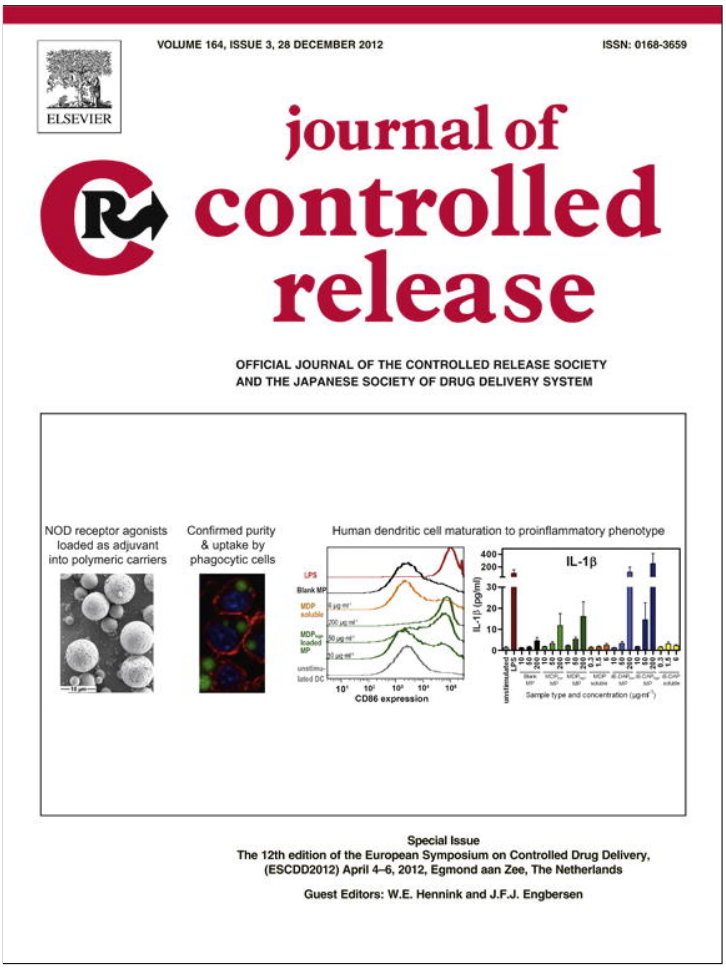

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Investigating the relationship between nanomaterial hazard and physicochemical properties: Informing the exploitation of nanomaterials within therapeutic and diagnostic applications

Helinor Johnston *, David Brown, Ali Kermanizadeh, Eva Gubbins, Vicki Stone

Nanosafety Research Group, School of Life Sciences, Heriot-Watt University, Edinburgh, EH14 4AS,UK

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ABSTRACT

Nanomaterials (NMs) have the potential to improve the treatment and diagnosis of disease as they are suitable candidates for a number of diagnostic and therapeutic applications. On entering the body via a variety of exposure routes, and during their translocation to secondary target sites it is inevitable that NMs interact with biological molecules, such as proteins. These interactions may influence the behaviour and toxicity of NMs following exposure. As the surface of NMs is what interacts with cells and tissues it is necessary to identify the influence of NM surface properties on their toxicity, and determine how this is influenced by the route of exposure, and physico-chemical characteristics of NMs. The term protein corona is used to describe the coating of the NM surface with protein. The protein corona is a dynamic and complex structure whose composition is dictated by the biological medium and the physico-chemical properties of NMs (such as their size, composition, hydrophobicity and charge) as this influences protein binding specificity and affinity. Depending on the route of exposure (e.g. inhalation or injection) NMs will encounter different proteins. We have observed that i) the composition of protein corona of NMs is likely to be dictated by their route of entry, ii) the translocation of NMs to secondary target sites may influence the composition of the protein corona (i.e. they encounter different proteins on their transport in the body) so that the composition of the protein corona evolves over time, iii) the physico-chemical characteristics of NMs dictate the composition of the protein corona, and the toxicity of NMs and iv) NMs can affect secondary target sites that vary according to delivery route and corona composition following exposure. These findings, and evidence from the wider literature has therefore led us to hypothesise that NM toxicity is dictated by the exposure route due to the acquisition of a surface coating (protein corona) that is determined by the route of entry and physico-chemical properties of the NM. This information can be exploited within the intelligent design of NMs in the future (e.g. to control protein adsorption and the subsequent cellular response), and be used to improve the design of toxicology investigations (e.g. to inform how NMs should be dispersed within *in vitro* experiments to more accurately reflect *in vivo* conditions).

