and intra-tracheally dosed with a fluorescent polystyrene particle suspension (0.5ml, 62 or 125µg dose, in saline, 18 hours) or were intravenously

(via the tail vein, 1mg, 2 or 4 hour exposure, in saline). Rats were administered a terminal dose of anaesthetic (pentobarbital, dosed intra-peritoneally) post treatment, and the liver was removed, weighed, homogenised and fractionated as described. A 2ml volume of each fraction was transferred to a quartz cuvette, and the fluorescence measured using a fluorimeter (10nm slit width) over a 15 minute period (figures A2 and A3).

It was apparent that polystyrene fluorescent particles (20 and 200nm diameter), administered by i.t. instillation, did not accumulate within the liver, at a detectable level, at the time point investigated. This conclusion was based on the fact that no increases in fluorescence within the tissue homogenate or liver fractions were associated with particle exposure, relative to the control (figure A2). This finding may result as a consequence of the fact that the polystyrene NPs do not translocate to the liver from the lungs, subsequent to i.t. instillation, as there are a number of barriers that act to restrict the transport particles to other organs, such as the liver, including for example the mucociliary escalator. Consequently, 20nm fluorescent polystyrene particles were administered intravenously, using a higher concentration of NPs than those within the i.t. instillations to increase the likelihood of liver accumulation subsequent to exposure. However, it was again found that the fluorescent polystyrene particles did not accumulate within the liver, at a detectable level, at the time points investigated. This conclusion was based on the evidence that there was no increases in fluorescent intensity within the tissue homogenates and sub-cellular fractions, when compared to the control (figure A3). Therefore, despite the fact that fluorescent polystyrene NPs could be detected within each liver fraction, using a fluorimeter, when added directly to fractions obtained from control animals, particle presence could not be detected after i.t. or i.v. administration. As a result, the detection method used may not have been sensitive enough to detect the small concentrations of particles that are expected to accumulate within the liver subsequent to i.t. or i.v. exposure. Therefore radiolabelled NPs could be used, whose distribution within the body could be more precisely quantified.

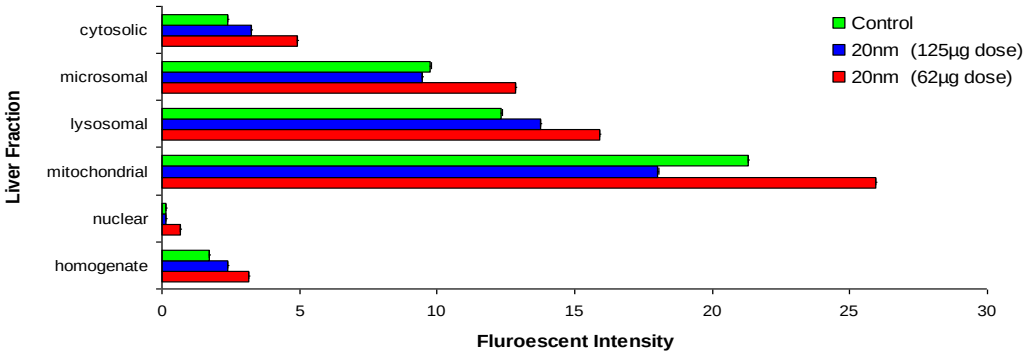

Figure A2. Detection of fluorescent polystyrene particles within the rat liver after i.t. instillation. The average fluorescent intensity measurements (taken over a 15 minute observation period) for each differential centrifugation liver fraction was determined using a fluorimeter (using a slit width of 10nm), subsequent to the exposure of rats to 62µg or 125µg 20nm fluorescent polystyrene particles or saline vehicle control for 18 hours.

