1.0 Considerations for the human health implications of nanotheranostics

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Abstract

The research field of nanotechnology promises to deliver copious advantages to the scientific, technological and industrial divisions globally. Most notably, and in focus in this chapter, is the contribution of nanotechnology to the medical sector where engineered nanoparticles (ENPs) and nanomaterials (ENMs) can be beneficially applied to monitor, diagnose and potentially treat human health. Despite heightened interest from industrial and consumer markets pertaining to the significant advantages proposed through ENM medical applications. There remains a substantial concern as to the adverse environmental and human health implications of novel ENMs particularly within nanomedicine, the clinical application of nanotechnology. Nanotheranostics, now an established science concerned with diagnosing and treating specific adverse human health conditions utilises a host of novel ENMs. Initially these ENMs require extensive physicochemical characterisation coupled with far-reaching biologically relevant testing encompassing complex *in vitro* (co-culture) cell models, *in vivo* and human trials before clinical approval and ultimate use. This chapter will provide an overview of the considerations as to the biocompatibility and toxicological impact of novel nanotheranostics building upon the foundation of metal and carbon-based ENMs as well as quantum dots (QDs) which hold tremendous potential owing to their extremely small size and biological marker capabilities.

1.1 Introduction

Nanotechnology is an exciting discipline in science and engineering defined as the design, production, characterisation and application of materials or devices which possess at least one physical dimension within the nanometre range (1-100 nm) (Silva, 2004). At this sub-microscopic scale, the fundamental properties (optical, structural, reactivity) of bulk materials have been shown to change dramatically resulting in novel uses for drug delivery and enhanced biological imaging technologies which, when applied to medical science, result in applications for disease diagnosis, therapeutic delivery systems and enhanced imaging capabilities (Saini et al., 2010). The size reduction of commonly sourced materials *e.g.* iron oxide particles to the nano-size range dramatically changes their surface area, increasing reactivity and how these particles absorb light thus giving rise to applications the bulk material does not possess. With an estimated $18.1 billion invested in nanotechnology in 2014 alone (LUX research) the United States plunges large donations towards the medical, technological and consumer aspects of nanotechnology. There are numerous examples of where the field of nanoscience has garnered much attention for consumer products containing elements of nanotechnology including skin-care products (Gupta et al., 2013), sporting equipment (Institute of Medicine Roundtable on Environmental Health Sciences, 2005), clothing (Kulthong et al., 2010) and food items (Srinivas et al., 2010). it is the medical field however which warrants the largest degree of scientific interest, continued research and most importantly concern where nanotheranostics is concerned. The great benefits of utilising nanoparticles, with their high surface area to volume ratio as opposed to larger particles, they possess unique bioavailability (Jia, 2005) i.e. they can be targeted to specific sites for specific applications. The advantage of this scenario, specific pharmacokinetics can be engineered tailored to each molecule to reduce the interaction between therapeutic nanoparticles and undesired cellular targets, healthy cells and tissue. A vast array of nanomaterials (*e.g.* quantum dots, carbon nanotubes, nanoparticles) have medical applications, and thus far have been incorporated into cell labelling, contrast agents, drug delivery vehicles and antimicrobial agents. Therein introduces the hybrid discipline of nanotheranostics the monitoring and treatment of human health through a combination of therapeutics and diagnostics, aims to increase drug efficacy, safety and biocompatibility (Wang et al., 2012).

Yet despite an elevated interest in nanotheranostics and the proposed advantageous applications there remains a growing public concern as to the potentially detrimental health hazards associated with engineered nanomaterial exposure (Digesu et al., 2016). Nanoparticles may access the body as a nanotheranostic via four main portals; inhalation, intravenous injection, translocation across the epidermal layer or ingestion (Medina et al., 2007). In each case of exposure route, there remains a risk of nanoparticles sequestering into the bloodstream and subsequent translocation and accumulation

at other tissues and organs of the body (e.g. liver and kidneys) (Reddy et al., 2010). Nanoparticle portal routes are of particular concern when examining the toxicological aspect of their nature (Yah et al., 2012), the specificity associated with entry into the body may lead to diverse biological effects from intravenous injection to direct skin-contact approaches. The rather limited data available on unintentional nanomaterial ingestion, due to the numerous host defence mechanisms in place to prevent such localisation, still advises that strong caution should be exercised as particle uptake is still a strong possibility in regions of the gut, Peyer's patches for instance (Bergin and Witzmann, 2013b). The approach for nanotheranostics ideally would emphasise the need for intravenous injection and skin portals for maximum efficiency, ease of access and localised treatment. The emphasis therefore is currently placed on the urgent need to assess the biocompatibility of medically-targeted materials with nanotheranostic potential, utilising nano(geno)toxicology as a screening tool. Summarising, the purpose of this chapter is to provide an overview of the current knowledge encompassing nanotheranostics, and incorporating nanotoxicology, outline the biocompatibility and subsequent hazard associated with current and projected nanotheranostic options available.

1.2 Nanotheranostics

In conjunction with nanomedicine (application of nanotechnology in a clinical setting) (Farrell et al., 2011) (Webster, 2006) we are striving for the next big development in managing human health. Of the nanomedicine sector, drug delivery dominates the market place with *in vitro* diagnostics the second leading field of nanomedicine (Morigi et al., 2012) which in 2012 was worth an estimated $4.8 billion (Zhao and Castranova, 2011). Given the extensive funds available to the nanotheranostic sector, product manufacture and distribution has increased exponentially; metal oxide, carbon-based and inorganic nanoparticles represent a select few examples. Nanomaterial physico-chemical characteristics, specifically engineered and medically-relevant ones, can be further functionalised with surface coatings bearing specific pharmacokinetics enhancing therapeutic potency or enabling molecule targeting maybe potential benefits of such modifications.