1. Introduction

Nanotechnology involves the employment of engineered nanomaterials within products, or processes performed at the nanoscale (generally considered to be 1–100 nm). The development, production, and exploitation of engineered nanomaterials (NMs) within diverse products are expanding rapidly. NMs have at least one dimension in the nanoscale (<100 nm) and their behaviour has been demonstrated to be strikingly different to that of their larger 'bulk' counterparts. Consequently, pharmaceutical, cosmetic, textile and electronic industries are harnessing the size related properties of NMs within a variety of applications such as medicines, clothes, sunscreens and food. The size of materials has been demonstrated to be instrumental to their toxicity, so that as particle size decreases, toxicity generally increases. This phenomenon has been consistently demonstrated for many different materials including carbon black, polystyrene, titanium dioxide, and silver [1–4]. The importance of particle size to NM toxicity suggests that their surface area drives their toxicity, as the surface area of materials increases with a decrease in particle size. In fact, the toxicity of particles is well correlated to their surface area [5]. As the surface of NMs interacts with cells and tissues following exposure, the surface properties of NMs are likely to be fundamental to their behaviour. However, although NMs share the commonality that they are 'small' they are a diverse population of NMs that can vary with respect to their size, composition, charge, surface area, solubility, crystal structure, surface chemistry and shape. It is therefore critical to identify the physical and chemical properties of NMs that confer toxicity.

Of particular interest to the 12th European Symposium on Controlled Drug Delivery is the potential for NM exploitation within

* Corresponding author.
E-mail address: h.johnston@hw.ac.uk (H. Johnston).

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various medical applications. The area of nanomedicine is concerned with the design and development of NMs with a medical application, and important uses of NMs in this area includes their exploitation as novel drug delivery devices, diagnostic and therapeutic agents to improve the diagnosis and treatment of disease. Furthermore, individual NMs can have both therapeutic and imaging activities to allow for the simultaneous diagnosis and treatment of disease, and these NMs are termed theranostics [6].

NMs offer numerous advances within the diagnosis and treatment of disease which has stimulated optimism surrounding the benefits associated with their exploitation. In fact, a number of NM based medical applications are in transition from the research to clinical phase [7]. However, critical to the development of NMs is the rigorous assessment of their safety. Therefore, in order to support innovation within the emerging field of nanomedicine, and realise the benefits to human health as well as the many financial gains promised it is necessary to address the potential adverse impacts of NMs to human health.

The inherent properties of NMs can be exploited for imaging (diagnostic) or for therapeutic purposes. For example iron oxide NMs can be used as contrast agents for magnetic resonance imaging (MRI) to detect tumours [8], and the optical properties of gold NMs allows for their exploitation as photothermal agents using hyperthermia to kill tumour cells [9]. Furthermore, the chemical and physical properties of NMs can also be tailored so that NMs perform specific functions. For example, NMs are excellent carriers of therapeutic or diagnostic agents. Typically, the therapeutic load is conjugated to the surface of the NM (which is facilitated by the high surface area of NMs), or encapsulated within the NM (hollow) core [10] and then released in a sustained or controlled manner. Surface modification of NMs is also frequently used to improve circulation time in blood (e.g. polyethylene glycol (PEG)), target NMs to specific sites, and prevent NM agglomeration so that NMs are delivered to the required location for a period that is sufficient to realise the desired therapeutic or diagnostic activity, but reduces adverse effects. The specific targeting of NMs to their required site of action is required to minimise adverse effects associated with systemic administration. This can be achieved through passive or active targeting processes. For example, the physical and chemical properties of NMs (e.g. size) can allow for the passive delivery to tumours due to the leaky nature of blood vessels that supply tumours [9]. In addition, modification of NM surface properties using carefully selected targeting moieties can enable the targeted delivery of NMs to the required target site [9]. It is therefore critical to understand how the surface properties of NMs influence their interaction with cells in the body, and their toxicity as this will dictate the biological and toxicological response to NMs.

Following exposure NMs have the ability to interact with a number of biological molecules (such as proteins) at the exposure site. Furthermore, NMs have the capacity to accumulate within secondary target organs, such as the liver, following exposure [e.g. 11]. The movement of NMs throughout the body therefore suggests that NMs will encounter, and interact with a variety of biological molecules which will be dictated by their exposure site and their biodistirbution. 'Protein corona' is the term that has been coined to describe the coating of the NM surface with proteins [12]. The formation of NM–protein complexes is able to influence the biological response to NMs [13]. The composition of the corona is driven by the physico-chemical properties of NMs, as these dictate protein binding specificity and affinity [12]. For example, the size, hydrophobicity, surface area and charge of NMs has been repeatedly demonstrated to affect protein binding to the NM surface [13–17]. It has been suggested that a stable 'hard' core of biological macromolecules interacts strongly with the NM surface (termed the hard corona), and a more loosely bound outer layer of biological macromolecules which associates more weakly to the particle surface and to the hard corona [12]. Importantly, binding of proteins to the NM surface is not simply related to their relative abundance within biological fluids [13] which suggests that different proteins vary in their affinity for binding which is likely to be related to the physico-chemical properties of the NM. However, the most abundantly bound proteins may not be the most influential to the biological response induced by NMs [18]. The composition of the corona may evolve over time, whereby proteins associate and disassociate from the NM surface as proteins compete for binding. The composition of the protein corona is therefore likely to change with time; for example proteins in the greatest abundance or those with the highest mobility may attach to the NM surface in the first instances and then are later replaced by less abundant or motile proteins that have a higher affinity for the surface, and this process may take several hours [19]. Furthermore, the movement of NMs within the body following exposure (e.g. the translocation of NMs from the lung to the liver via blood) means that the composition of the protein corona is likely to evolve throughout the NM's lifetime in the body [12]. The rates of protein association and dissociation from the NM surface are likely to vary considerably with protein and NM type. The protein corona is therefore a complex and dynamic structure.

