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Review Article

Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION)

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Abstract

Superparamagnetic iron oxide nanoparticles (SPION) are being widely used for various biomedical applications, for example, magnetic resonance imaging, targeted delivery of drugs or genes, and in hyperthermia. Although, the potential benefits of SPION are considerable, there is a distinct need to identify any potential cellular damage associated with these nanoparticles. Besides focussing on cytotoxicity, the most commonly used determinant of toxicity as a result of exposure to SPION, this review also mentions the importance of studying the subtle cellular alterations in the form of DNA damage and oxidative stress. We review current studies and discuss how SPION, with or without different surface coating, may cause cellular perturbations including modulation of actin cytoskeleton, alteration in gene expression profiles, disturbance in iron homeostasis and altered cellular responses such as activation of signalling pathways and impairment of cell cycle regulation. The importance of protein–SPION interaction and various safety considerations relating to SPION exposure are also addressed.

Keywords: *SPION*; *cellular stress*; *cytotoxicity*; *DNA damage*

Superparamagnetic iron oxide nanoparticles (SPION), the only clinically approved metal oxide nanoparticles (NPs), hold immense potential in a vast variety of biomedical applications such as magnetic resonance imaging (MRI), targeted delivery of drugs or genes, tissue engineering, targeted destruction of tumour tissue through hyperthermia, magnetic transfections, iron detection, chelation therapy and tissue engineering (1–6). The SPIO agents have a unique property of superparamagnetism that confers advantages such as the generation of heat in alternating magnetic fields; or an ability to be guided to a specific tissue or organ by an external

Neenu Singh completed her Master's in Medical Biochemistry in 2003 (with distinction), and in 2007 she obtained her PhD in Biomedical Science at the University of Wales Institute, Cardiff (UWIC). She worked on the megakaryocyte development for a year before moving to the School of Medicine, Swansea University in 2008 as a post-doctoral researcher in nanomedicine. In 2009, she obtained a grant from the Texas-UK Collaborative to visit and work at Rice University and Texas A&M. Currently her project involves investigating the genotoxic potential of iron oxide nanoparticles with different surface coatings and their mechanism of action.

Gareth J.S. Jenkins is currently employed as a Professor, leading a multidisciplinary research group investigating DNA damage induction and the genetic basis of GI tract cancer (mainly oesophageal). He has written 50 papers on these subjects over the past 10 years. He has been investigating the molecular events driving cancer development in Barrett's oesophagus patients, whilst also being involved in basic molecular research in the fields of DNA damage and mutagenesis. He is particularly interested in the mechanisms of DNA damage induction (by chemicals and novel nanomaterials), the dose responses to DNA damage, and the protective mechanisms that reduce the impact of DNA damaging agents. He has a growing interest in risk assessment of DNA damage induction and has recently joined the UK government's Committee on Mutagenicity (COM), which assesses risks posed to the UK population by DNA damaging (genotoxic) agents.

magnetic field. This property is therefore central to the exploitation of SPIO in many of the above-mentioned technological and biomedical applications.

Common to all NPs, SPION are associated with unique physico-chemical features, such as nanometre sizes and a large surface area to mass ratio that also facilitate novel applications. On the other hand, the same nanoscale properties (e.g. large surface area coupled with enhanced reactivity, increased propensity to diffuse across biological membranes, and tissue barriers due to nano-size can cause cellular stress) can potentially induce cytotoxicity that can manifest itself by impairing the functions of the major components of the cell, namely mitochondria, nucleus and DNA (7–9). In fact, exposure to SPION has been associated with significant toxic effects such as inflammation, the formation of apoptotic bodies, impaired mitochondrial function (MTT), membrane leakage of lactate dehydrogenase (LDH assay) generation of reactive oxygen species (ROS), increase in micronuclei (indicators of gross chromosomal damage; a measure of genotoxicity), and chromosome condensation (10–15) (Fig. 1).

SPION are divided into three main categories based on their hydrodynamic diameter: oral SPION, 300 nm–3.5 μm; standard SPION (SSPIO), 50–150 nm; and ultrasmall SPION (USPIO), <50 nm (16). SPION that are 10–100 nm in size are considered to be optimal for intravenous administration whereas particles >200 nm and <10 nm are sequestered by the spleen or removed through renal clearance, respectively (16). However, routes of entry and surface properties of SPION govern their ultimate fate in terms of the efficiency of

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Shareen H. Doak currently holds a prestigious Research Councils UK (RCUK) Academic Fellowship supported by both Schools of Medicine and Engineering at Swansea University, where she has established a nanogenotoxicology research program focusing on the genotoxic profiles of engineered nanomaterials, their mechanism of action, and subsequent consequences upon human health. Dr. Doak is involved in the development of a new £21.6 million Centre for NanoHealth, where she is a member of the research and development executive team and leads research into the safety assessment of nanomaterials destined for biomedical use. Her research career to date in the fields of genotoxicology and translational cancer research has recently led to the UK (2008) and European (2009) Environmental Mutagen Society 'Young Scientist Awards.' Dr. Doak is an active member of the Texas-UK Collaborative (ongoing nanogenotoxicology projects with Rice University and Texas A&M) and the Food & Drug Administration – Alliance for NanoHealth Nanotechnology Initiative (FDA-ANNI) Scientific working group.

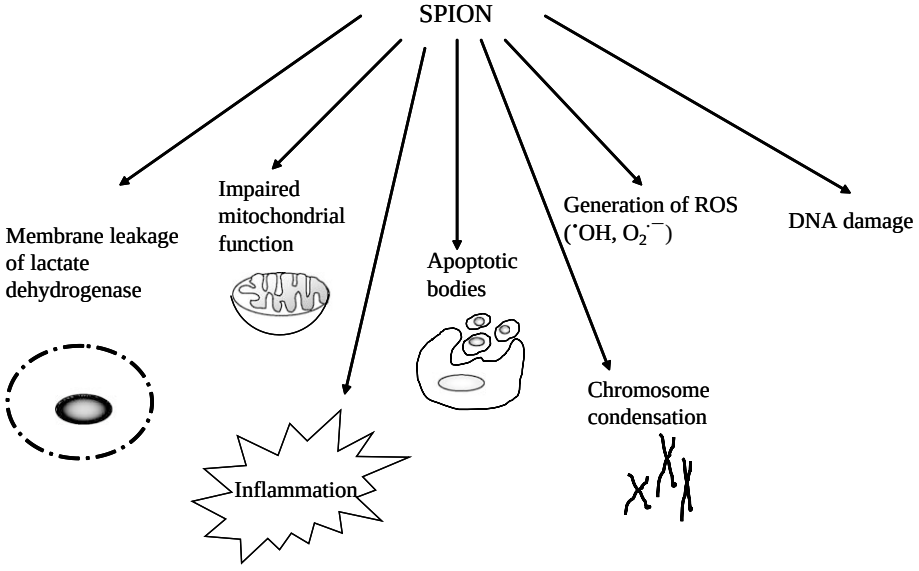

Fig. 1. Cellular toxicity induced by SPION. Exposure to SPION could potentially lead to toxic side effects such as membrane leakage of lactate dehydrogenase, impaired mitochondrial function, inflammation, formation of apoptotic bodies, chromosome condensation, generation of reactive oxygen species (ROS) and DNA damage.