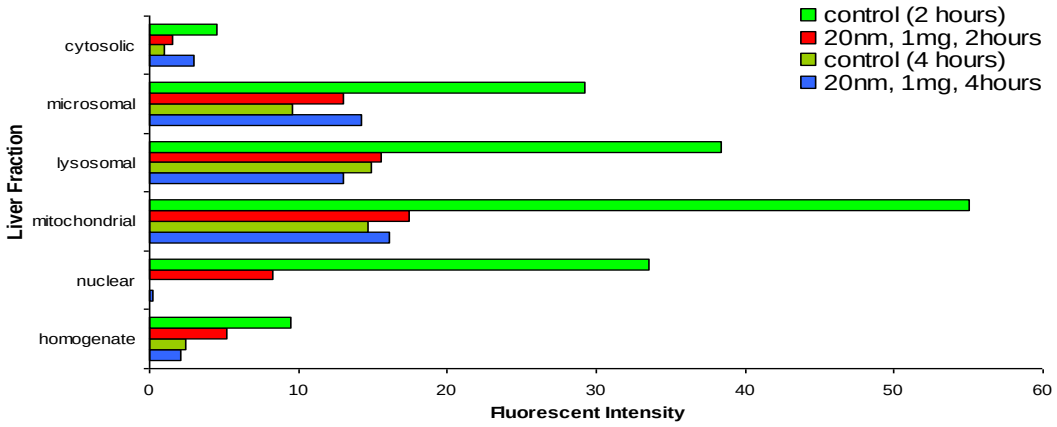

Figure A3. Detection of fluorescent polystyrene particles within the rat liver after i.v. administration, via the tail vein. The average fluorescent intensity measurements (taken over a 15 minute observation period) for each differential centrifugation liver fraction was determined using a fluorimeter (using a slit width of 10nm), subsequent to the exposure of rats to 1mg, 20nm fluorescent polystyrene particles or saline vehicle control for 2 or 4 hours.

A2. GSSG measurement

A decrease in GSH concentration and increase in GSSG concentration is indicative of a pro-oxidant state, so that a GSH:GSSG ratio is often obtained to quantify oxidative stress development within cells. However, it is known that GSSG is present within cells at a low concentration, and consequently difficult to measure. Therefore, as part of the experimental procedure it was necessary to prevent any inter-conversion of GSH and GSSG during the extraction or measurement of GSH or GSSG. This was achieved by using N-Ethylmaleimide (NEM), which forms a stable complex with GSH to prevent its participation in

the assay, and is also known to inhibit glutathione reductase which ordinarily converts GSSG to GSH. It is also thought that a higher pH facilitates the measurement of GSSG, hence the use of NaOH as a buffer within the experiment. Both GSH and GSSG are quantified using the fluorchrome OPT, which forms a highly fluorescent product.

GSSG concentration within the cell extract (see section 5.2.3.2 for the protocol for generating the cell extract) was determined through the generation of a GSSG standard curve. A stock solution of 0.05M GSSG (in 0.1M NaOH) was diluted 1 in 100 to give a 0.5mM GSSG working solution. The top standard of 50µM was then diluted from the 0.5mM GSSG working solution, and 1 in 2 serial dilutions completed to obtain a concentration range of 12.5µM to 0.78µM GSSG. The samples (100µl) or standards (100µl) were incubated with 0.04M NEM (40µl, in GSH buffer) for 30 minutes at room temperature, after which time NaOH (430µl) was added. The NEM-sample/standard mixture (180µl) was added to the wells of a 96 well clear bottomed plate (Porvair sciences, Middlesex, UK) in triplicate. OPT (10µl, 1mg/ml in methanol) was then added to the wells, and left to incubate for 15 minutes at room temperature. The fluorescence was then determined using the FLUOstar Optima microplate reader with an excitation wavelength of 350nm and emission wavelength of 420nm. The concentration of GSSG within the samples was extrapolated from the standard curve, and the concentrations adjusted for any dilutions made within the experiment.

It was found that the protocol used was unable to measure accurate levels of GSSG within cells as the concentration of GSSG was below the limit of detection.

A3. Determining CYP2E1 activity within hepatocyte cell lines

To determine if the PARTICLE_RISK particle panel was able to affect CYP450 function, the activity of CYP2E1 within the hepatocyte cell lines was evaluated. CYP2E1 is found within hepatocytes and is responsible for the metabolism of a number of substrates, including alcohol and paracetamol, and its activity can be induced by isoniazid. Chlorzoxazone is a model substrate for CYP2E1 as it is exclusively metabolised by CYP2E1, and is converted to 6-hydroxyxhlorzoxazone (product). The conversion of chlorzoxazone to 6-

hydroxychlorzoxazone was determined using HPLC, using the following protocol.

HepG2 and C3A cells were plated at a concentration of $1x10^5$ cells/ml, in a 24 well plate (1ml/well) and incubated at 37^oC, 5%CO_2 overnight. The next day, cells were exposed to 200µM isoniazid (in complete cell culture medium) for 24 or 48hours. Cells were then washed with PBS and exposed to 200µM chlorzoxazone (in complete phenol red free cell culture medium) for 20 hours. Product formation within the cell supernatant was then analysed with HPLC, using the parameters outlined in table A2.

Table A2 HPLC Gradient used to detect CYP2E1 activity. A C18-120-5µm, 150x4.6mm column was used to detect 6 hydroxychlorzoxzaone product formation within cell supernatants using the gradient outlined within the table.