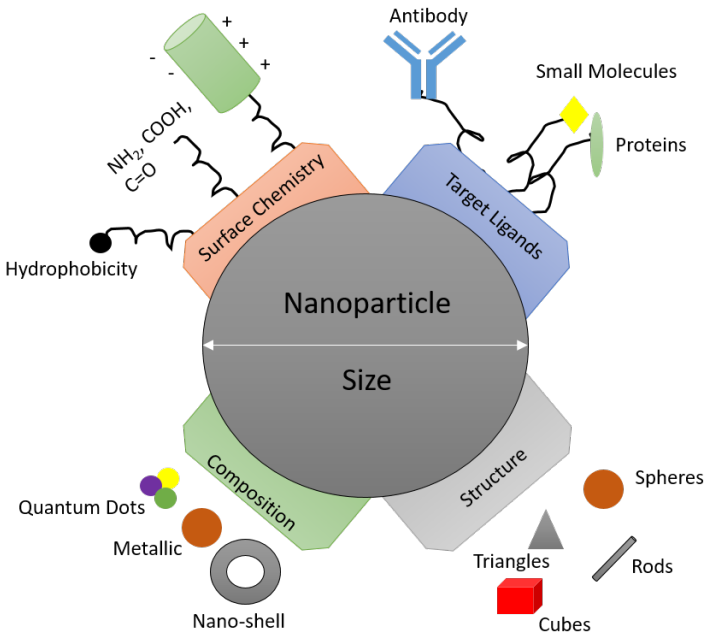

Figure 1. ENP & ENM design can be highly dynamic in shape, surface coating, structure and function. Resulting nanotheranostics can therefore adopt unique and highly specific forms to suit the medical application whether that be drug delivery or translocation to desired cells for optical purposes. Image adapted from (Chou et al., 2011).

Current applications in nanotheranostics rely heavily on the physico-chemical characteristics (PCCs) of the nanomaterials involved being known beforehand. Furthermore, the successful application of these nanomaterials as a theranostic tool is inherently linked to the ability to manipulate the PCCs of the nanomaterial (De Jong and Borm, 2008) particularly where passage across the blood-brain barrier or enhanced distribution are required. The successful application of nanoparticles as a nanotheranostic could be entirely based upon their biodistribution capabilities, this quality of nanoparticles is dependant however on features such as morphology, shape and charge, fortunately modern advances in nanoparticle functionalisation means additional targeting biodistribution can be engineered or 'programmed' into the material. Gold nanoparticles for instance have been explicitly studied due to their low toxicity, bio-imaging, delivery and conjugation characteristics (Tiwari et al., 2014). With simple modifications to the surface of these particles with cell penetrating peptides (CPP) derived from glycoproteins the intracellular uptake and biodistribution were vastly enhanced in mammalian cell lines (HEp-2 &HeLa) quantified with flow cytometry and transmission electron microscopy (TEM). In the same study the bio distribution in mice was then monitored using both functionalised and non-functionalised gold nanoparticles administered via intravenous injection at low doses of 63.72 μg (200 μl) and high doses of 79.65 μg (250 μl), the results showed a higher accumulation of functionalised particles in the liver and spleen of the mice indicative of higher biodistribution capabilities allowing

for their translocation throughout the body resulting in their eventual deposition in the liver primarily where nanoparticle clearance can be initiated (Tiwari et al., 2014).

Nanotheranostic materials can greatly differ in reactivity as opposed to their macroscopic counterparts, examples of each being superparamagnetic iron oxide nanoparticles magnetic potential increases as size decreases (Lu et al., 2007) and graphene edge reactivity increases as atomic layer number approaches one (Sharma et al., 2010). It is these novel, tuneable properties on which theranostics is based however these features could be inadvertently altered (reprogrammed) in a biological environment, potentially corrupting their intended therapeutic function. Size, morphology, surface area and surface charge may become significantly changed once the theranostic enters a biological environment (Gatoo et al., 2014), the now-altered nanomaterial may exhibit unintentional toxicology through increased cellular affinity or an innate ability to trigger an immune response (Zolnik et al., 2010). Considering the degree of variability between nanomaterials (size, shape, aspect ratio functional groups e.g.) the safety of these materials is paramount, it follows therefore that all nanomedicines should be screened for their ability to induce biological damage at a cellular, immune-compromising and genetic level before they can be disclosed for managing, treating and curing human health conditions. At present the risk assessment of engineered nanomaterials and exposure hazards may still present significant risks after clearing Phase I, Phase II and Phase III clinical trials (Resnik and Tinkle, 2007). Associated with oral, dermal, inhalation and injection routes of exposure there are currently three crucial elements to toxicity screen testing nanomaterials, namely; physico-chemical characterisation, *in vitro* assays both cellular and acellular and *in vivo* assays (Oberdorster et al., 2005). Each present unique advantages in robustly screening nanomaterial hazard; physico-chemical characteristics may inherently be associated with toxicity dependent upon shape, charge, distribution and porosity however many of these features are routinely absent in some nano publications, *in vitro* techniques provide opportunity for specific mechanisms or pathways to be elucidated in a controlled environment, aspects which are not feasible when introducing *in vivo* testing. Finally Tier 1 *in vivo* testing (oral, dermal and injection) and Tier 2 (inhalation) exposures provide a more true-to-life scenario accounting for elements not present under *in vitro* conditions. Tier 1 studies tend to focus on inflammation, proliferation and oxidative stress, which can be specifically monitored and quantified with *in vitro* experiments, Tier 2 may then elaborate on deposition, translocation, bio-persistence and toxicokinetics (Oberdorster et al., 2005).

Currently, there exists a battery of assays which can successfully screen nanotheranostics toxicology in cellular systems both *in vitro* and *in vivo.* The number of genetic toxicology assays has grown in recent years to the present day where there exists a tiered screening system for new chemicals and nanomaterials. The HPRT forward mutation assay can be performed in mammalian cells, this requires

more time and a more complex operating procedure but provides reliable results (Johnson, 2012). Especially useful assays are able to quantify genetic damage but also perform analyses in a high throughput manner, the cytokinesis block micronucleus (CBMN) assay being a key example. The CBMN assay relies on cell division to exclude micronuclei from the parent nucleus, the frequency in which micronuclei are captured during cell scoring provides an indicator of genetic damage, and can be applied to nanomaterial exposures (Doak et al., 2012). It follows, there is a clear necessity for nanotoxicology research to inform the scientific development of nanotheranostic therapies as the ever-growing demand for more efficient and potent theranostic treatments with higher efficacy increases in the near future.

1.4 Nanotheranostic biocompatibility

The vital feature of a nanotheranostic material is its biocompatibility i.e. is ability to be introduced into a biological environment without the induction of adverse toxic endpoints. Ensuring a biomedical NM is biocompatible can however be an issue, as the physico-chemical properties that evolve due the material being on the nanoscale can make it inherently toxic. Due to this issue the first step of nanotoxicology study is full physico-chemical characterisation of the test material. These characteristics are defined as being either primary or secondary, referring to properties of the pristine material and the properties when the material is under experimental/biological conditions respectively. Primary NM characteristics include size, morphology, surface area, composition/purity, surface charge, and surface chemistry. Surface charge and chemistry however, will become altered upon introduction into a biological environment, and so infer new secondary characteristics alongside agglomeration state and protein corona formation (Johnston et al., 2012). It is essential that both primary and secondary physico-chemical characteristics of a nanotheranostic material are evaluated. This ensures it possesses the properties required for its intended function but also allows a correlation to be drawn with any adverse toxicological endpoints.