The formation of NM–protein complexes can have a variety of implications on the biological and toxicological response following NM exposure; i) the attached proteins may block the NM surface, and thereby reduce NM reactivity and toxicity; ii) protein binding to the NM surface may result in the bolus delivery of active protein to the cell surface, iii) the attached proteins may undergo changes in their structural conformation and be denatured preventing active protein activity; iv) the toxicity of NMs may be underestimated due to their interference with the assays used to assess their toxicity (e.g. cytokine production detection [14]) and, v) the adsorption of proteins onto the NM surface can improve the dispersion of NM suspensions (i.e. prevent against NM agglomeration and aggregation). It is therefore essential to identify the biological molecules which 'coat' the surface of NMs, and how the formation of NM–protein complexes can impact on NM toxicity, and studies are ongoing in this area. NMs are readily coated *in vitro* with proteins of various compositions in a size dependent manner [14]. Pro-inflammatory cytokines (e.g. IL-8 and TNF-α) can lose some of their activity when adsorbed to 14 nm ultrafine carbon black (ufCB) NMs [14]. Also of importance is that the biological effect of ufCB is reduced during this interaction [14]. The surface of NMs is routinely modified with the intention of controlling NM–protein interactions. For example, the modification of the NM surface with polyethylene glycol is a commonly used strategy within the area of drug delivery which increases the circulation time of drugs by preventing recognition by the reticuloendothelial system through the introduction of 'stealth' properties and reducing protein absorption (i.e. opsonisation) [10]. NMs with a PEG coating have reduced uptake by macrophages *in vitro* [20] which is therefore likely to increase their circulation time in the body.

2. Investigating the relationship between the protein corona and NM toxicity

On entering the body, it is likely that NMs will interact with a variety of proteins and other biological molecules, which is influenced by the route of exposure. The importance of NM–protein interactions to NM toxicity were investigated, and how this could be related to the route of exposure and physico-chemical properties of NMs. We were primarily interested in the injection of NMs directly into the circulation (as this exposure route is likely to be used widely within the area of nanomedicine), the pulmonary exposure of NMs (due to the large number of studies that have assessed the pulmonary toxicity of NMs), and subsequent accumulation and toxicity of NMs within the liver (due to the preferential accumulation of NMs in the liver following exposure via a variety of routes [e.g. 11]). The findings of three independent research studies are outlined in order to identify the importance of NM–protein interactions with regard to NM toxicity. These studies were concerned with investigating; i) the interactions that occur between NMs and proteins contained in relevant biological media/dispersants, and how the physico-chemical characteristics of NMs influence the affinity and degree of protein binding, ii) whether

the inclusion of physiologically relevant dispersants are able to influence the toxicity of NMs *in vitro*; and iii) whether NMs can manifest toxicity at sites that are distal to the exposure site *in vivo*. Taken together these studies can provide a greater understanding of how the route of exposure, physico-chemical properties of NMs, and interactions with proteins are able to influence NM toxicity. The findings of these studies have allowed us to develop a hypothesis that relates NM toxicity to the formation of a surface coating (protein corona) on the NM surface that is determined by the route of entry, biodistribution and physico-chemical properties of the NM.

3. Identification of hard corona composition

Following injection, NMs are likely to interact with proteins contained in blood. Iron oxide NMs may be used as contrast agents for magnetic resonance imaging (MRI) within the detection of disease. The proteins contained in serum or plasma or lung lining fluid (LLF), that were bound to the surface of iron oxide (45 nm or 280 nm) was investigated in order to determine the nature of the protein corona that is formed following the injection of NMs into blood (using plasma and serum to suspend the NMs), or exposure via the lungs (using lung lining fluid to suspend the NMs). The OECD has identified a panel of NMs whose safety should be assessed with highest priority (due to their anticipated widespread commercial use or due to concerns regarding their safety) within the OECD sponsorship programme for NM safety assessment. The iron oxide materials investigated were part of the OECD priority group of materials. The NMs selected are being used at an international level to develop standardised approaches for testing NM safety (for a variety of endpoints in eco and mammalian toxicity). Two sizes of iron oxide were selected in order to investigate the size dependency of any observed effects.

