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Engineered superparamagnetic iron oxide nanoparticles for externally controlled hyperthermia, drug delivery, and therapeutic toxicity

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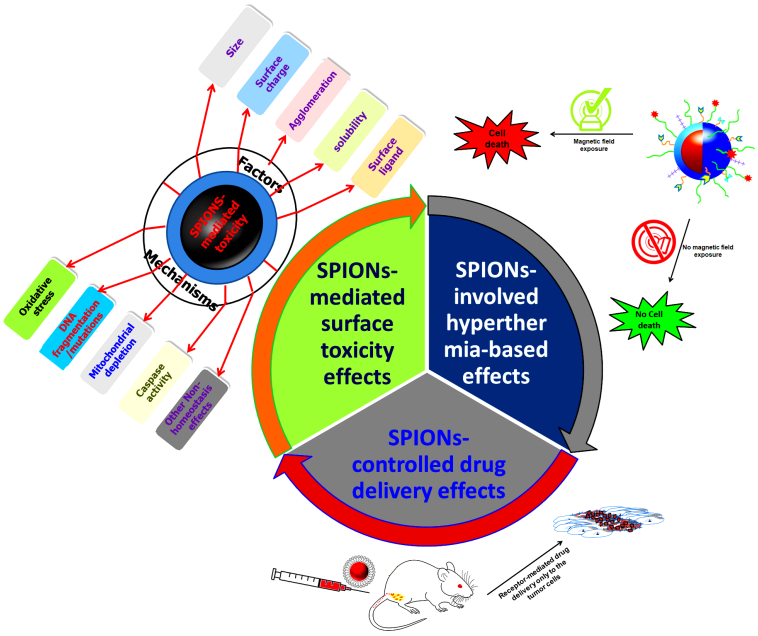
Graphical Abstract



Keywords:

Engineered nanoparticles, therapeutic toxicity, cancer, cell death, hyperthermia, controlled drug delivery, surface coatings.

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Abstract

Nanotechnology and materials science are highly developing sectors where several new materials are investigated. One area includes iron oxide nanoparticles with superparamagnetic behavior. Since nanomaterials are prone to be associated with high levels of intrinsic toxicity, they may have adverse effects if not properly guided. So, toxic mechanisms associated with nanomaterials are like that of a therapeutic drug or any other toxic compound. In that way, by ignoring the general pathways of cell death followed by the nanomaterials, the present report covers the points to control the growth of cancer cells by employing engineered nanoparticles (NPs) to induce therapeutic toxicity. We discuss the pathways for the induction of toxicity to the cancer cells using the superparamagnetic iron oxide nanoparticles (SPIONs) with and without surface ligands, and the ligand efficiency towards controlling toxicity is covered. Also, the therapeutic means of controlling the cancer cells, such as generating heat and releasing anticancer drugs in an externally organized manner, are also discussed. Overall, the report links the physical properties of SPIONs related to their natural or therapeutical toxicity by connecting physicochemical and toxicology principles.

Rationale, Purpose, and Limitations:

Hyperthermia, drug delivery, and therapeutic toxicity are important nanomedicine areas that necessitate extensive investigation. We have presented pathways for toxicity induction to cancer cells using the SPIONs with and without surface ligands. The present work depicts the ligand efficiency towards toxicity control. In addition, we have discussed the links of physical properties of SPIONs to generate toxicity naturally or therapeutically by following the physicochemical and toxicology principles. One of the limitations is our hypothesis on the surface-controlled toxicity effects of SPIONs, particularly the reaction of oleic bond and thiol groups. It is also revealed that the concept is challenging to prove due to difficulty isolating SPIONs sample post-cell culture testing.

1. Introduction

"Nanotoxicology" refers to the science of the study of adverse effects of chemicals or agents (typically in the nano-to-micrometer size range) from environmental, industrial, and/or therapeutic sources. In recent years, the rapid increase in the research and development of nanoscience and nanotechnology from both the academic and industrial side is due to advancements in synthesis and characterization methods. This development allowed for the introduction of many hybrid composites with novel applications. Such introduced nanomaterials are very small and possess high surface areas; in that way, they are prone to be associated with high levels of toxicity [1]. The common sources of these particles are air, water, food, drugs, cosmetics, or other consumable goods, and from which they can enter the human body organs through inhalation, ingestion, skin, or oral routes. It has been noted that the physicochemical properties of nanoparticles (NPs), such as the size, shape, morphology, surface charge, bioconjugation, and agglomeration, play a key role in the adsorption and subsequent penetration of the particles into the skin [2]. Such introduced particles can directly or indirectly affect the living cells with known or unknown mechanisms. For any particle to involve in the toxicity mechanism, it must be thoroughly distributed first within the system, and the particle's distribution property is entirely governed by size, surface charge, and other colloidal properties [3]. Also, some engineered NPs introduced into the body for therapeutic purposes must be excreted completely from the body without any permanent localization. One example of such a nanoparticulate system is the superparamagnetic iron oxide nanoparticles (SPIONs), which are prone to precipitate in the liver organ and can induce continuous toxicity [4]. The common toxicity pathways of NPs are the reactive oxygen species (ROS) induced oxidative stress, intracellular protein oxidation, mitochondrial dysfunction, DNA mutations, and inflammation, where all of these pathways finally lead to cell death [5-6]. Figure 1 shows the schematic representation of different kinds of toxic pathways mediated by the SPIONs, indicating that the SPIONs-generated toxicity can be mainly of two types: natural toxicity and engineered toxicity. The natural toxicity is provided by SPION's physicochemical factors, while engineered toxicity is therapeutic and occurs through the release of a surface-loaded drug or induction of hyperthermia by external control.