Upon NM administration to the human body, secondary characteristics will evolve upon interaction with biological fluid and biomolecules prior to initial cellular contact (Monopoli et al., 2011). Biomolecules including proteins, lipids and sugars are adsorbed on to a NM surface resulting in the formation of bimolecular interface roughly divided into two components known as the hard and soft coronas (Monopoli et al., 2012). The soft corona is highly dynamic, interchangeable and attached to the tightly bound hard which is comprised of those biomolecules with the greatest affinity for the material. Although the hard corona is tightly bound the material, dissociation does occur, however its association does ensue for many hours and so is regarded as inferring the identity of a NM within a

biological environment (Carrillo-Carrion et al., 2017). The cascade of interactions that occur during the process of corona formation at the NM surface can cause alteration of the secondary structure of attached proteins resulting exposure of new epitopes, potentially giving rise to unexpected biological response (Fleischer and Payne, 2014). Moreover, corona formation will be a determining factor in NM hydrodynamic diameter, agglomeration state and surface charge. Consequently, NM-corona formation is a vital consideration when evaluating nanotheranostic biocompatibility as it will be determining factor in regard to cell surface interactions, cellular uptake and translocation though the body.

A prime example of how corona formation how can influence cellular interaction/uptake of a NM is the absorption of opsonins such as immunoglobulins and complement factors, which facilitates phagocytic uptake by leukocytes (Chen et al., 2016, Schöttler et al., 2016). If a nanotheranostic is designed to specifically target leukocytes e.g. vaccine delivery, then this opsonisation may enhance the materials function (Lameijer et al., 2013). Conversely, enhanced leukocyte uptake may cause the initiation of a chronic immune response potentially leading to downstream toxicity (Evans et al., 2016). Various studies have concluded that NM-corona formation in fact inhibits cellular uptake rather than enhance it (Doak et al., 2009, Cheng et al., 2015, Guo et al., 2016). For example, Cheng and colleagues (2015) investigated how protein corona formation influenced uptake of Au NPs in RAW 264.7 (murine) macrophages and HepG2 cells (human hepatocytes). Corona formation by incubation in serum resulted not only in reduced NP uptake but also alteration of the primary uptake mechanism by obstructing NP-scavenger receptor recognition and promoting recognition by clathrin.

Alteration of NP uptake mechanism due to corona formation introduces a major challenge in the design of targeted therapeutic NPs. In order to facilitate cell specific targeting by a NP its surface is typically functionalised with antibodies or other biomolecules (Mout et al., 2012). NP-corona formation on a functionalised NP is likely to block or impair targeting moieties by causing structural or conformational disruption and obscuring surface recognition (Zanganeh et al., 2016, Saha et al., 2014). Consideration is also required of the effect of corona formation on the drug release profile from nano carriers with surface loaded drugs. For example, it has been demonstrated that corona formation on gold nanorods capped with cetyltrimethylammonium bromide causes a greater hold capacity on the drug payload compared to nanorods without the presence of a corona (Kah et al., 2012).

1.5 Exposure routes and translocation

The typical routes of nanotheranostic exposure are via inhalation, intravenous injection, via dermal exposure and the gastrointestinal (GI) tract (El-Sherbiny et al., 2015, Shi et al., 2017, Sim et al., 2016, Jatana and DeLouise, 2014). For medical applications the degree of this exposure will vary depending on the treatment or diagnostic required, varying from a one off dose (e.g. for an imaging technique) to extensive long term treatment (e.g. for treatment of a chronic condition) (Singh et al., 2012, Nalwa, 2014).

Prior to the advent of nanomedicine, inhalable drugs have been widely available not only to specifically target the lung but also as an alternative to intravenous injection for systematic delivery (Corkery, 2000). Drug delivery via the lung offers the advantages of avoiding the pharmacokinetic issue of pre-systemic metabolism, being easy to self-administer and non-invasive (Rau, 2005). When NPs are administered via the respiratory tract they offer the advantage over their micro counterparts of being able to penetrate deeper into the alveolar region and potentially avoid clearance by alveolar macrophages (Thorley and Tetley, 2013). This ability to penetrate deeper in to the respiratory tree than micro particles mean that there are numerous potential applications of nanotheranostic via the inhalation route. An exciting example of this is the development of vaccine nanoformulations such as alternatives to the standard BCG vaccine (Ballester et al., 2011). The study entailed conjugating the TB antigen Ag85B to 30 nm polypropylene NPs and demonstrated increased vaccine efficacy when administered by inhalation compared to intradermal delivery in murine models. Nanomedicines can also be constructed to facilitate drug delivery in the bronchial regions of the lung e.g. polyethylene glycol (PEG) NP coatings have been shown to permit NP mucus penetration if it is constructed with a high density and low molecular weight (Liu et al., 2015). There is however a significant toxicological risk when administering nano medicine via the respiratory tract therefore full toxicity assessment and physico-chemical characterisation is essential. Once within the lung a NM has the potential to promote a chronic inflammatory response and subsequently lung injury. And at the cellular level promote oxidative stress, inflammatory signalling, mitochondrial damage, protein denaturation, impairment of phagocytosis, endothelial dysfunction, cell cycle alteration and DNA damage (Bakand et al., 2012).

Self-medication is most commonly undertaken by oral administration due to its convenience and non-invasiveness. However, taking drugs via the gastro intestinal tract is not particularly efficient due to low bioavailability because dissolution rate-limited absorption typically of many drugs, first pass metabolism effects, food effects and short half-lives (Gershanik and Benita, 2000). These are particular challenges when treating chronic conditions such HIV, diabetes or psychiatric illnesses (e.g.

schizophrenia), challenges that may be resolved by the use of nano-drug delivery systems (Dening et al., 2016, Giardiello et al., 2016). A suitable example is the use of solid lipid nanoparticles (SLNs) which offer the ability to encapsulate lipophilic and hydrophilic drug molecules for controlled release and also offer protection from the environment of the gastro intestinal tract (Das and Chaudhury, 2011). Indeed, SNLs have been developed that provide a notable increase in drug bioavailability; by encapsulating the antipsychotic clozapine in SNLs the drug was absorbed by lymphatic transport pathways thereby avoiding the hepatic portal vein system, preventing the first pass effect (Dening et al., 2016). A further example of a NM increasing oral drug bioavailability is the development of insulin conjugation chondroitin sulphate caped AuNPs, which like SNLs provide protection against the harsh environment of the GI tract (Cho et al., 2014) . From a toxicological stand point however there is significant potential for an adverse response as with the airway tract with the additional consideration of the guts microbiome and the effect of its perturbation on the host (Bergin and Witzmann, 2013a).