NMs were incubated in biological media; 10% serum, 10% plasma, or 10 μg/ml LLF lung lining fluid (LLF) at 37 °C for one hour in a shaking water bath. The 10% serum is of relevance as it reflects the *in vitro* medium used in a majority of cell culture models, the 10% plasma is relevant as following injection NMs will enter blood and encounter serum proteins, likewise, following inhalation into the lung, NMs may become coated with LLF. The proteins that were strongly associated with the nanomaterials (i.e. those that remained attached after a series of centrifugation/washing steps) were then identified using a combination of SDS-Page electrophoresis and mass spectroscopy. A comparison was then made to the proteins that were bound to NMs when they had been pre-coated with lung lining fluid, and then exposed to serum or plasma, and the composition of the 'hard corona' investigated. This was included to encompass the possibility that inhaled NMs entering the lungs can then translocate and enter the circulation.

The proteins contained within the 'hard corona' of NMs were dictated by both the particle and the biological media used, insinuating that the characteristics of the particle surface and the composition of biological media determines the extent, specificity and affinity of protein binding to the NM surface. For example, mass spectroscopy findings indicated that the most abundant protein found in the plasma corona (of 280 nm Fe_2O_3 particles) was apolipoprotein B-100 and complement C3 proteins, and in the serum corona was serum albumin (Gubbins et al., manuscript in preparation). Importantly, protein binding to the NM surface was not related to their abundance in the biological media, as observed previously [13]. NMs that had been pre-coated in LLF and then suspended in plasma or serum had a greater amount of protein within the corona (Gubbins et al., *manuscript in preparation*). Pre-coating of NMs with LLF was therefore able to influence the composition of the protein corona, whereby a different profile of proteins was bound to the NM surface. A larger panel of NMs has now been tested, as well as different concentrations of biological media (e.g. plasma), in order to include more physiologically relevant exposure conditions (Gubbins et al., *manuscript in preparation*). However, the preliminary findings suggest that the route of entry into the body is able to dictate the nature of the NM protein corona. In addition, it is evident that the composition of the protein corona can evolve following the transit of NMs within the body which has the potential to influence their behaviour and toxicity.

The finding that complement proteins bind to the NM surface is of great interest due to the important role of the complement system within the recognition, opsonisation and removal of foreign material from the body. Following complement protein binding to the NM surface, complement activation may result in the generation of inflammatory peptides that stimulate an inflammatory response, which if persistent could result in tissue damage. In addition, complement activation could promote the opsonisation of NMs to reduce their circulation time, which may be detrimental within the exploitation of NMs within nanomedicines. Alternatively, complement protein sequestration by NMs may diminish complement activation. It has been demonstrated that complement proteins can adsorb onto the surface of NMs (carbon nanotubes) to result in complement activation [21,22]. Furthermore Barlow et al. [23] observed that carbon black NPs activate serum to induce macrophage migration *in vitro* via a mechanism that involved reactive oxygen species and may involve complement. Further studies are required to investigate the surface properties of NMs that determine whether complement activity is affected by complement protein binding to the NM surface. This information will promote the safe design of NMs within the area of nanomedicine in the future to circumvent problems associated with complement activation, such as reduced NM circulation time, or stimulation of an inflammatory response which could result in tissue damage.

The isolation and identification of the proteins that are associated with the NM surface is a challenging task. It is essential that the methodologies employed do not disrupt the NM–protein complex or promote additional protein binding [18]. In order to separate NM–protein complexes from unbound and weakly bound proteins, centrifugation has been routinely used [18], as described for this study. This allows for the separation of NM–protein complexes but this process is associated with a number of limitations. For example, centrifugation has the ability to perturb the composition of the protein corona as high centrifugal force may encourage the association or dissociation from the particle surface, and the number of washing steps (used to separate weakly bound proteins or unbound proteins from the protein corona) can influence the composition proteins of the protein corona [24]. Alternative approaches are available to separate NM-protein complexes (e.g. size exclusion chromatography) and to characterise the protein corona (e.g. differential centrifugal sedimentation) but despite the limitations associated with centrifugation it is the most commonly employed approach (for a review please see [24] Walkey and Chan, 2012). Following separation of the NM–protein complexes, the proteins are then isolated from the NMs (using, for example, detergents, or high temperatures) and then SDS PAGE and mass spectroscopy are used to identify the attached proteins (for a review please refer to [24]). In addition to the complex analysis of the composition of the protein corona, complementary approaches (such as differential centrifugal sedimentation (DCS), dynamic light scattering (DLS), and transmission electron microscopy have been used to characterise the nature of the protein corona of NMs following dispersion in biological media. Whilst these approaches may not provide information about the composition of the corona it can provide information on the structure of the corona e.g. thickness, size, and density.

It is also necessary to investigate how the protein corona evolves with time [18,19]. Therefore, as well as obtaining information on binding affinity (i.e. protein abundance within the protein corona) it is necessary to consider protein binding kinetics (rates of association and disassociation), as this will inform whether the bound proteins are attached for long enough to elicit a significant biological or toxicological response [18,19,25]. Furthermore, the exchange of proteins on the NM surface will also be affected by the route of exposure and subsequent movement of NMs throughout the body, as each of these

compartments will have a different composition of proteins and this needs to be investigated further in the future. Such detailed knowledge on the NM protein corona may allow for predictive modeling studies to be performed (that predict the interaction between proteins and NMs and the subsequent biological response) and also inform the intelligent design of NMs to control protein adsorption and subsequent cellular response. In order to enable this, a more diverse array of NMs needs to be tested, and a more diverse array of biological media that are representative of different exposure sites (and subcellular compartments). Furthermore, such combinations then need to be tested using *in vitro* cell models to see whether they better relate to *in vivo* responses than the simple serum dispersions that have been used widely to date.