Time (minutes)	0.5% Acetic Acid (Mobile Phase A)	Acetonitrile (Mobile Phase B)
0	80%	20%
12	80%	20%
15	30%	70%
18	30%	70%
23	10%	90%
23.1	80%	20%
25	80%	20%

To quantify product formation a 6hydroxychlorzoxazone standard curve was created, where concentrations ranged from 1.057 to 100µM. It was observed that the product peak could be identified at 6.2 minutes (figure A4) within the HPLC chromatogram, which was equivalent to that obtained by other investigators (Lucas *et al.*, 1996). The area under the curve data for each concentration of product investigated was then used to generate a standard calibration curve which could be used to delineate product concentration within samples. It was observed that CYP2E1 activity within hepatocyte cell lines could be induced by isoniazid, which was identified by product formation (at 6.2 minutes, figure A5), and it was apparent that the quantity of product formed did not vary with the different concentrations of inducer used. A substrate peak of unconverted chlorzoxazone could also be identified at 16.5 minutes (figure A6). However, on repetition of the experiment (n=3), product formation was not

reproducibly demonstrated by the hepatocyte cell lines subsequent to inducer formation. Consequently, different concentrations of substrate and inducer (100, 200 and or 500µM) were then used to determine if it was possible to measure CYP2E1 activity in a reproducible manner, but this was unsuccessful. Therefore the assay was believed to be insufficiently sensitive to accurately measure CYP2E1 activity within hepatocyte cell lines. However, perhaps the use of primary cells would allow for the measurement of CYP activity within hepatocytes, however due to time limitations this could not be completed.

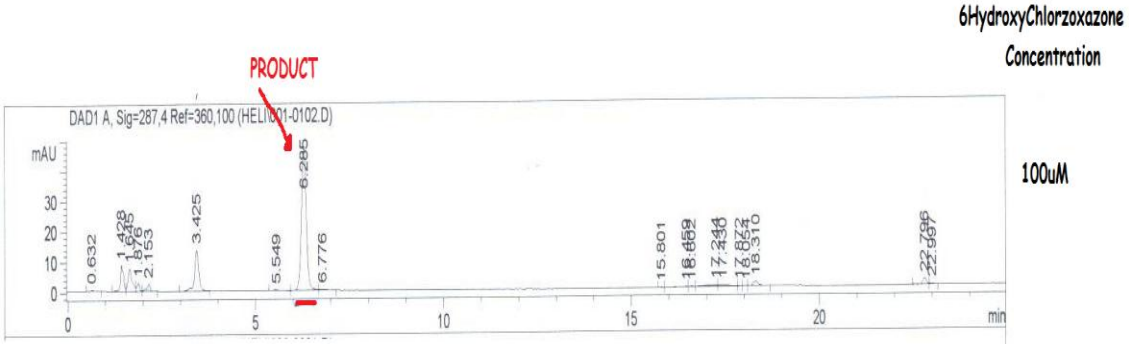

Figure A4 6HydroxyChlorzoxazone HPLC chromatogram. The peak for 6Hydroxychlorzoxazone (100µM) occurred at 6.2 minutes.

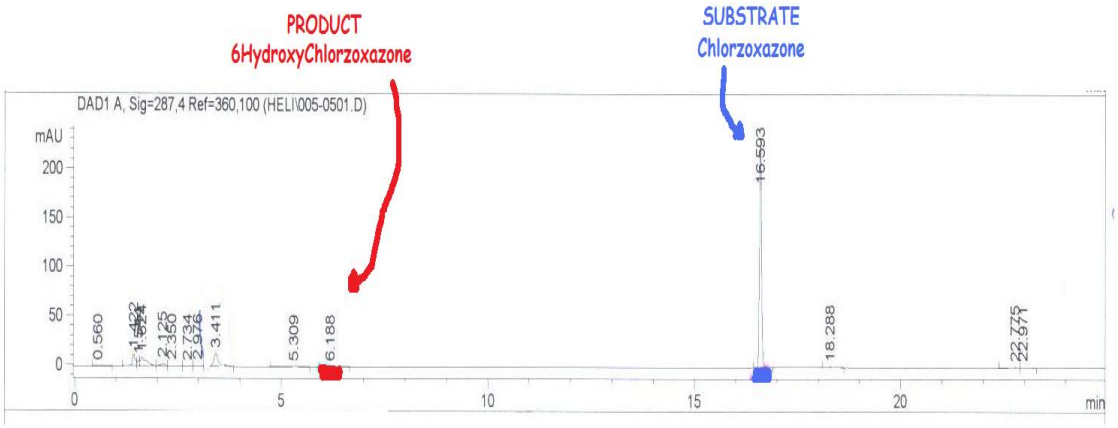

Figure A5 HPLC Chromatogram: Determining CYP2E1 activity. 200µM isoniazid was exposed to the C3A hepatocyte cell line for 24 hours, the cells were then washed with PBS and 200µM chlorzoxazone was added for 20 hours. The cell supernatant was analysed using HPLC. The product peak was evident at 6.156 minutes and the substrate peak was apparent at 16.5 minutes

References

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