Medical application of NMs to the skin maybe either for a topical treatment or by transdermal drug delivery (TDD) for systematic treatment. AgNPs for instance maybe applied as topical antimicrobial agents to control microbial growth and consequently would be required to stay on the dermal surface (Pal et al., 2009, Jacobs et al., 2010). Conversely, TDD is required to cross the dermal barrier and reach the circulatory system or be retained in the skin. TDD offers the benefit of being less painful than standard needle injection and the ability to bypass first pass metabolism (Palmer and DeLouise, 2016). The toxicological risk a NM possesses is dependent on its potential to penetrate the dermal layer. Typically, NMs may potentially penetrate the skin via three different pathways intracellularly, intracellularly or via dermal structures such as hair follicles (Baroli et al., 2007).

Depending it physico-chemical characteristics NM may not be confined to its target region and maybe capable of distributing from the site of exposure to a number of secondary organs via the cardiovascular, central nervous, renal or hepatic systems (Kermanizadeh et al., 2015). Depending on the NMs intended function this may or may not be a desirable outcome. For example, if the intended function of a NM was the treatment of a systemic decease by inhalation then translocation across the air/blood barrier would be essential (Thorley and Tetley, 2013). It is of note however that the exact mechanism of NM translocation though the air/blood barrier is unclear. It is thought that alveolar macrophages play a role in NM translocation by transporting NMs to the thoracic lymph nodes (Zhao et al., 2011, Shaw et al., 2016). It is also speculated that formation of a NM-protein corona via interaction with lung lining proteins may allow shuttling of the NM across the air/blood barrier (Kermanizadeh et al., 2015). As with airway administration nanomedicines administered via the GI tracts also have to utilise transepithelial absorbance mechanisms to facilitate systemic distribution. The major pathways that have been implicated in the uptake of NMs across the GI wall are through

M-cells lining intestinal Peyer's patches and through intestinal enterocytes (Bellmann et al., 2015). Following translocation through the GI epithelial layer a NM will enter into the lymph fluid and potentially drain into the systemic cardiovascular system or be absorbed into the capillaries of the cardiovascular circulatory system (Cuong and Hsieh, 2011). In comparison the rate of NM translocation following intravenous injection will differ significantly from the inhalation and oral ingestion. Intravenous administration permits direct access to the circulatory system and consequently rapid distribution throughout the body. A recent three part *in vivo* study by Kreyling et al. has evaluated the quantitative biokinetics of radioactive titanium dioxide TiO_2 NPs administered in rats by intravenous injection, oral application and inhalation (Kreyling et al., 2017a, Kreyling et al., 2017c, Kreyling et al., 2017b). This large study noted that that 24h following intravenous administration 95.5% of the NPs administered located to the liver, 2.3% to the spleen, 0.7% the skeletal system, 0.5% remaining in blood circulation and with detectable NPs in all other organs, interestingly NPs were still undergoing hepatic clearance 28 days after treatment (Kreyling et al., 2017a). Comparatively part 2 of the study demonstrated a very different biokinetic patters when the same NPs were administered orally with <0.6% of the applied dose absorbed across the GI barrier, a NP faction >0.001% was present still in most organs 7 days after treatment (Kreyling et al., 2017c). Finally, part 3 of the study again demonstrated different biokinetic patters compared to the intravenous administration and one more similar to the GI tract (Kreyling et al., 2017b). After one hour, 4% of the applied NP dose was able to pass through the air/blood barrier and 0.3% was retained in the animal after 28 days with the highest fractions present in the liver and kidneys (0.03%). This study demonstrated the variation NP translocation depending on exposure route however also importance is the biopersistence of the test material regardless of how it was administered. This consequently shows how toxicological consideration must also be given to regions of the body other than the specific target of a nanotheranostic material due to its potential biokinetic distribution. In particular, its effect on the hepatic and renal systems need to be considered (He et al., 2015).

1.6 Metal based nanomaterials

In recent years' significant emphasis has been on the development of metal and metal oxide based nanotheranostic materials. There is a great variety of different metal based NMs being developed for usage in the medical field for purposes such as photodynamic therapy, targeted drug delivery and the enhancement of imaging techniques (He et al., 2015, Jain et al., 2015, Estelrich et al., 2015). This discussion will however focus on the use and potential toxicity of iron oxide NPs there has been extensive focus on their development for nanotheranostic approaches in addition assessment of their toxicology profile.

1.6.1 SPION uses and characteristics

The unique physico-chemical characteristics of Superparamagnetic iron oxide nanoparticles (SPION) have made them an ideal candidate for several biomedical applications. These include magnetic resonance imaging (MRI) enhancement, targeted drug/gene delivery systems and hyperthermia therapy (Bulte and Kraitchman, 2004, Gupta and Gupta, 2005, Dizaj et al., 2014, Laurent et al., 2011, Mahmoudi et al., 2011). SPION for MRI enhancement can be used as both a negative and positive contrast agent by allowing for reducing signal deterioration that results in darker or brighter images respectively compared to more traditional contrast chemicals (Dolci et al., 2013). This is achievable due to the superparamagnetic behaviour SPIONs exert. The induced magnetic movement of the particles creates a local magnetic field that affects nearby water molecules. The latitudinal and longitudinal relaxation times of those water molecules are detected by the MRI equipment such that the resulting relaxation time map produces an image of the tissue under investigation (Neuwelt et al., 2015). In terms of targeted drug delivery SPION's superparamagnetic properties can allow localisation of a drug to a specific target site within the body using a magnetic field. This enhances the specificity of drug release at a defined pathological site, allowing the reduction of dosage and potential adverse drug toxicity (Mahmoudi et al., 2011). Similarly, the ability of the material to be localised in this manner potentially makes it suitable as a gene delivery vector; SPIONs coated with PEG grafted polyethylenimine have been shown as an efficient ancosd MRI-visible vector for gene delivery into human adipose derived mesenchymal stem cells (Pang et al., 2014). Aqueous SPION suspensions exist for the most part as Fe_3O_4 (magnetite) or γ-Fe_2O_3 (maghemite) (Hamm et al., 1994, Dias et al., 2011). Fe_3O_4 has a cubic crystalline lattice inverse spinel structure comprised of closely packed O-2 ions, Fe^{2+} ions are in half of the octahedral sites and Fe^{3+} ions are in the remaining octahedral and tetrahedral sites (Gawande et al., 2013). Pure γ-Fe_2O_3 has a similar cubic structure but with the absence of Fe^{2+} ions (Erlebach et al., 2014). Due to the presence of Fe^{2+} in Fe_3O_4 it is considered to be thermodynamically unstable as the Fe^{2+} can readily undergo oxidation (Wei et al., 2015). It is therefore

not uncommon for SPION to exist in a non-stoichiometric state in between γ-Fe_2O_3 and Fe_3O_4 when in suspension. SPION chemical state amoung its other physico-chemical poperties is a vital consideration when evulating its toxcioligcal profile.