4. Influence of physiologically relevant dispersants on NM toxicity *in vitro*

The preparation of NMs within different biological media is able to influence their toxicity due to the nature of the protein corona that is formed. Gold NMs have many biomedical applications. Specifically, the optical properties of gold allow for imaging and exploitation as a diagnostic and therapeutic agent. The impact of gold NM dispersion within different biological media on their *in vitro* toxicity was investigated. Gold NMs (2, 20, and 100 nm) were suspended in cell culture medium alone, or cell culture medium supplemented with LLF to represent exposure via the lung, bovine serum albumin (BSA) or fetal calf serum to represent exposure via blood. Serum (in blood) and LLF (in the lungs) are complex mixtures of proteins and other materials (such as lipids), which when bound to the surface of NMs may modify the reactivity and toxicity of NMs. Gold NMs were suspended in cell culture medium that was supplemented with different physiological dispersants, and then exposed to primary rat hepatocytes for 4 h at concentrations up to 250 μg/ml. Hepatocytes were included due to evidence of NM accumulation in the liver [e.g. 11]. This study aimed to evaluate how the use of physiologically relevant media which represent different exposure routes can modify the toxicity of NMs.

It was demonstrated that the inclusion of serum, lung lining fluid or bovine serum albumin within cell culture medium was able to improve the dispersion of the gold NMs, which was confirmed using electron microscopy and dynamic light scattering (Brown et al., *manuscript submitted*). As such, it is suggested that the proteins contained within the different dispersants interact with, and coat the particle surface to prevent against NM agglomeration. It would now be of interest to investigate the composition of the protein corona, as this may help in understanding the mechanism by which the stability of the NM suspension is improved through the incorporation of protein within the biological media. Importantly, this study is able to influence the means by which NMs are dispersed within future toxicology investigations. This is important as the most appropriate means to disperse NMs is of great topical interest and has been demonstrated to impact on the toxicity of NMs as NMs have a tendency to agglomerate or aggregate to form larger structures within biological media. To improve the dispersion of NMs a number of approaches have been attempted, including the incorporation of dispersants (such as proteins, detergents and solvents), and/or the utilisation of mechanical and physical processes (such as sonication). This study demonstrated that the effectiveness of different physiologically relevant dispersants to improve the dispersion of NMs within biological media is dictated by the dispersant and physico-chemical properties of the NM under investigation.

The ability of the NMs to impact on the viability of hepatocytes was investigated using the lactate dehydrogenase (LDH) assay. The release of pro-inflammatory cytokines (including interleukin (IL)-6, macrophage chemotactic protein (MCP)-1, tumour necrosis alpha (TNFα), IL-10 and IL-1β) following NM exposure was investigated using commercially available multiplex cytokine kits. LLF greatly enhanced the cytotoxicity and pro-inflammatory cytokine production following gold NM treatment when compared to serum as a dispersant (Brown et al, manuscript in preparation, Figs. 1 and 2). In contrast, preparation of NM suspensions using albumin and serum decreased their toxicity to hepatocytes. This suggests that the inclusion of physiologically relevant dispersants influences the toxicity of NMs, which may be mediated by the interactions that occur between proteins contained in the different biological media and the NMs. This is extremely important as hepatocytes *in vivo* are unlikely to encounter 'naked' particles, and instead the NMs that they are exposed to will have a protein corona that is dictated by their route of entry into the body, and the physico-chemical properties of the NM. This study therefore demonstrates the potential importance of NM–protein interactions to NM toxicity. As such we hypothesise that the exposure route can influence the subsequent toxicity of NMs due to the acquisition of a surface coating that is determined by the route of entry. Further investigations are required to investigate the importance of NM–protein interactions to NM toxicity. For example, the findings demonstrated that complex media that contain proteins or mixtures of proteins and lipids are able to modify particle induced cytotoxicity, perhaps by coating the particle surface. It is now necessary to model 'real-life' exposure conditions more accurately within *in vitro* investigations. Specifically, pre-coating NMs with LLF and then serum proteins prior to the exposure of hepatocytes would more realistically represent the exposure of hepatocytes *in vivo* following pulmonary exposure. Exposure of animal models *in vivo* will also be required to validate the findings obtained from the *in vitro* study, and these are ongoing.