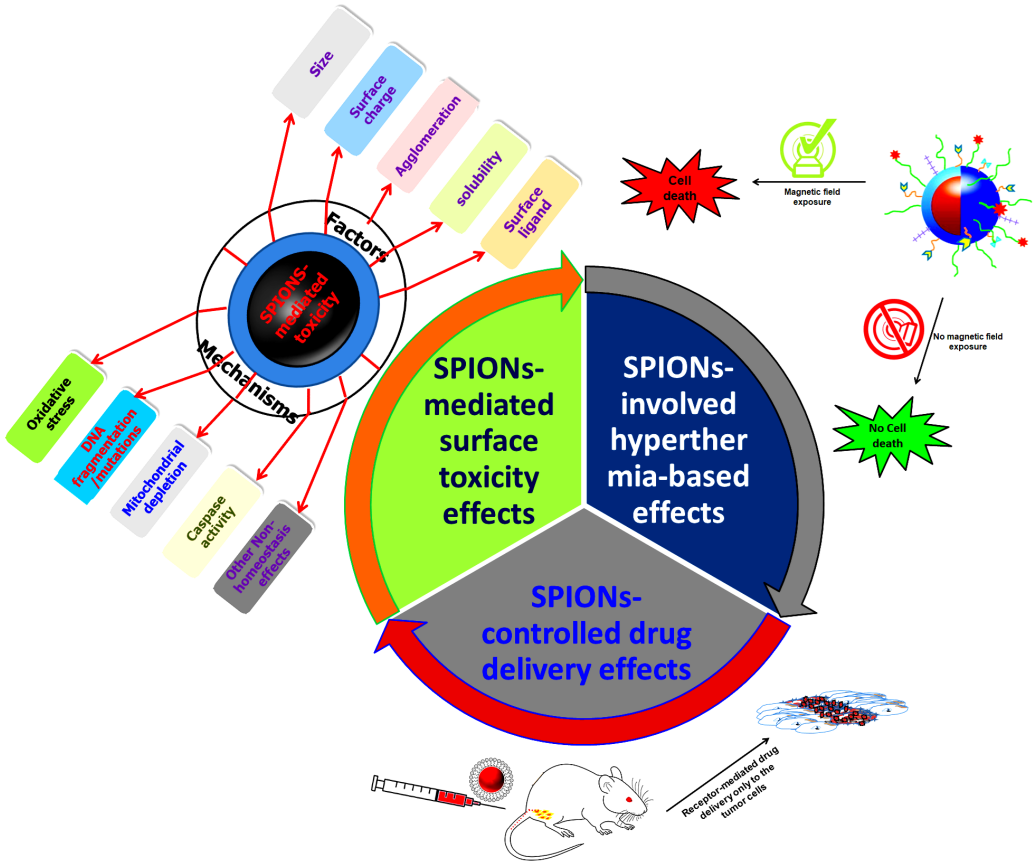


Figure 1. Schematic representation of the role played by SPIONs in future nanomedicine for the development of sustainable therapeutic approaches.