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cellular uptake, biodistribution, metabolism and potential toxicity (17).

Given that iron oxides – both magnetite (Fe_3O_4) and maghemite (γFe_2O_3) – occur naturally as nano-sized crystals in the earth's crust generated by various environmental sources such as volcanoes and fires, it would seem that there is no intrinsic risk associated with these NPs per se. However, the major concern is the increased exposure (via different routes) level to humans and the ecosystem as more and more NPs are being manufactured to meet the demands of the rapidly proliferating field of nanomedicine (18). The dramatic growth and the therapeutic benefits that SPION have to offer, accompanies the risks and concerns associated with their exposure (19). Therefore, there is a considerable need to address biocompatibility and biosafety concerns associated with their usage in a variety of applications. This review focuses on one of the most widely used NPs in medical diagnostics, aiming to highlight the potential adverse biological effects and safety issues associated with SPION.

Methods of preparation

Iron oxide NPs are found naturally in the environment as particulate matter in air pollution and in volcanic eruptions. SPION, either Fe_3O_4 (magnetite) or γFe_2O_3 (maghemite), can not only be generated as emissions from traffic, industry and power stations but can be specifically synthesised chemically for a wide variety of applications (20, 21). Various methods can be employed in their fabrication such as classical synthesis by co-precipitation, reactions in constrained environments, polyol method, flow-injection synthesis and sonolysis (22–25) (Fig. 2). Once the iron oxide core has been generated, a second tier of SPION preparation may be utilised to coat their surfaces with biocompatible molecules such as polyethylene glycol, dextran, albumin or dendrimers (6, 26–28), which can either be performed in situ or via post-synthesis methods that require additional steps (22). The magnetic behaviour is an important parameter that needs to be considered when designing and synthesising the SPION in order to maximally facilitate their imaging and therapeutic efficacies as high magnetisation values are required. Although this can be accomplished by applying a maximum magnetic field acceptable under the clinical settings, the reaction conditions during the synthesis processes can be modulated to generate particle size with a large surface area, which in turn allows these particles to exhibit high magnetic susceptibility (29, 30). Other alterations in the conditions of SPION synthesis, specifically an increase in temperature, have been shown to significantly affect not only their magnetic properties but also hydrodynamic diameter and shape (31). Hydrodynamic particle size is an important parameter that influences magnetisation values, dissolution and stability (32). Consequently, chemical methods allow for numerous variations in reaction conditions that can be modulated in order to achieve SPION with desired physico-chemical properties.

A major challenge in SPION design is adequate control of the reaction conditions to enable synthesis of particles

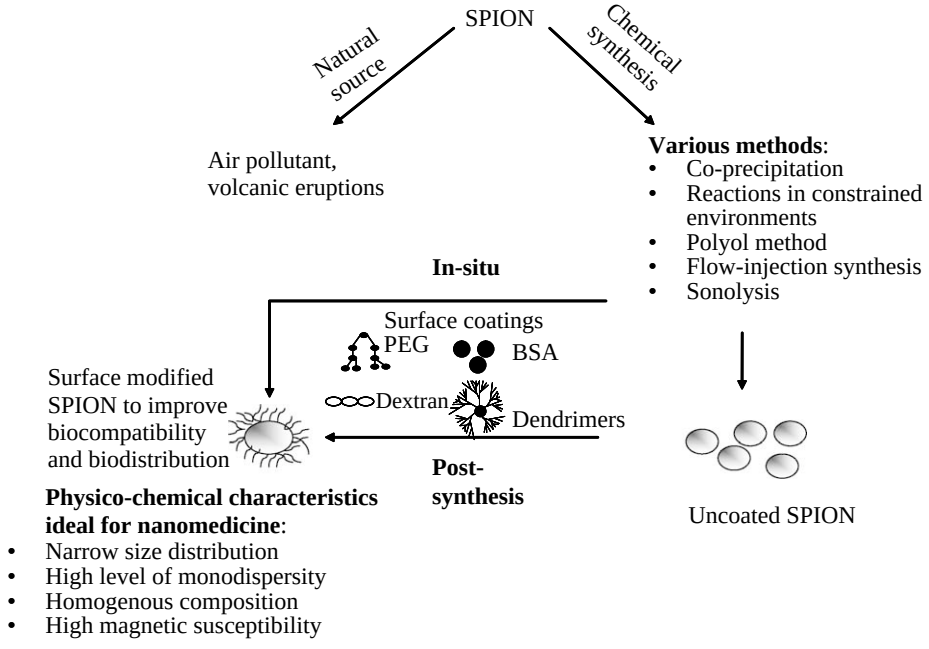

Fig. 2. Methods of SPION synthesis. Various methods can be employed for the synthesis of SPION with the desired physico-chemical characteristics. These can be also coated with biocompatible molecules either in situ or via post-synthesis methods wherein the uncoated SPION are surface-coated subsequent to their synthesis.

with a narrow size distribution, high level of monodispersity and homogenous composition (6). With regards to the materials used for surface coating, factors such as stability, biocompatibility, biodegradability and surface chemistry need to be taken into consideration in order to modestly predict their cellular interactions, cell uptake mechanisms and steric/electrostatic interactions with intracellular macromolecules. All these characteristics are vital to the success of the application that the SPION is destined for.

Surface modification

Uncoated iron oxide NPs have very low solubility that can lead to precipitation (if not sufficiently small) due to gravitation forces and also a high rate of agglomeration under physiological conditions that can impede blood vessels particularly in a clinical exposure setting. Thus, to be used effectively for any clinical application and to improve biocompatibility and biodistribution, SPION are coated with an amphiphilic layer; they can also be designed to bind to complex biological molecules such as antibodies, peptides, hormones or drugs (15). Commercially available SPION are surface coated with materials such as silicon, dextran, citrate and PEGylated starch and are mainly used as contrast agents in target organs such as the gastrointestinal tract, liver, spleen and lymph nodes (33). The derivitisation of iron oxide plays an important role in internalisation efficiency as well as cytotoxicity (34–36).

But the stability of these coatings with regard to their shelf-life and the consequences of their breakdown *in vitro* or *in vivo* have not been thoroughly investigated. Interestingly, many of the commercially available contrast agents such as Ferridex, Resovist, Supravist and Sinerem are coated with dextran or carboxy dextran (33), but there is evidence to indicate that dextran coatings on iron oxide NPs are not strongly bound and therefore more prone to detachment leading to aggregation and precipitation (37, 38). Although some reports have emerged that suggest there may be a significant problem associated with these coatings (see section on *Toxicity studies*) as yet, there is still insufficient information on the effect of these coatings on cytotoxicity associated with DNA damage and oxidative stress.