1.6.2 SPION saftey assessment

With potential wide spread usage of SPION, assessment of the NM potential toxicity is vital. Indeed, various studies have demonstrated SPION to promote the formation of apoptotic bodies, activate an immune response, generate reactive oxygen species and cause DNA damage (Singh et al., 2010). Early studies of SPION toxicity focused primarily on evaluation of cytotoxicity; a study by Gupta and Gupta (2005) for instance evaluated the toxicity of pullulan coated SPION (40 – 45 nm). The investigation determined the test SPION to be cytotoxic and a promotor of cytoskeleton disruption in human dermal fibroblasts (Gupta and Gupta, 2005a). Similarly, dimercaptosuccinic acid (DMSA) coated SPIONs promoted acute cytotoxicity in growing rat neurons (PC12) (Pisanic Ii et al., 2007). Various studies have however also shown SPIONs to be non-cytotoxic (Yu et al., 2008) (Yu et al., 2008, Mahmoudi et al., 2009, Laurent et al., 2008).

A multitude of investigations have shown SPIONs to be capable of inducing an immunoloigcal respone. For example, elevated IL-8 and IL-6 expression occurs in normal epidermal keratinocytes (HEK) following exposure to SPION (Murray et al., 2013). Furthermore, whole blood treated with both polyacrylic acid (PAA) coated and non-coated SPION induced an increase of 6 cytokines; interleukins 1β,6,8, 10 and the tumor necrosis factor (IL-1β, IL-10, IL-6, IL-8, and TNF) via activation of the TAK1, p38 MAPK and JNK pathways (Couto et al., 2014). *In vivo*, a dose dependent increase in IL-1β and TNF expression was quantified in the lungs of Sprague-Dawley rats following inhalation of bare γ-Fe2O3 NPs (~72 nm) (Zhong et al., 2010). The same study noted increased lung oxidative stress identified by elevations in a glutathione disulphate (GSSG) to glutathione (GSH) ratio and a reduction in ferric/reducing antioxidant power. Similarly, aerosol exposure of uncoated Fe_3O_4 NPs of Wistar rats over 4 weeks resulted in substantial neutrophil influx into the alveolar region of the lung alongside oxidative stress quantified by increased 8-hydroxy-2' -deoxyguanosine (8-OHdG) levels (Ahamed et al., 2013). The ability of SPION to activate immunological pathways demonstrates the importance considering interaction of the material with the cellular components of the innate immune system and the consequential influence of this on other cell types within tissue, particularly in relation to the onset of genotoxicity.

Iron has long been associated with the induction of carcinogenesis (Valko et al., 2006). Iron ions are able to catalyse the formation of •OH radicals via the Fenton reaction, potentially causing a redox imbalance and consequently oxidative stress (Weinberg, 1996). Certainly, studies have proved SPIONs to be capable of inducing genotoxicity in this manner; Singh and colleges (2012) demonstrated SPION genotoxicity to be redox state dependant. Investigating genotoxic potential of dSPION in both γ-Fe2O3 and Fe3O4 forms via the in vitro micronucleus assay showed only γ-Fe2O3 capable of inducing double stranded DNA breaks as a direct consequence of oxidative stress in the MCL-5 B- lymphoblastoid cell line (Singh et al., 2012). This was correlated with the ability of γ-Fe_2O_3 dSPION to undergo cellular uptake by endocytosis and ion dissociation to occur within lysosomes. Similarly, non-coated γ-Fe2O3 SPION have been shown to cause oxidative DNA damage in liver hepatocellular cells (HEPG2) (Sadeghi et al., 2015). Moreover, a recent study comparing metal oxide NP genotoxicity in primary human lymphocytes, uncoated γ-Fe2O3 promoted ROS generation and subsequent genotoxicity quantified by the single-cell gel electrophoresis assay (comet assay) (Rajiv et al., 2016). Fe3O4 SPIONs have however also be shown to be genotoxic, differing from the study conducted by Singh et al (2012). Oleate-coated Fe3O4 for instance promotes DNA damage in the TK6 lymphoblastoid cell line (measured by the comet assay), the same study did however state that identical SPION without the oleate coating did not induce genotoxicity (Magdolenova et al., 2015).

Not all invetsigations have proven SPIONs to be genotoxic for example L-glutamic acid coated γ-Fe2O3 that underwent genotoxic assessment by the comet and micronucleus assay demonstrated no significant genotoxic response (Zhang et al., 2015). Similarly, an investigation comparing iron oxide NPs with bulk iron oxide claimed that neither nano-sized or bulk γ-Fe_2O_3 and Fe_3O_4 caused genotoxicity in Syrian hamster embryo cells (Guichard et al., 2012). Differences between SPION genotoxicity studies is the result of numerous factors; the most prominent being variation between physico-chemical characteristics. It can however be summarised that SPION (dependent upon form) pose a substantial risk of causing genotoxicity and ultimately potential downstream carcinogenesis, hence, assessment of is risk is essential.

A vast number of nanotoxicology studies in the literature determine metal and metal oxide NPs such as SPIONs are inherently toxic and as such imply they are fit for their intended biomedical purpose. It should however be of note that rather than uses these studies to dismiss the use of a NM, they should in fact be used to identify the specific properties that cause toxicity so they can be rectified in future designs.

1.4 Carbon-based nanomaterials

Carbon-derived nanostructures arguably, are the most diverse in application and design with respect to drug delivery and cancer treatment within the field of nanomedicine (Elhissi et al., 2012) (Madani et al., 2011). Carbon nanostructures are typically highly ordered and possess characteristics engineered for a specific purpose, carbon black pigments in printer toner (Pirela et al., 2015), carbon nanotube rigidity as cell scaffolds in tissue engineering (Edwards et al., 2009), lastly graphene and few-layer graphene with hydrogen storage capabilities (Kostoglou Nikolaos, 2015). Graphene also holds tremendous potential as a smart therapeutic delivery system of photodynamic treatments (Wei et al., 2016) which can be targeted to tumour growths with monoclonal antibodies to deliver doses of photo-toxicity. Carbon black and particularly carbon nanotubes have garnered significant attention for their ability to elicit genotoxic responses (Patlolla et al., 2010) (Toyokuni, 2013). Recently however graphene-based genotoxicity is being reported in literature (Ma-Hock et al., 2013), largely due to the physico-chemical characteristics and aspect ratio similarities shared with carbon nanotubes. Carbon-based nanomaterials exhibit sp^2 and sp^3 electronic valences commonly which provides opportunities for fluorescence and absorbance exploration in biological imaging. Nano-diamond and carbon dots by virtue of optoelectronic properties may display natural fluorescence (Wang et al., 2012), beneficial to theranostic tracking *in vivo*.