5. Investigating the distal effects of NMs following pulmonary exposure

The FP7 funded programme ENPRA is currently investigating the toxicity of a panel of 10 NMs (including zinc oxide (ZnO), titanium dioxide (TiO_2), silver, and multi walled carbon nanotubes (MWCNTs)) at different target sites using both *in vitro* and *in vivo* models. The ranking of NM toxicity was consistent across *in vitro* models of the lung epithelium, renal epithelium, fibroblasts, macrophages, hepatocytes and endothelial cells. The NMs investigated could therefore be grouped into low and high toxicity groups which suggest that the physico-chemical properties of NMs were important to their toxic potency (e.g. [26]). Furthermore, the mechanism (e.g. pro-inflammatory, or oxidant driven response) by which NMs produce a toxic effect was also reliant on their physical and chemical properties [26]. This study also revealed that systemic effects were observed in response to NM exposure. Specifically,

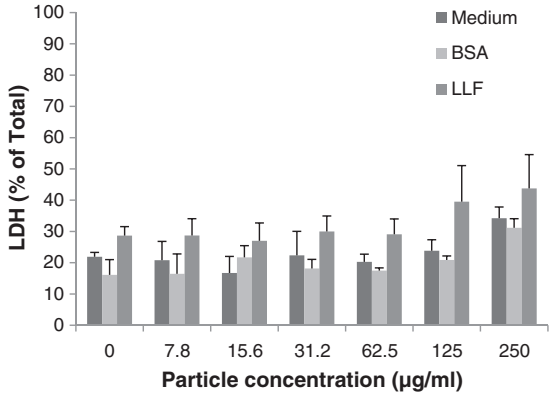

Fig. 1. The cytotoxicity of gold NMs (20 nm) to primary rat hepatocytes when dispersed in cell culture medium containing LLF (1 μg/ml), BSA (0.1%) or serum (0.1%) was investigated using the LDH assay (n = 3). Gold NMs elicited a dose dependent increase in hepatocyte cytotoxicity when dispersed in cell culture medium 4 h post exposure. The toxicity of the gold NMs was enhanced by the inclusion of LLF within the cell culture medium used to disperse the NMs. The toxicity of gold NMs was reduced with the inclusion of serum or BSA within the cell culture medium used to disperse the NMs.

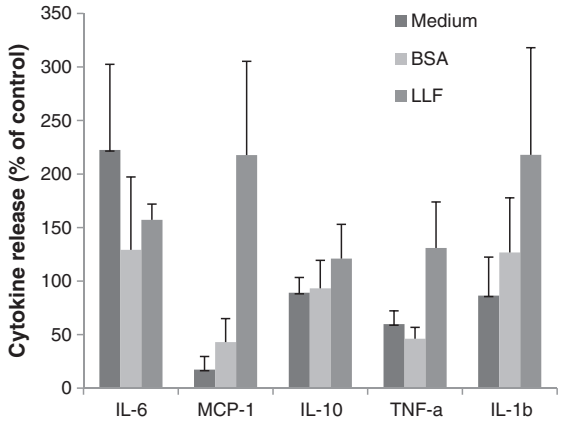

Fig. 2. The release of pro-inflammatory mediators from primary rat hepatocytes following exposure to gold NMs (20 nm, 125 μg/ml, 4 h) when suspended in different biological media/dispersants was investigated using Bioplex multiplex assays (n = 3). NM-induced release of pro-inflammatory cytokines was enhanced by LLF inclusion within the cell culture medium.

following intratracheal instillation (doses administered ranged from 1 to 128 μg/mouse) it was observed that glutathione (GSH) levels were depleted in the liver, but that this response was not evident for all NMs tested (Gosens et al., 2012, *manuscript in preparation*, Fig. 3). This correlated with the findings of *in vitro* investigations where glutathione depletion was observed within hepatocytes (Kermanizadeh et al., manuscript submitted). Surprisingly, nanomaterials that did not induce a significant lung inflammatory response (e.g. TiO_2) were still able to induce glutathione depletion in the liver. The inflammatory and oxidative responses within the liver were further investigated using PCR. It was demonstrated that there was a significant decrease in the levels of C3 gene expression while higher levels of both IL-6 and IL-10 were detected and no change in the expression of TNFα and CXCL2 was observed (Gosens et al., 2012 *manuscript in preparation*, Fig. 4). It is now necessary to elucidate whether these effects are manifested due to the translocation of NMs to the liver, or whether systemically acting factors are produced at the exposure site.

6. Discussion

On entering the body, it is inevitable that NMs will interact with biological molecules. The small size and large surface area of NMs allows for the adsorption of biological molecules such as protein. The composition of the protein coating (or corona) is likely to be dictated by the route of exposure. The translocation of NMs from their exposure site to secondary target sites is likely to influence NM behaviour and toxicity due to the acquisition of different surface coatings during their movement in the body, indicating that the protein corona is a complex and dynamic structure. The widespread use of NMs within diverse applications means that exposure via inhalation, ingestion, dermal application and injection is expected within occupational, consumer and environmental settings. Within the described research a focus has been placed on the fate and toxicity of NMs following inhalation and injection. However, future studies should assess the fate of NMs following ingestion and dermal exposure, as well as how this influences the composition of the NM–protein complexes that are formed and how this relates to NM physico-chemical characteristics to affect NM toxicity.