Mechanisms associated with toxicity

SPION have attracted much attention not only because of their superparamagnetic properties but also because they have been shown to be associated with low toxicity in the human body (12, 13, 39, 40). A study comparing several metal oxide NPs *in vitro* demonstrated iron oxide NPs to be safe and non-cytotoxic below 100 μg/ml (41). Another study on normal, glia and breast cancer cells revealed that the toxicity of Fe_3O_4 NPs coated with a bipolar surfactant (tetramethylammonium 11-aminoundecanoate) is concentration dependent with the particles being non-toxic in the concentration range of 0.1–10 μg/ml while cytotoxicity could be seen at 100 μg/ml (42). There are, therefore, several reports in the literature that demonstrate that a range of SPION with varying physico-chemical characteristics primarily demonstrate low toxicity or cytotoxicity at doses of 100 μg/ml or higher. The number of *in vivo* studies performed in humans is however very limited, but one investigation found that Ferumoxtran-10, a dextran-coated USPIO, only induced side effects such as urticaria, diarrhoea and nausea, all of which were mild and short in duration (43, 44). It is thought that this is primarily because they can be degraded and cleared from circulation by the endogenous iron metabolic pathways. Iron released from SPION is metabolised in the liver and subsequently used in the formation of red blood cells or excreted via kidneys (43).

Though the dose of SPION administered intravenously accounts for 1.25–5% of the total body iron stores (16), SPION are required to be magnetically targeted to a particular tissue/organ in order to maximally benefit a therapeutic or diagnostic application, leading to high concentrations in a localised area. Consequently, this iron overload can have toxic implications as excessive accumulation of the SPION, and in particular, high levels of free Fe ions in the exposed tissue can lead to an imbalance in its homeostasis and can cause aberrant cellular responses including cytotoxicity, DNA damage, oxidative stress, epigenetic events and inflammatory processes (6, 10, 11, 42). More importantly, in the absence of cytotoxicity, this exposure can still lead to subtle but deleterious cellular disruption in the form of DNA damage that may initiate carcinogenesis or have a significant impact on future generations if the fidelity of the genome in germ cells is not maintained (45). Indeed, iron has long been associated with cancer and several mechanisms for iron-induced carcinogenesis have been suggested including a generation of ROS that can potentiate direct damage to DNA, proteins and lipid peroxidation (46–49). Iron-overload following intramuscular injections of an iron–dextran complex has been associated with spindle cell sarcoma and pleomorphic sarcoma in rats (50). It is possible that these neoplasms could be the result of a phenomenon known as solid-state carcinogenesis, whereby implantation of a foreign body (SPION in this case) leads to tumour formation (51).

Another mechanism by which SPION can induce (geno)toxicity is via the generation of ROS. Following internalisation via a number of possible mechanisms (Fig. 3), SPION are presumably degraded into iron ions within the lysosomes by hydrolysing enzymes effective at

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low pH (26, 52, 53). This 'free iron' can potentially cross the nuclear or mitochondrial membrane and in the latter case the free iron in the form of ferrous ions (Fe^{2+}) can react with hydrogen peroxide and oxygen produced by the mitochondria to produce highly reactive hydroxyl radicals and ferric ions (Fe^{3+}) via the Fenton reaction:

$$Fe^{2+} H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

Therefore, hydroxyl radicals generated by the free iron could damage DNA, proteins, polysaccharides and lipids *in vivo* (54). Indeed, iron overload is associated with the production of hydroxyl radicals in rats (55), which react with membrane lipids giving rise to breakdown products including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), both of which can bind to DNA and are mutagenic (56, 57) (Fig. 3). In fact, incubating iron with rat liver nuclei or mitochondria results in the formation of DNA strand breaks, an effect that can be abrogated by using an iron chelator (58). Furthermore, *in vivo* an increased number of DNA breaks have been demonstrated in rats subjected to dietary iron overload, whilst oxidative damage to DNA (measured by the presence of 8-OH-dG adducts) have been observed in mice administered with iron–dextran (59). An *in vivo* study on Swiss mice using polyaspartic acid-coated magnetite NPs demonstrated a time and dose-dependent increase in micronucleus frequency (15).

In addition to the Fenton reaction, structural damage to ATP-generating mitochondria by SPION localisation or iron overload, could potentially result in anomalous functioning of the mitochondria such as altered membrane potential, cytochrome *c* release, ${}^{\bullet}O_2^{-}$ production, and uncoupling of oxidative phosphorylation (60, 61), which may also contribute to the underlying mechanisms associated with cytotoxicity. Therefore, iron overload as a result of SPION-exposure could potentially result in deleterious cellular consequences eventually leading to cell death.