i) ***Graphene***, an inorganic nanomaterial is becoming more commonly associated with drug delivery, and has been extensively explored as a chemotherapeutic agent and theranostic (Rahman et al., 2015) due to the highly tuneable high surface area and adsorption properties (Li et al., 2016). A large number of molecules, polymers, antibodies may be functionalised with graphene in order to enhance targeting or potency as a theranostic treatment. With every atom exposed, graphene exposes an extremely large surface area (>1000m^2/g) efficient for therapeutic loading and bioconjugation (Yang et al., 2013). As a nanotheranostic the PCCs of graphene have been exploited in particular the near-infra-red (NIR) optical absorbance which when utilised during in vivo treatments can deliver photothermal therapy, which has already achieved excellent anti-tumour effects (Yang et al., 2013). As with all nanotheranostics however, significant attention is being placed on the toxicity of such treatments, where it is abundantly clear that size, and surface chemistry directly influence toxicity (Yang et al., 2013) (Nezakati et al., 2014) (Chang et al., 2011). Graphene also shows ultrahigh in vivo tumour uptake, only being surpassed in uptake potential by PEGylated CNTs using mouse models, utilising the NIR absorbance properties in phototherapy.

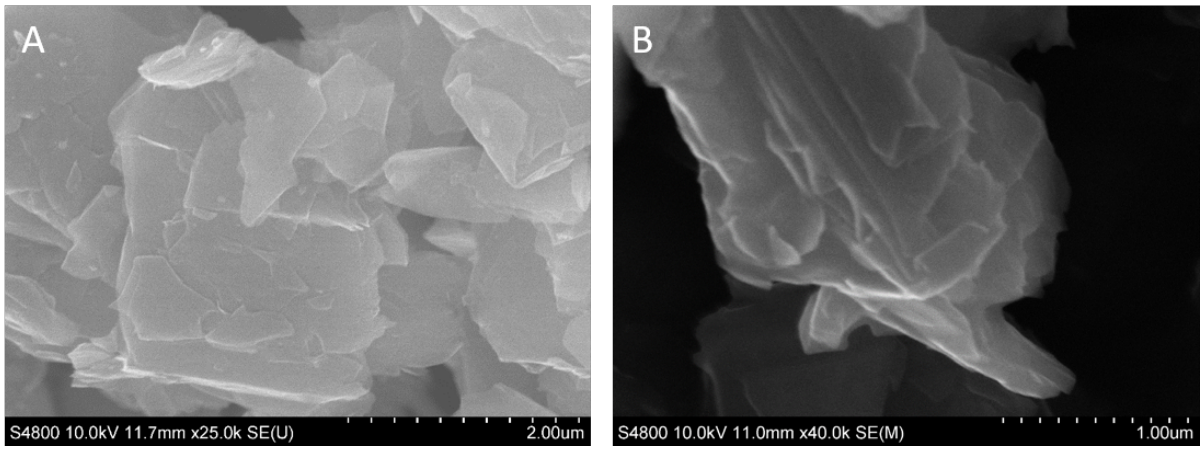

Figure 2. Scanning electron micrographs of dry few-layer graphene as presented immediately post-manufacture within an occupational environment. Analysis was performed using the Hitachi Ultra High Resolution field emission (FE)-SEM model number: S-4800 at Swansea University, images captured and provided by Michael J Burgum.

The functionalisation of graphene, graphene-oxide (GO) and reduced GO (rGO) have been widely explored as potential anti-cancer therapeutics with coatings including polyethylene glycol (PEG) to maintain biocompatibility as well as acutely potent drugs such as doxorubicin. The various combinations of graphene and targeted applications can be summarised below as each pertain to the treatment of cancer (Orecchioni et al., 2015).

Table 1. The various applications of functionalised graphene as nanotheranostics, adapted from (Orecchioni et al., 2015). The nature of the material and the therapeutic agent it carries can specifically alter the targeting of the material as well as the function and target cell type.

Application	**Cancer Type**	**Model**	**Graphene Therapeutic**
Drug Delivery & Imaging	Burkitt's Lymphoma	Human *In Vitro*	GO-Doxorubicin + Rituxan (Yang et al., 2010)
Imaging & Photothermal Therapy	Breast Cancer	Mouse *In Vivo*	PEGylated nano-graphene sheets (nGS-PEG) (Yang et al., 2012)
Drug Delivery	Breast Cancer	Human *In Vitro*	GO-Adryamicin (Wu et al., 2012)

Using current literature as a guide, three-times the number of publications in 2014 as opposed to 2012 (Orecchioni et al., 2015) there appears to be an increasing interest in the use of graphene as an anti-cancer theranostic, the double exposed surface sides of graphene presents unique opportunities for modifications via π-π stacking however if nanomaterial clearance cannot be achieved by host immune cells, potent toxicity issues are likely. Moreover, graphene surfaces present a platform for multiple anti-cancer drug functionalisation for targeted therapeutics, initially the graphene is decorated with sulfonic acid groups rendering the molecule stable in physiological solution, followed by folic acid (FA) conjugation allowing targeted delivery to human breast cancer MCF-7 cells and subsequent release of two commonly used therapeutic treatments doxorubicin (DOX) and camptothecin (CPT) (Zhang et al., 2010). The folic acid-conjugated GO bearing DOX and CPT demonstrated high affinity for MCF-7 cells and high cytotoxicity as opposed to FA-GO bearing only one of the anti-cancer drugs concluding that in future, the combined use of DOX and CPT aboard FA-GO delivers a far more potent cytotoxic dose to breast cancer cells (Zhang et al., 2010).

ii) ***Carbon nanotubes (CNTs)***, similarly to graphene possess numerous PCCs which make them choice selections as theranostics, high surface area for functionalisation and NIR optical properties (Wang et al., 2012) in delivering phototoxic doses to tumour cells (Robinson et al., 2010). In the study carried out by (Robinson et al., 2010) the first dual function for PEGylated CNTs was exploited whereby the biologically non-toxic nanotubes administered intravenously delivered photothermal treatment for tumour elimination at 808 nm and acted as photo-luminescent agents for *in vivo* imaging. In the experiment 70 μg CNTs/mouse (equivalent to 3.6 mg/kg) was administered and revealed significant tumour elimination with no toxic side effects, notably this result closely resembled a comparable experiment repeated with gold nanorods however the nanorod dose required 10 times the concentration to achieve similar results lower and showed more irradiation suggesting significant benefits of utilising CNTs for combined cancer therapy and treatment.