Taken together the findings of the three independent research studies described demonstrate that the surface properties of NMs are important to their toxicity, and that the formation of NM–protein complexes is influenced by the exposure route and physico-chemical properties of NMs. Specifically, the following important observations were made; i) the composition of the protein corona of NMs is likely to be dictated by their route of entry into the body, ii) the translocation of NMs to secondary target sites can influence the composition of the protein corona (i.e. they encounter different proteins on their movement in the body) so that the composition of the protein corona evolves throughout NM's lifetime in the body, iii) the physico-chemical characteristics of NMs dictate the composition of the protein corona, and the toxicity of NMs, and iv) NMs can affect secondary target sites following exposure. We therefore hypothesise that NM toxicity is dictated by the exposure route, due to the acquisition of a surface coating (protein corona) that is determined by the route of entry and that is modified on the transit of NMs in the body (Fig. 5). This hypothesis therefore needs to be tested further, in order to determine its applicability to more diverse forms of NMs, and exposure routes. In this respect, a number of lessons can be learnt from the drug delivery field (e.g. opsonisation).

Binding of proteins to the NM surface (to form the protein corona) is able to influence the toxicity of NMs. Not only is the biological activity of the NM modified by the adsorption of proteins onto the NM surface, but structural changes in adsorbed proteins have also been observed, which can have implications for normal protein function (e.g. [27,28]). Ongoing research in this area will help improve our understanding of the composition of the protein corona, and the implications of NM–protein complex formation.

7. Physico-chemical characterisation

There is increasing pressure from multiple sources (e.g. reviewers, journals, and peers) to conduct physico-chemical characterisation of nanomaterials used in toxicology studies. There are multiple purposes for such characterisation. The first involves characterisation of the pristine particles as provided by the supplier. This characterisation allows the researcher to verify the properties of the substance that they are about to investigate. In addition, this characterisation

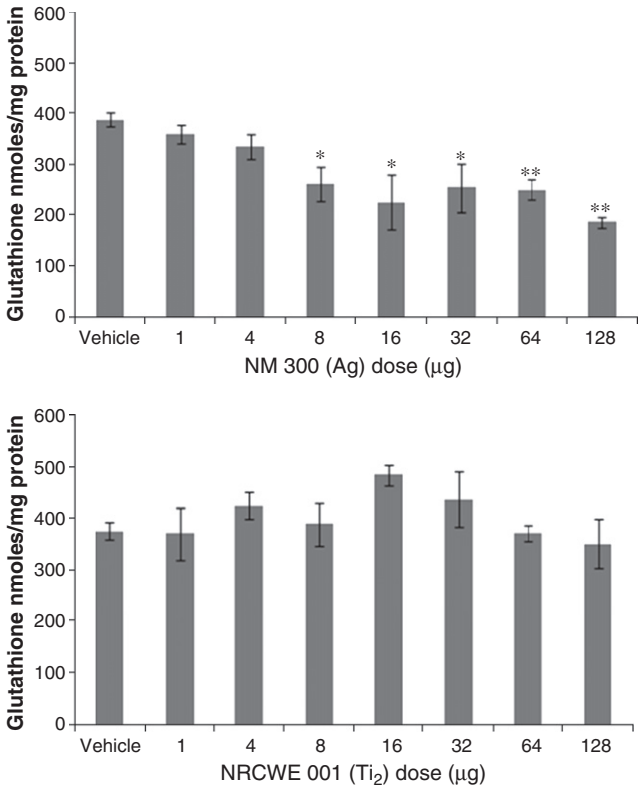

Fig. 3. Effects of NM intratracheal instillation (24 h, 1–128 μg/mouse) on the total glutathione content of C57/BL6 mouse liver tissue. Values represent mean ± SEM (n = 3), significance indicated by * = $p<0.05$ and ** = $p<0.005$, when NM treatments are compared to the control.

Gene	Ag NMs	TiO_2 NMs
Complement factor 3	---	--
IL-6	+	++
TNF-α	0	0
CXCL2	0	0
IL-10	+++++	+++++

Fig. 4. Changes in gene expression in the liver was detected using real time PCR following the intratracheal instillation of C57/BL6 mice to silver (Ag) or titanium dioxide (TiO_2) NMs (24 h, 128 μg/mouse). The findings are representative of data from three animals. An increase in gene expression is represented by '+', and a decrease in gene expression is indicated by '−'. The extent of change observed is indicated by; 0 = no change, −/+ = 50% change, −−/++ 70% change, −−−/+++ 90% change, −−−−/++++ more than 100% change when NM treatments are compared to the control group.

allows identification of the properties that will ultimately determine how the particle will behave in the body. For a structure–activity relationship, it is these properties that are likely to be required. The second 'type' of characterisation is to determine the nature of the nanomaterial once dispersed in the relevant media to be used in the investigation. This allows the researcher to investigate phenomena such as agglomeration and how this might influence cellular responses such as uptake into cells or settling in the culture media. The third 'type' of characterisation relates to the characteristics of the nanomaterial once in contact with the biological system (*in vitro* or *in vivo*) and how these characteristics are modified over time. This characterisation relates to investigation of the mechanisms by which the particles interact with the biological system that might result in a positive or negative impact. It is impossible to do all types of characterisation for all types of study. In an ideal situation, once we understand the mechanism of toxicity and we have established robust structure activity relationships, it may be possible to revert to the relatively 'simple' analysis of the characteristics in the sample as provided by the supplier. Such progress is essential if nanotoxicology is to be financially viable.