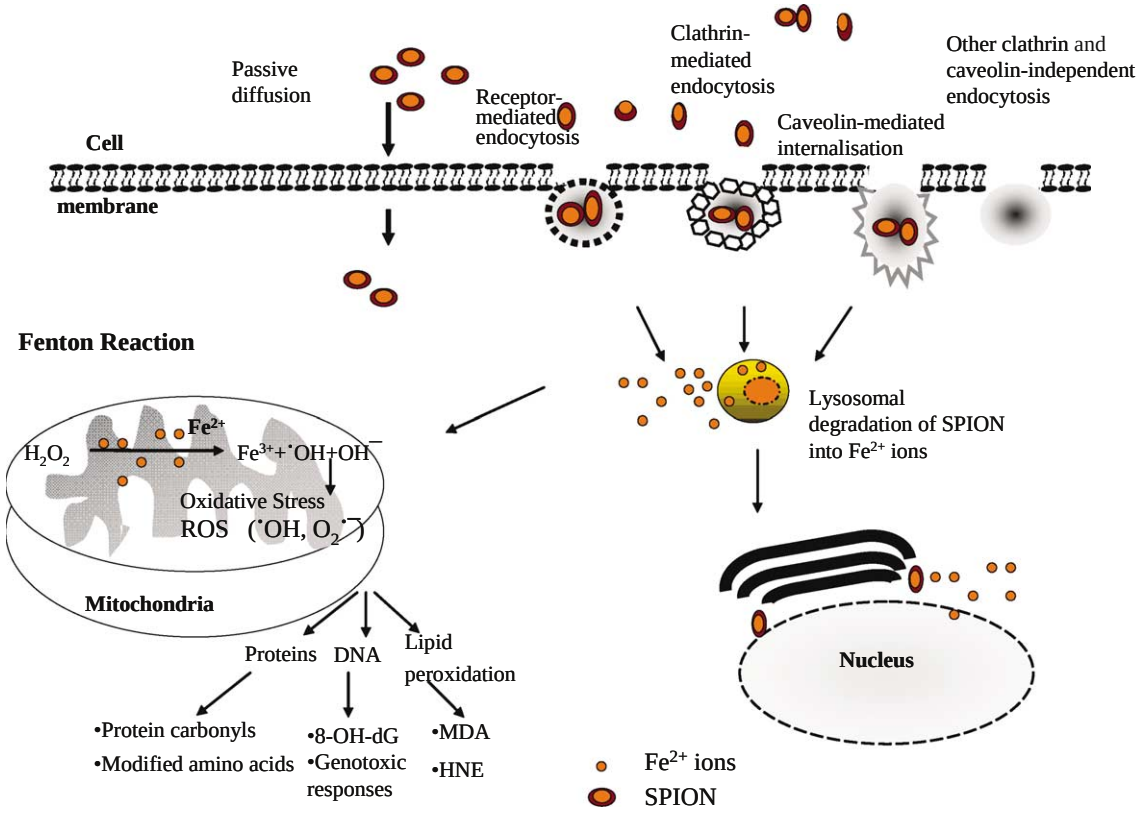

Fig. 3. Schematic representation of different intracellular uptake pathways of SPION. Possible mechanisms of uptake include passive diffusion, receptor-mediated endocytosis, clathrin-mediated endocytosis, caveolin-mediated internalisation, and other calthrin and caveolin-independent endocytosis (105, 106). Upon internalisation, the SPION may presumably be degraded into iron ions in the lysosomes. This 'free iron' can potentially cross the nuclear or mitochondrial membrane and in the latter case the free iron in the form of ferrous ions (Fe^{2+}) can react with hydrogen peroxide and oxygen produced by the mitochondria to produce highly reactive hydroxyl radicals and ferric ions (Fe^{3+}) via the Fenton reaction. Hydoxyl radicals (${}^{\bullet}OH$) generated could indirectly damage DNA, proteins and lipids (8-OH-dG = 8 hydroxydeoxyguanosine, MDA = malondialdehyde, HNE = 4-hydroxy-2-nonenal).

Toxicity studies

Several studies have examined the cytotoxic potential of several different types of SPION with a range of surface coatings and have generally found low or no cytotoxicity associated with these NPs until high exposure levels (>100 μg/ml). The toxicity was also found to be dependent on various factors such as type of surface-coating or its breakdown products, chemical composition of cell-medium, oxidation state of iron in SPION and protein–SPION interaction (27, 28, 62, 63).

A study investigating the effect of different surface coatings on cell behaviour and morphology have shown that dextran-magnetite (Fe_3O_4) NPs result in cell death and reduced proliferation similar to that caused by uncoated iron oxide particles (27). The reason behind the observed cytotoxicty with dextran-magnetite was attributed to the breakdown of the dextran shell exposing the cellular components to chains or aggregates of iron oxide NPs. However, the cell behaviour and morphology of cells treated with dextran-magnetite was different from the uncoated NP, with the former showing more prominent membrane disruptions (27, 28). They also reported membrane disruption after exposure to albumin derivatised iron oxide NPs that was attributed to the interaction between albumin and membrane fatty acids and phospholipids. However, though the uncoated and dextran-coated iron oxide NP resulted in cytotoxicity (at 50 μg/ml), the albumin-coated particles did not result in cell death.

Another cytotoxicity study by Mahmoudi et al. (62) on a mouse fibroblast cell line showed that the uncoated particles induce greater toxicity than the biocompatible polyvinyl alcohol (PVA)-coated particles. However, the toxicity induced by uncoated particles was significantly reduced by substitution with surface-saturated uncoated SPION. The latter SPION were prepared by pre-incubating the media with the NPs prior to exposure, which may have resulted in adsorption of biomolecules onto the SPION surface. Surface saturation with experimental cell medium presumably masks the reactive surface of the NPs thus preventing the unfavourable cell–nanoparticle or serum protein–nanoparticle interactions that may in turn cause reduced cellular uptake leading to lower toxicity. The authors also reported the formation of gas vesicles after exposure to the uncoated particles; resulting in altered protein functions and changes in ionic equilibrium within the cells, which also promotes toxicity. The vesicles were presumed to be caused by protein–nanoparticle interactions as the surface-saturated SPION did not cause the formation of these gas vesicles.

Additionally, the toxicity seemed to be governed by compositional changes in the media as a result of the serum proteins binding to the negatively charged uncoated SPION. This resultant altered composition of the cell medium to which the cells are exposed presumably results in the observed cytotoxicity (63). This effect may not be seen *in vivo* because homeostasis maintained by the liver and kidneys efficiently regulates any changes in pH, ionic strength and chemical composition of the blood plasma.

Apart from the type of surface coating, the tail length of a coating (for e.g. length of polyethylene oxide, PEO, in PEO-coated SPION) can bear a negative correlation with toxicity with the shortest tail of 0.75 kD causing chromatin condensation, nuclear blebbing and formation of apoptotic bodies (11). It can be argued that the longer tails can undergo degradation into shorter tails within the intracellular milieu and cause toxicity, but the authors believe that the stable moieties such as ether groups on PEO and the amide ester bonds on the surface of SPION could potentially protect the coating from degradation and enzymatic attack.

Although one of the key mechanisms responsible for cytotoxicity involves oxidative stress, citrate-coated very small superparamagnetic iron oxide nanoparticles (VSOP) have been shown to lead to cellular oxidative (14) stress in rat macrophages (as shown by a significant increase in the levels of malonyldialdehyde and protein carbonyls) without causing any cytotoxic effects. Interestingly, this increase was only transient, as 24-h post-incubation resulted in a decrease to control levels. This increased oxidative stress was, however, eliminated by the iron chelator desferal and the intracellular spin trap PBN suggesting that iron may have been released from VSOP at the early stages of incubation and was responsible for the effects observed.

Similarly, genotoxicity (using the comet assay) using meso-2,3-dimercaptosuccinic acid (DMSA) coated SPION was observed at concentrations of 10–100 μg/ml where no significant cytotoxicity occurred (64). The group also suggested that a surface-coating such as (DMSA) can inhibit a potential cytotoxic effect by preventing direct contact between maghemite (Fe_2O_3) NPs and human dermal fibroblasts. They showed a significant decrease in cell viability of fibroblasts upon exposure to DMSA-coated maghemite (NmDMSA) at concentrations between 10^{-6} and 10^{-3} mg/ml. Interestingly, the higher concentration of 100 μg/ml did not show reduced cell viability; instead it showed a significant increase in metabolic mitochondrial activity. This finding was attributed to the increase in aggregate size from $\sim$30 nm to $\sim$70 nm at higher concentrations resulting in less effectual contact between NmDMSA and the cells.