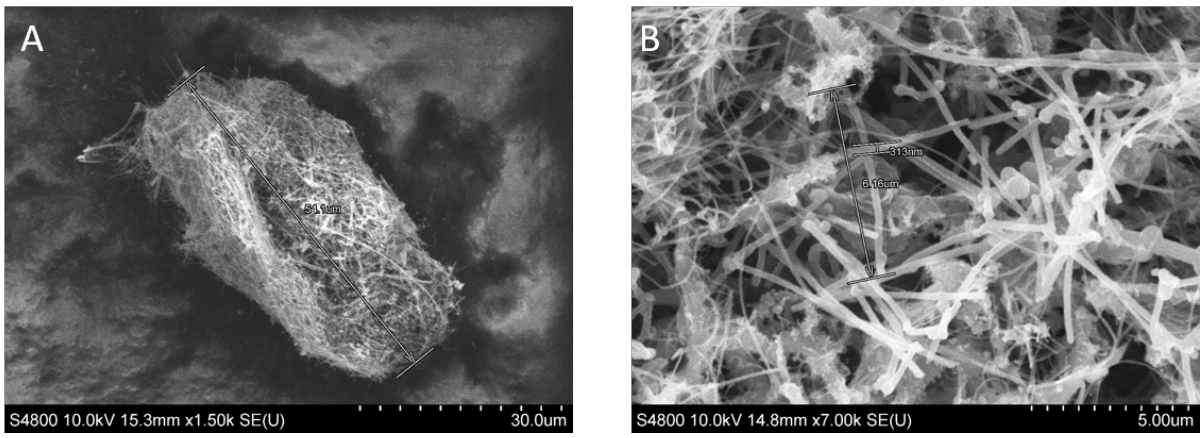

Figure 3. Scanning electron microscope images of **A)** water-dispersed CNTs 30 μm and **B)** 5 μm resolution. CNTs dispersed in this manner commonly associate into 'bird nest' agglomerates and pose a significant biological hazard in this biopersistent arrangement making enzymatic clearance difficult. Analysis was performed using the Hitachi Ultra High Resolution field emission (FE)-SEM model number: S-4800 at Swansea University, images captured and provided by Michael J Burgum.

It has been established that introducing nanomaterials to the human body, or *in vivo* animal testing poses toxicity issues, which can stem from nanoparticle size, surface area and functionalisation. Carbon nanomaterials, particularly graphene and CNTs possess these toxicity capabilities, however they pose greater risk owing to their morphology and aspect ratio which can induce frustrated phagocytosis (Boyles et al., 2015), hinder nanomaterial clearance (Roberts et al., 2016) and potentially exacerbate immune responses. The question remains, should these nanostructures be introduced as nanotheranostics when the majority of literature hints at their toxicity capabilities and bio persistence tendencies? In vitro exposure studies with graphene and CNTs have revealed cytotoxicity, genotoxicity, immune-cell activation and oxidative stress to be common themes throughout with bio persistence likely a contributing factor (Sanchez et al., 2009). The manipulation of carbon-based nanotheranostics *in vivo* therefore becomes pivotal at two control points; bio-persistence and clearance both of which tightly associate with their pathogenicity, similar to asbestos. The bio-persistence of these materials is fundamentally correlated with their tendency to agglomerate thus making nanomaterial clearance by macrophages and neutrophils much more difficult. The degradation, largely in the form of peroxidase enzymes released by immune neutrophils (Kotchey et al., 2013) is generally accepted in literature as the primary means of carbon-based material degradation *in vivo* however other mechanisms likely play a role in assisted clearance. It has also been reported that controlled respiratory burst by macrophages containing lignin peroxidase can induce biodegradation in single-walled carbon nanotubes (SWCNTs), it was later hypothesised that the

biodegraded products would induce a weaker immune response (Hou et al., 2016). Following the use of respiratory burst the authors noted that in vitro mimicking the enzymatic reactions of the biodegradation the SWCNTs order of accelerated degradation was heavily reliant on functionalisation of the order: OH-SWCNTs, > ox-SWCNTs, >> pristine-SWCNTs, it should also be noted that defect sites on the CNTs were likely to accelerate the biodegradation process by allowing the enzymes access into the carbon-based structures. These findings are likely to be beneficial during the design and safe manufacturing of clinically-relevant CNTs in the future whereby the increased biodegradability, efficient clearance and lower toxicity will allow them to be potential theranostic agents.

It is evident that size, aspect ratio, morphology, composition and functionalisation play a crucial role in altering carbon-based theranostics toxicity both in positive and in some instances detrimental ways. As a result carbon-based nanomaterials should always be treated with extreme caution and extensive theoretical investigation coupled to robust toxicological testing prior to their use in clinical applications.

1.5 Quantum Dots

Where modern biological imaging is concerned there are few choices available which surpass quantum dots, brightly fluorescent nano crystals spanning just a few nanometres in diameter and can be engineered across a broad spectrum of light frequencies (Smith and Nie, 2009). These semiconductor nano quantum dots encompass an exciting part of inorganic fluorophores with use in optoelectronics as well as biological imaging (Chen et al., 2008) with unique advantages over molecular dyes with highly resistant photo-bleaching capabilities (Medintz et al., 2008). In addition to the increased stability of quantum dots over conventional fluorescent dyes used in traditional biological assays, quantum dots are engineered to exhibit a wider range of excitation and emission wavelengths (Lovric et al., 2005). Microscopic observations of these particles have revealed an on-off blinking omission characteristic however current advances in this technology specifically engineering alloyed composition from the core to the quantum dot surface can greatly enhance the omission to now remain consistently “on” (Smith and Nie, 2009). Quantum dot composition can fluctuate widely in accordance with intended use and manufacture preference, fundamentally a single quantum dot consists of a fluorescent core and an outer crystal shell which can vary in thickness, the function of which is to insulate the core from ionisation processes within a biological environment (Chen et al., 2008). As Chen et al concluded however, there are limitations as to the photoluminescence of single and bulk nano crystal quantum dots (NQDs) under experimental conditions. These problems stem from the surface chemistry of the crystalline shell which after interacting with the environmental physiology can greatly affect the emission spectra of these molecules. This study overcame the issue

by manufacturing 'giant' Cadmium selenide (CdSe) quantum dots possessing an overly large shell consequently placing a sufficiently large gap between photo luminescent function and surface chemistry. Herein however lies a major issue with quantum dots, they quite often contain core components, chemicals, nanostructures which can offer a degree of toxicity, cadmium in this case has been extensively studied for its genotoxicity (Celik et al., 2009). The paradox remains therefore, that to utilise these nanotheranostics overcoming the innate toxicity associated with nanostructures and chemicals is crucial.