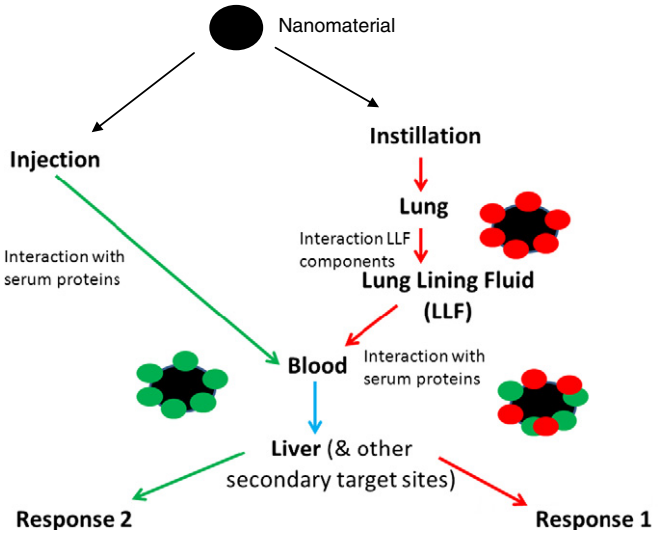

Fig. 5. It is hypothesized that NM toxicity is dictated by the exposure route, due to the acquisition of a surface coating (protein corona) that is determined by the route of entry and that is modified on the transit of NMs in the body. Specifically, following injection into blood, NMs will interact with serum proteins that are absorbed onto the surface of the NMs. The proteins bound to the NMs will be dictated by the physico-chemical properties of the NM. The NMs are likely to accumulate within the liver and exhibit a response that is dictated by the physico-chemical properties of the NMs and the composition of the protein corona. Alternatively, when NMs are instilled into the lungs, proteins within the lung will interact with the NM surface (which is dictated by the physico-chemical properties of the NM). The translocation of the NMs into blood, and their subsequent accumulation will change the composition of the protein corona (and this will be different to the protein corona of injected NMs). NMs exposed via instillation may therefore exhibit a different response in the liver to those that are injected, which derives from differences in the composition of the protein corona.

8. Conclusions

NMs can be exploited within diverse therapeutic and diagnostic applications that can improve the diagnosis and treatment of disease. The types of NMs utilised within the area of nanomedicine are diverse and include the types of materials investigated within the various research projects described. As the surface of NMs is what interacts with cells and tissues it is necessary to identify the influence of NM surface properties on their biological response, and determine how this is influenced by the route of exposure, and physico-chemical characteristics of NMs. Therefore to promote the intelligent and safe design and responsible development of NMs, there is a need to understand how the chemical and physical properties of NMs can be controlled to modify their toxicity. The route of exposure may be able to modify the toxicity of NMs due to the coating of the NM surface with protein, and this is dictated by the physico-chemical properties of the NM. A better understanding of this interaction and the impact on toxicity may also lead to improved protocols for investigation of nanomaterial toxicity *in vitro*. However, this will require that the findings from *in vitro* studies are validated *in vivo*. Specifically, knowledge of the importance of NM–protein interactions may influence how NMs are prepared within hazard investigations. Thus, the findings from *in vitro* cell models need to be compared to *in vivo* responses in order to evaluate whether the simple serum dispersions that have been used widely to date to prepare NMs for the exposure of cells *in vitro* are appropriate or whether the NM suspensions generated should better reflect the exposure route, and transit of NMs in the body. Importantly, this manuscript has used the findings from 3 independent studies as well as evidence from the wider scientific literature on NM biodistibrution, toxicity, and protein corona to generate a hypothesis that relates the toxicity of NMs to the formation of NM–protein complexes that is dictated by the physico-chemical properties of NMs and their route of exposure. It is clear that when NMs are coated with proteins, the properties of the NMs are altered which can modify NM toxicity. A systematic evaluation of the contribution of the protein corona to NM toxicity in different scenarios (e.g. exposure routes, NM physico-chemical characteristics) is required in order to support the development of models that can be used to predict NM–protein interactions and their influence on NM toxicity, and to inform the intelligent design of NMs in the future so that NM–protein interactions and subsequent cellular response are controlled. There are currently no standardised procedures available to study NM–protein interactions, and thus further research is required to overcome the limitations associated with current approaches.

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