Another crucial aspect that demands attention is the safety of these SPION with regards to the oxidation state and compositional changes that might occur over time and affect its shelf-life and toxicity. Magnetite, which is a

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mixture of FeO and Fe_2O_3, is not very stable and can readily undergo oxidation (from Fe^{2+} into Fe^{3+}) to form maghemite in the presence of air, light and moisture (22):

$$Fe_3O_4 + 2H^+ \rightarrow \gamma Fe_2O_3 + Fe^{2+} + H_2O$$

Magnetite (Fe_3O_4) and maghemite (Fe_2O_3) can show different cellular responses because of their ability to undergo oxidation/reduction reactions. In fact, magnetite has been shown to cause higher levels of oxidative DNA lesions (using comet assay) in A549 human lung epithelial cell line in the absence of decreased cell viability as compared to maghemite owing to its potential to undergo oxidation (40, 41). It is hypothesised that the toxicity can, however, be decreased by coating magnetite particles resulting in fewer oxidative sites that are less reactive and thereby produce less DNA damage (63).

Interestingly, significant differences in cellular uptake and DNA damage have been demonstrated depending on the oxidation state of iron (Fe^{2+} or Fe^{3+}) in dextran-coated iron nanoparticles as analysed by X-ray photoelectron spectroscopy (XPS) (45). Dextran-coated maghemite (Fe_2O_3) with a Fe^{2+}/Fe^{3+} ratio of 0.118 showed significant genotoxicity, which correlated with cellular uptake as compared to the dextran-coated magnetite (Fe_3O_4) with a Fe^{2+}/Fe^{3+} ratio of 0.435, showing that Fe^{3+} ions are more potent in inducing DNA damage. Since human exposure to ferrofluids is predicted to increase in nanomedicine-based therapeutics, these findings warrant the need to devise adequate testing strategies in order to ensure that a given ferrofluid has not incorporated changes in its valence shell that might influence its cellular interaction and the ensuing downstream toxicity. Alternatively, it may be necessary to design iron NPs that are highly stable chemically and oxidation resistant without compromising on cellular damage.

Altered cellular responses

Cellular stress

To date, most studies have only focused on cytotoxicity induced as a result of exposure to SPION, with very few considering the effect on other normal cellular and physiological functions. This is of concern given that important cellular processes could be impaired but go unobserved if the focus of most research studies is confined to more noticeable determinants of gross toxicity. Since SPION can be coated to cause increased cellular uptake, Soenen et al. (65) designed a cationic amphiphile with the aim to maximise internalisation of SPION without inducing any cytotoxic effects on neural progenitor cells and human blood outgrowth endothelial cells. However, high doses of these NPs caused interference with the actin cytoskeleton resulting in decreased cell proliferation indicating the possibility that non-toxic doses could cause other forms of cellular stress.

The same group have demonstrated that magnetoliposomes can affect actin cytoskeleton architecture, formation of focal adhesion complexes and impair cell proliferation (which took 7 days to return to normal post-SPION exposure) indicating a longer-term effect of SPION (66). Another case of SPION-mediated cellular stress involves disruption of a cytoskeleton protein, tubulin that has been shown to be associated with the uptake of transferring-derivatised SPION (67). Apopa et al. (68) have also reported a similar finding upon SPION (uncoated maghemite) exposure; they noted that the dynamic cortical meshwork of F-actin in human microvascular endothelial cells rearranges and undergoes polymerisation rapidly in response to extracellular signals that impinge on the plasma membrane resulting in increased cell permeability. They demonstrated that production of ROS by iron-NPs induced GSK-3β inhibition via activation of the Akt signalling pathway, which is involved in changes in actin dynamics such as cell migration that in turn effect chemotaxis, locomotion and invasion. Apart from its role in altering the cytoskeleton and its associated processes, Akt is also known to play a key role in insulin signalling and in linking growth factor signalling through PI 3-kinase to basic metabolic functions, such as protein and lipid synthesis, carbohydrate mechanism and transcription. Therefore, activation of the Akt pathway by SPION could potentially lead to perturbation of these normal biological processes. Another problem associated with this remodelling is the formation of gaps between endothelial cells that could lead to extravasation of unwanted macromolecules or drugs into adjoining areas, thus having the potential to result in adverse patholgoical responses.

Changes in gene expression

Organisation of the cytoskeletal structure of F-actin filaments is an essential element in maintaining and modulating cellular morphology and structural integrity (69, 70) and any disruption in its architecture can lead to alteration in the expression of cytoskeleton-associated proteins. In fact, microarray analysis subsequent to a 48 h exposure of SPION on primary human fibroblasts showed an upregulation in expression of genes involved in the modulation of actin remodelling. In addition, SPION exposure also caused the increase in expression of genes involved in cell signalling, including integrin subunits, tyrosine kinases and several members of the protein kinase C family (α/δ/θ/ζ), suggesting that SPION can have an impact on signalling transduction pathways. Other genes that were upregulated included genes related to cell movement and interaction such as growth hormones, ion channels, and Ras-related proteins.

Furthermore, there were significant increases in ECM proteins and matrix metalloproteinases, suggesting that the SPION exposure caused the fibroblasts to reorganise their matrix material (67).

A study conducted on pancreatic islet cells labelled with Resovist (carboxydextran-coated SPION; commercially available MRI agent), revealed that insulin expression in labelled islets was significantly elevated ($\sim$2 fold) (71). Beta-cell E-box trans-activator (BETA2) an important transcription factor for insulin gene transcription, was also increased in labelled islets (1.7 fold). However, no difference was observed between labelled and unlabelled islets in terms of the ability to secrete insulin, as determined by the glucose stimulation index. There could be several ways to explain this: Firstly, before concluding that iron overload does not influence insulin secretion it is important to rule out any defects involving desensitisation of glucose-induced insulin secretion such as expression levels of glucose transporter-4 that helps in the facilitated diffusion of glucose. Secondly, since the primary mechanism of SPION uptake is endocytosis and insulin is secreted via exocytosis there may be a possibility that insulin secretion is compromised as a result of vesicular trafficking in either direction. Finally, an increase in insulin expression without a concomitant increase in insulin secretion suggests that longer incubation times with Resovist may be required for an observable effect on insulin secretion. On the other hand, considering that insulin expression is significantly increased in response to Resovist, it is tempting to speculate that the incorporation of MRI contrast agents into pancreatic islets may prove beneficial in diabetic patients undergoing islet imaging.