Biological applications of quantum dots requires stable dispersion in aqueous media, tolerability to neutral, acidic and basic pH ranges and ideally are water soluble. With advances in nanotheranostics in mind there now exist numerous effective strategies for producing hydrophilic quantum dots post-synthesis, these two categories are; complete cap exchange and native surface modification. Complete cap exchange encompasses hydrophobic ligand displacement in favour of hydrophilic groups which coordinate the outermost chains in the surrounding shell of the quantum dot. Typically this can be as primitive as the removal of amines and addition of thiols conjugated to hydrophilic moieties which act both to improve water solubility and enhance stability (Chan and Nie, 1998). Designing a multifunctional nanotheranostic utilising quantum dots can theoretically accomplish precision diagnosis with effective treatment typically as anti-tumour agents, this is the case with Tungsten sulphide quantum dots which remain under optimisation in delivering radiotherapy/photothermal synergistic treatment (Yong et al., 2015). Quantum dots, in clinical applications would be administered intravenously for maximum efficiency, with tumour targeting in mind to deliver acute doses of radiotherapy and photothermal exposures, moreover in the study by Yong et al, the administration of 3 nm Tungsten sulphide quantum dots revealed no toxicity, confirmed through eosin staining, blood haematology and biological assays either in vitro or *in* vivo which demonstrates high levels of biocompatibility and therapeutic efficacy. Quantum dot toxicity appears to be inherently linked to size, as is the case with cadmium telleride (CdTe) which at sizes below 4 nm showed a greater ability to localise and induce toxic effects on PC12 and N9 cells at concentrations as relatively low as 10 μg/ml, quantified with chromatin condensation and membrane blebbing. Moreover the surface charge of quantum dots bearing positively charged groupings induced higher toxicity opposed to neutral charge quantum dots (Lovric et al., 2005). In the 2005 study by Lovric et al, the conclusions drawn were that size as well as surface chemistry greatly influences the quantum dot localisation and subcellular distribution, however with the addition of antioxidant reagents (N-acetylcysteine) and bovine serum albumin, the toxicity of 3-4 nm quantum dots can be mitigated significantly.

Since it has been well established that the relative toxicity of nanotheranostics can stem from their bio-persistence, the degradation of quantum dots presents a unique challenge especially if this process takes place in vivo as the potential bi-products would be heavy metals along with potentially modified nano structures of the shell. The following study by Mancini et al offers novel methods of quantum dot degradation which have been encapsulated in a polymer coating to better target the therapeutic, in which reactive oxygen species (ROS) a collection of short lived, high energy radicals produced naturally through cellular metabolism actively breakdown the quantum dot's (Mancini et al., 2008). This particular finding also facilitates fluorescence quenching with hypochlorous acid (HOCl) and hydrogen peroxide proving the most efficient at crossing the polymer coating and instigating the degradation and simultaneous quenching, leading to the oxidation of either selenium ions in the core or other forms of heavy metal constituent. This may prove to be a hindrance as a nanotheranostic tool however as quantum dots, either at the time of delivery or over a chronic exposure period may generate ROS or activate an immune response capable of introducing more oxidative stress to a localised site promoting the premature removal of the quantum dots.

1.5 Summary

Presently, despite numerous studies displaying medical promise with a vast array of nanotheranostics ranging from metal oxide, quantum dots and carbon based materials there remains a pressing issue. While the mechanistic science of toxicology and (pro)-inflammation (pathway elucidation) is well understood from *in vitro* exposures in monoculture and co-cultures as well as basic in vivo exposures (mice, rats) the pressing issue of nanomaterial fate still remains. As this is often driven by physico-chemical characteristics during in vitro exposures, extrapolating this to in vivo conditions rarely yields similar trends. Currently however there appears to be much conflict of interest within publications as to the potential toxicity of the nanomaterials discussed and their intended use in nanotheranostics. Ideally, a nanomaterial which boasts low toxicity provides a good platform when considering nanotheranostic applications which incorporates high biocompatibility, no bio-persistence and can be specifically targeted. A summary of the advantages and disadvantages posed by the nanotheranostics discussed in this chapter can be seen in Table 2 below which also details the use of various cell models used in nano(geno)toxicology to provide a robust risk assessment posed by novel ENMs.

Table 2. Suitability of ENMs applied as nanotheranostics

Criteria for assessing nanotheranostic potential	Formulation for potential nanotheranostic		
	Metallic NPs	Carbon-based ENMs	QDs
Advantages for use in human clinical trials	Superparamagnetic behaviour would allow for development of targeted drug delivery systems and enhanced medical imaging techniques with high resolution capabilities.	Highly diverse in structure, chemistry and potential applications. High surface area (Graphene & CNTs) provides ample functionalisation space, targeted therapeutics. Resilient to damage, long-lasting therapeutic potential of slow release drug	Exceptional fluorescent capabilities, vast array of excitation and emission wavelengths possible permitting tailored applications. Surface modifications can enhance uptake and targeted theranostics.
Disadvantages for use in clinical trials	Evidence within the literature that transition metal NP's can possess adverse toxicological profiles. The primary risk highlighted is the induction of oxidative stress, which can cause an adverse immunogenic response and genotoxicity.	Largely untested in complex cell models, with conflicting data in the literature regarding genotoxicity. Surface chemistry greatly governs the overall toxicity of carbon-based ENMs, problematic in targeted therapeutic design. Imaging, tracking of carbon-based ENMs	Outer shell encapsulating the nano crystal core can be greatly affected by physiological conditions. Nanocrystal composition typically contains structures, chemicals which offer a degree of toxicity (Cadmium).

		difficult, require additional surface modifications which alter toxicity.	
Cell model advised for nanotheranostic development	Co-culture model representative of the region(s) of exposure. It is vital that these models incorporate immune cell types to elucidate complex cell to cell interactions.	Extensive airway co-culture modelling required to elucidate complex cell-cell interactions (immuno-toxicity).	Complex 3D structures, highly representative, pre-made specific tissue/organ. Allows screening potential for drug delivery within a specific tissue whilst maintaining optical properties for diagnostics.

Unfortunately, it seems at the nano-scale the majority of nanomaterials pose some biological hazard, either through toxicity or bio-persistence in non-target cells inducing downstream, damaging effects. With the heightened interest and influx of funding, nanotheranostics will likely receive far more exposure and attention moving forward, however, it is essential that the correlation between successful application and minimal detrimental health effects remains paramount.

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