However, insulin causes a marked stimulation of iron uptake by fat cells; also, it can cause an increase in ferritin synthesis and localisation of transferrin receptors to the membrane leading to increased iron uptake. Normally the free iron within cells is stored as an iron–ferritin complex to negate the high toxicity associated with free iron. However, under pathological conditions (such as cancer, atherosclerosis, hypertension and arthritis), which are associated with the generation of ROS, iron may effectively be released from ferritin (49, 72). These radicals act as reducing agents that convert Fe^{3+} to Fe^{2+}; the latter in turn can result in the formation of superoxide anion and highly reactive $^{\cdot}OH$ radical via the Fenton reaction. This can cause a vicious circle whereby increased insulin expression by Resovist could potentially cause enhanced iron uptake that can prove detrimental particularly in a pathological condition. This could result in iron overload within the cell and the production and accumulation of highly toxic free radicals that may in turn attack cell membranes, DNA and proteins.

Although the expression of two other endocrine hormones, somatosatin and glucagons, did not change and the study indicated the safety of using Resovist to image islets, in our view a watchful eye is warranted for any deleterious effect particularly where underlying disease pathologies are involved.

Dextran-coated SPION also have the potential to cause perturbation in the regulation of iron homeostasis. Indeed preliminary studies within our group have found that although ferritin (iron storage protein complex) and ferroportin (iron export molecule) were not altered following exposure to dextran-coated SPION, transferrin-receptor 1 (TfR1) and hepcidin were significantly down-regulated in HepG2 hepatocellular carcinoma cells. The TfR1 is a cell-surface receptor that controls iron uptake (73); its down-regulation following exposure to dextran-coated SPION therefore suggests that the cells were reacting as though they were in a state of iron overload (Fig. 4A). Hepcidin is a peptide hormone secreted by the liver responsible for regulating intestinal iron absorption. Normally hepcidin blocks ferroportin inhibiting iron export into the plasma so in normal iron homeostasis if there is an increase in plasma iron, it stimulates increased hepcidin release from the liver to inhibit further release of iron into the plasma from the

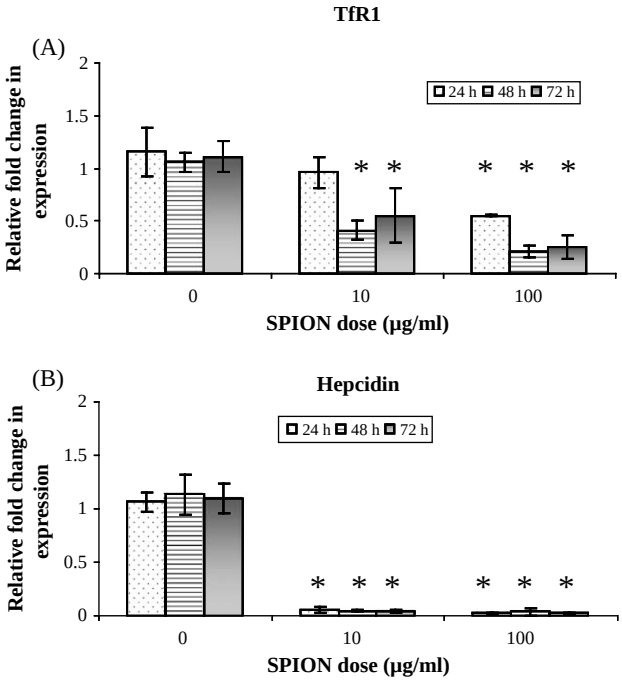

Fig. 4. Preliminary data to demonstrate the effect of dextran-coated SPION on the expression of genes involved in iron homeostasis using real-time RT-PCR. (A) TfR1 and (B) hepcidin. The students' paired *t*-test was used to determine if down-regulation proved to be significant change in expression (with error bars representing standard deviation ($*P < 0.05$); as compared to the control where water was used in place of the nanoparticles.

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duodenum and hepatocytes, whilst promoting iron storage in reticuloendothelial macrophages (74). Our results are contrary to what would be expected when hepatocytes are incubated with SPION as a dramatic down-regulation of hepcidin was observed following exposure to dextran-coated SPION (Fig. 4B). However, this is not an isolated observation and several *in vitro* based studies have found that iron overload of hepatocytes results in decreased hepcidin mRNA, where as iron-overload *in vivo* would result in increased hepcidin expression (75). It has therefore been speculated that there may be other iron-sensing signals to hepatocytes that induce the production of hepcidin during iron-overload situations, which is not mimicked in cell culture (74–77). Although further studies are required to elucidate the mechanisms involved, it can be speculated that the body might act to reduce dietary iron uptake, which may in turn lead to conditions such as anaemia if these nanomaterials aren't cleared from the circulation when they're administered. Therefore, changes in expression of genes involved in iron metabolism necessitate the need for more studies in order to ensure the safety of these agents in various biomedical applications.

Impact on cell proliferation

Generally, the internalisation of NPs within cells is likely to occur in a time-dependent manner and after a certain threshold is reached the uptake is expected to plateau off when cells have reached a point of maximum saturation. Similarly, cell viability subsequent to nanoparticle uptake is expected to be either unaffected or decrease as a function of time. However, it has been shown by Soenen et al. (78) that cell viability of 3T3 fibroblasts in response to cationic magnetoliposomes that contained distearoyl analogue was augmented as compared to control cells. The transient cell growth and proliferation observed in the study may be due to additional nutrients present within the cells in the form of iron and phospholipids for the viable cells stimulating cell growth (79). In another study (67), an increase in cell proliferation in primary human fibroblasts (hTERT-BJ1) was observed in response to transferrin-coated SPION; transferrin, a major iron transport protein, has been demonstrated to be important for cellular proliferation (80, 81).

Soenen et al. (78) also provided an explanation for the bell-shaped curve that was observed in the study that can be explained as follows: initial lag phase in cellular activity as the cells have to deal with sudden exposure and internalisation of NPs, middle log phase marked by a period (~6–10 h) of cellular growth as the cells resume their cellular activities and finally the cell number either stabilises or there is reduced cell viability as a result of cell toxicity. This stresses the need to follow cell viability over an extended period of time in order to avoid possible cell viability artefacts and to accurately ascertain cytotoxicity of a given nanoparticle. Another important point to consider is that as cells divide over time the daughter cells are exposed to relatively low concentrations of NPs due to the fact that SPIO concentration gets diluted with successive cell divisions; this could affect various cellular processes such as cellular uptake, cell viability and cell toxicity.

A study investigating the toxic effects of Ferucarbotran (Resovist) on mesenchymal stem cells showed augmented cell growth and an increase in cell cycle progression via alterations in the expression of cell cycle regulatory proteins and by reducing intracellular hydrogen peroxide (H_2O_2) (82). The group reported an increased expression of hyper-phosphorylated retinoblastoma tumour suppressor protein pRb, cyclins and cyclin-dependent kinases, namely cyclins B, D1, E, CDK2 and CDK4. On the other hand, Ferucarbotran caused decreases in the expressions of $p21^{Cip1}$, and $p27^{Kip1}$, members of the CIP/KIP family that are negative regulators of the cell cycle. Furthermore, these SPION decreased the expression of the tumour suppressor gene, p53. The effects of SPION labelling on cell cycle regulatory proteins were opposite to those of iron depletion indicating the involvement of these proteins in regulating cell cycle progression and cell growth in response to free iron within the cells (83). Since SPION can also induce AKT activation (68), there is a possibility that this pathway is involved in cellular proliferation and viability following exposure to SPION, given that the PI3/AKT pathway can cause the cells to escape apoptosis.

SPION uptake can induce signalling events such as Akt pathway. Conversely, modulation of signalling molecules like MAPK pathway can affect its uptake**.** In a study conducted on aortic macrophages in a mouse atherosclerosis model, uptake of intravenously administered Feridex (dextran-coated SPION; commercially available MRI agent) was attenuated by inhibiting the p38 MAPK demonstrating that SPION uptake or the macrophage phagocytic activity seeem be regulated by MAPK signalling pathway (84). This reflects the potential strategy of using inhibitors (in this case SB239063) for signalling molecules to modulate SPION-induced macrophage toxicity resultant to SPION uptake (85). However, the pitfall of tampering with signalling molecules in therapeutic approaches is that the inhibition of one signalling pathway could lead to the activation of another pathway. For example, it has been identified that rapamycin analogs that reduce tumour growth by inhibiting the mTOR protein complex 1 (mTORC1), also activate the MAPK (mitogen-activated protein kinase) pathway; this inhibits the antitumour activity of rapamycin by encouraging cell survival (86). Further analysis on mechanisms of SPION uptake will provide insights into the use of these NPs in therapeutics.

Ambient exposure

Besides the application of SPIO in a clinical scenario, safety and toxicological issues with respect to their presence within our environment also needs to be considered. SPION can accumulate in organs such as liver, brain, spleen and lungs subsequent to inhalation and penetration through hair follicles (87, 88). With regard to this, Karlsson et al. (20) have shown that subway particles that are composed of a high percentage of magnetite can cause genotoxicity via mitochondrial depolarisation and induction of oxidative stress. Interestingly, even though the iron-rich subway particles caused a significant increase in oxidative stress (measured by intracellular ROS) and mitochondrial depolarisation, the magnetite particles on their own failed to elicit such an increase in both assays used in this study. Genotoxicity, as measured by the DNA damage (comet assay), was shown to be significantly increased in both cases but more so in the subway particles as compared to the magnetite particles (240 and 40%, respectively). Despite the subway particles consisting primarily of iron oxide (as analysed by X-ray energy dispersive spectrometry), there were other metal components in the subway particles that might have interacted and therefore influenced the effects observed in this study. Hence, the true impact of human exposure to SPION in the local environment remains to be determined.

Nanoparticle–protein interactions

Given that the nanoparticle–protein corona is an inevitable entity in both *in vitro* and *in vivo* biological exposure scenarios, the transient nature of these associations as well as the SPION–protein affinities needs to be thoroughly determined (89). This is important especially in a clinical scenario where the disease status of an individual (undergoing treatment by ferrofluids) and the associated altered protein levels governs such interactions. These interactions may prove unfavourable possibly resulting in an inefficient uptake or even overload of SPION depending on the presence of certain proteins in the blood plasma that may act synergistically with the behaviour of NPs. Thus, it can be speculated that NPs undergo two main processes that are important determinants of their cellular uptake: transient NP–protein binding (in culture media *in vitro* or body fluids *in vivo*) and NP–protein interaction with cell surface/membrane macromolecules (89).

Given the possibility that NPs may be small enough to traverse the blood-brain barrier, there has been concern that they may induce or abrogate neurological disease, but this ability may also be used beneficially through the development of tailor-made nanomedicine for the treatment of such disorders (85). Indeed, a study on protein amyloid aggregation (a pathology seen in diseases such as Parkinson's, Alzheimer's and type II diabetes) found the protein aggregates were significantly reduced by magnetic Fe_3O_4 NPs. These NPs not only inhibited lysozyme amyloid aggregation by blocking the nucleation process but also induced depolymerisation of lysozyme aggregates by interacting with and interrupting the adjoining protein sheets; lysozyme adsorption seemed to govern both these processes (90). Currently there are no clinical drugs to reverse or prevent the formation of aggregates and based on this study the Fe_3O_4 NPs could potentially be used as novel therapeutic agents in the treatment of protein amyloid aggregation-associated human pathologies (91). This is an interesting finding warranting further exploration into NPs–protein interactions since protein adsorption, although beneficial in this example, may also prove to be detrimental in cases where the protein phenotype is altered by such interactions in normal cells.

To identify the proteins that associate with SPION, dextran-coated SPION were incubated with mouse plasma and despite rigorous washing and their low abundance in plasma, histidine–proline-rich glycoprotein (HMWK), high molecular weight kininogen and plasma prekallikrein (KLK) demonstrated a significant strong affinity towards the NPs (92). All three proteins have histidine-rich domain and therefore, bind to the negatively charged iron oxide core in SPION with none binding to neutral dextran coating (93, 94). In order to identify the weakly bound proteins the washing step was eliminated, and the analysis of mouse plasma showed significant amounts of an altogether different profile of proteins, namely mannose-binding lectin (MBLs), MBL-associated serine proteases (MASPs), apolipoproteins, beta-2 glycoprotein and clotting factors FXI and FXII. Under these less stringent conditions, the most abundant plasma proteins, albumin and transferrin, did not show any significant attachment to SPION indicating that the protein interactions were a selective process. Interestingly, the study does not support the involvement of plasma opsonins in the removal of SPION from the circulation by mice liver macrophages and also indicates that the plasma protein coating does not interfere with the interaction between the SPION and macrophage receptors. Thus, in the light of these findings, it is suggested that the nanoparticle surface is available for interaction and subsequent uptake by receptors, such as the scavenger receptors. Contrary to these findings, Xie et al. (95) have shown that in the human serum albumin (HSA) coated SPION, the HSA-sheath may be directly responsible for the cellular uptake by macrophages as it has been shown to interact with surface receptors such as glycoprotein (gp60) receptor and secreted protein acid and rich in cysteine (SPARC) receptor present on a range of cell types (96, 97).

The SPION-bound proteins may also impinge on various biochemical pathways involved in the complement system activation; MBLs and MASPs could stimulate the

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lectin-complement pathway, and surface bound immunoglobulins could activate the classical complement pathway, hence playing a role in nanoparticle toxicity (98, 99). Additionally, strongly bound proteins (i.e. kininogen and kallikrein in conjunction with coagulation factors XI and XII) could potentially trigger the intrinsic pathway of the coagulation cascade (100).

Conclusion

Given that the variety of medical applications of SPION require sufficient intracellular uptake for efficient diagnosis and treatment, understanding the potential risks associated with exposure to these NPs and the effect that the range of surface coatings utilised for functionality is crucial. In many cases these treatments may be adequately cleared from the body, but there is the possibility that cellular SPION overload may trigger adverse cellular responses and the long-term impact of these acute exposures are not well understood, thus there is a clear need to comprehensively investigate and elucidate the biological consequences of exposure to SPION. It is critical to design functionalised SPION that can not only be effectively and sufficiently internalised and are appropriately magnetisable, but also meet the demands of a particular application without compromising on cellular toxicity. For example, biomedical applications, like drug delivery, require high doses of internalised particles while for extracellular drug delivery these amounts are not favourable (101). Improved understanding of biological impacts will therefore lead to the design of more biocompatible nanomaterials that are fit for their function.

It is plausible that internalised SPION may corrode over a long period of time by releasing metallic ions that in turn bear a long-established correlation with DNA damage. Ideally, it would be worthwhile to decipher the stability and breakdown products of coatings because a 'biocompatible coating' that is considered stable initially may eventually break down into an unfavourable product or expose the iron oxide core, thereby eliciting adverse cellular responses. Hence, the stability of functionalised SPION is another crucial issue to be taken into consideration.

Numerous studies on SPION including those on commercially available and clinically approved MRI contrast agents such as Feridex and Resovist have reported that these NPs are biocompatible and lack cytotoxicity. At present the measure of biocompatibility largely focuses on the extent of cytotoxicity observed. However, the criteria to define toxicity of NPs needs to be clearly defined (82), particularly as emerging studies have begun to highlight aberrant cellular responses including DNA damage, oxidative stress, mitochondrial membrane dysfunction and changes in gene expression as a result of SPION exposure, all in the absence of cytotoxicity.

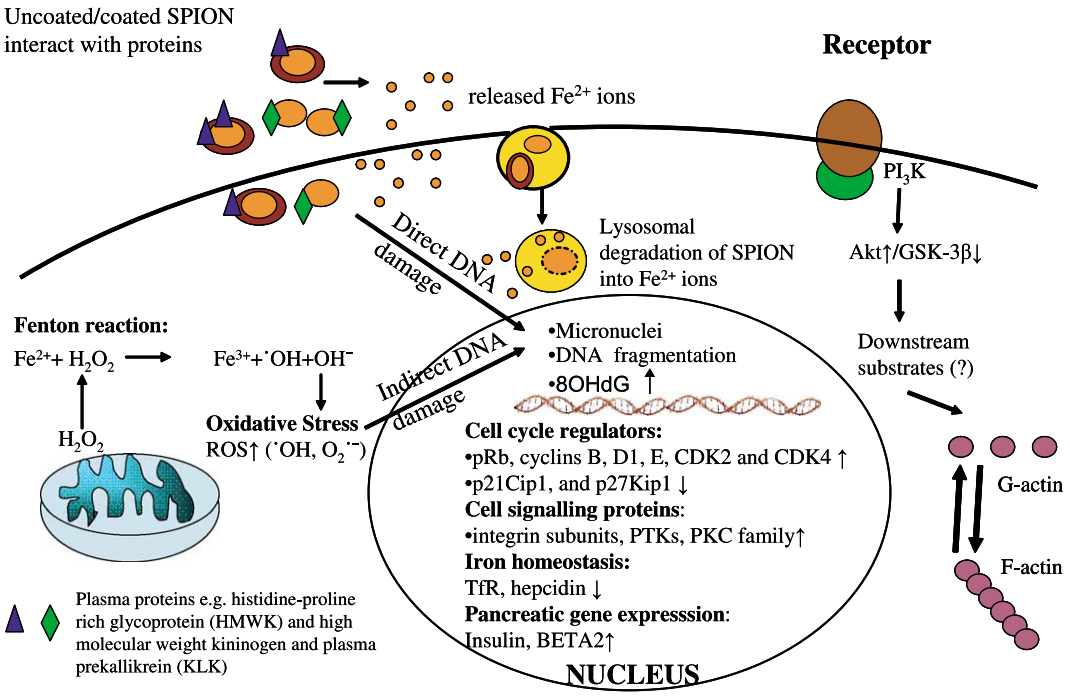

Fig. 5. Schematic representation of SPION-induced toxicity at cellular level. SPION may cause direct DNA damage or result in the generation of oxidative radicals that in turn have the potential to cause DNA damage (indirect), have an impact on actin cytoskeleton by modulating the Akt signalling pathway, and also alter the expression of various genes such as those involved in cell cycle regulation, iron homeostasis and pancreatic functioning.

Hence, terms such as 'biocompatibility' need to be re-evaluated when commenting on the safety of these SPION agents.

Our current lack of understanding of the health impacts following exposure to nanomaterials requires adequate testing strategies. Where there is clear evidence of hazard or the people involved are endangered, regulations need to be addressed and continually assessed as each new nanomedicine application evolves. With regards to SPION, there are a number of issues that clearly need be addressed by the scientific community prior to approving their clinical use:

1. How much access do SPION have to tissues and organs other than the ones being treated or investigated and how long after are they eliminated from the different tissues/organs and the body in general?
2. What impact would the SPION have on the morphology (e.g. actin polymerisation) and/or functions (e.g. gene expression) of the exposed cells and are the subtle but deleterious alterations, such as DNA damage and oxidative lesions (if any) being thoroughly investigated (Fig. 5)?
3. Could the SPION degradation that presumably occurs in the lysosomes, with the resultant generation of iron ions, have impact on various cellular processes (26, 55, 60, 61, 82)? Chen et al. (102) have recently demonstrated that Ferucarbotran can promote cell migration, activate signalling protein molecules, and inhibit osteogenesis in human mesenchymal stem cells, all of which were attributed to the generation of free iron from Ferucarbotran degradation.
4. Are the internal organs and the cellular machinery equipped to deal with the processing of SPION (both coated and uncoated) with a range of physicochemical characteristics?
5. Does iron homeostasis play the same role every time it encounters a SPION irrespective of its physicochemical characteristics?
6. Are the different SPION being processed by the intracellular pathways (e.g. endocytotic pathway) in the same sequential manner in all tissues or is their fate cell/tissue dependent?
7. Do they have a precise location upon entry within the cells and is the uptake and subcellular localisation dependent on size and/or surface coating (103)? An interesting observation noted by Song et al. (104) was that short iron nanowires accumulated in the vesicles of HeLa cells by non-specific pinocytosis, while long iron nanowires perforated and diffused through the lipid bilayer membrane and only penetrated as far as the cytoplasm thereby demonstrating the impact of physical features on uptake mechanisms and sites of accumulation.
8. How does the coating of SPION influence their interaction with the proteins and other biological entities within the cellular milieu?

These issues demand attention not only to ensure the safer use of SPION in nanomedicine, but are essential in establishing novel targeted therapies with improved design that are able to deliver their beneficial promises to the medical world.

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