SPIONs are mainly categorized into two classes per their origin sources: natural and synthetic. The NPs originated from natural sources, including the volcano eruption, dust storms, air particles decomposition in the sunlight, or ambient atmospheric conditions, where the bodily systems are very well amended to defend against most harmful intruders except for particulate pollution as it is associated with cardiovascular diseases and various type of cancers. Similarly, synthetic or engineered NPs can enter the bodily systems through therapeutic sources, cosmetics, drinking water, and food that is grown in contaminated soil [6-7]. The engineered SPIONs with fine-tuned magnetic and surface properties are being developed for day-to-day applications in nano/biomedicine sector by involving the following components are major ingredients: iron oxide (γ-Fe_2O_3 and Fe_3O_4) [8], silver (Ag) [9], gold (Au) [10], copper (Cu) [11], manganese (Mn) [12], platinum (Pt) [13], cobalt (Co) [14], nickel (Ni) [15], cerium (Ce) [16], titanium dioxide (TiO_2) [17], zinc (Zn) [18], gadolinium (Gd) [19], quantum dots (QDs) [20], and carbon nanotubes (CNTs) [21], just to mention a few. When remaining in the body after their application, these materials can exert lethal effects on the living cells. To avoid such effects, the researchers have made substantial efforts to reduce the interactions between the particles and the biological entities. One way to achieve such effects is by coating the NP's surfaces with suitable ligands where the surface coatings improve the safety of the particles by protecting them from being involved in the agglomeration, neutralizing surface charges, and further confining the particles to specific sizes [22-23].

SPIONs are a typical class of materials and are found to have extensive applications in the biomedical sector as contrasting agents of magnetic resonance imaging (MRI), targeted drug delivery, hyperthermia, cell sorting, and other fields like wastewater treatment, oil separation, etc. [5-6, 22]. For these materials, 'superparamagnetism' is the special property that can be observed in a magnetic field when the size of the magnetic material falls below d=15 nm when the particle's magnetic susceptibility becomes very high. Superparamagnetic materials

can especially be applicable in many biomedical and industrial applications where the particle's magnetic moment can be used effectively for the generation of heat, resonance imaging, and specific delivery of therapeutic agents, efficient binding of water contaminants and oil particles. Keeping in view the availability, ease of synthesis, and specific advantages offered by the SPIONs, the literature on iron oxide-based composites in recent years indicate that the intrinsic properties linked to its application, such as the magnetic, surface charge, size, agglomeration, and toxicity are exclusively controlled by its surface physical and conjugated ligands. In addition, it has also been noted that the oxidation of iron in the SPIONs also typically influences its toxicological effects and further decides their fate where they can be suitable or not for many biomedical applications and thus responsible for the induction of natural toxicity [1, 22-24]. Therefore, the objective of the present work is to provide an in-depth understanding of the influencing physical properties of iron, and in that way, it can be possible to link the engineered toxicity effects with that of cancer cell physiology. In addition, the means of improving the SPIONs role in developing sustainable cancer therapeutic approaches, controlled hyperthermia, and drug delivery are also discussed.

SPIONs: Properties and Biomedical applications

Recent advances in theranostics provided a significant breakthrough in the diagnosis-based therapy of many cancerous diseases at biological entities of both cellular and subcellular levels by simply exploiting the unique characteristics of SPIONs as contrasting agents [25]. The SPIONs as contrasting agents are widely used in the current imaging modalities of MRI, optical imaging (fluorescence/photoluminescence), and further in combinatorial treatments, which include photodynamic therapy (PDT), photothermal therapy (PTT), and hyperthermia-based therapy [26-28]. For most of these applications, the other NPs and SPIONs involved are Co, Ni, Fe_3O_4 or Fe_2O_3, Gd, Au nanoshells, CNTs, and QDs. Since the combination of these NPs can emit/scatter an absorbed light or magnetic energy with specific signals that are used for the diagnosis or therapeutic issues in medical sciences [27-28]. For example, alternating magnetic fields (AMF) are employed to generate heat from the SPIONs. The released heat can either have a direct effect on the cancer tumor cells through hyperthermia therapy or, alternatively, it can be transformed for the release of encapsulated (or conjugated) anticancer drugs to have an indirect effect on tumor cells or a combination of both (i.e., hyperthermia-based therapy and hyperthermia-based drug delivery to the tumor cells) [22, 29-30]. Moreover, the use of AMF and SPIONs for controlled hyperthermia and drug delivery has significantly reduced the current challenges associated with traditional methods such as ultrasound, radiofrequency, microwaves, infrared radiation, and hot water tubes. The limitations of traditional approaches employed for hyperthermia and drug delivery include [24]:

(i) uncontrolled and unregulated dissipation of heat that induces burns, blisters, and discomfort to the healthy normal tissues and non-targeted organs,

(ii) microwave, laser, and ultrasound-mediated generation of heat has limited penetration capacity towards the tissues and for the organs located deep inside the body and hence limited treatment efficacy, and

(iii) designing an efficient system (formulation) for the therapeutic delivery of heat or drugs is nearly an unsolved problem for the deep shielded tissues such as the bone pelvis or the scull.

Hyperthermia-based cancer therapy is an approach in which the tumor-developed tissues are exposed to a temperature that is a little above the normal body temperature, thereby activating the cell death mechanism. In addition, this hyperthermia approaches, when applied in combination with other treatment modalities like cancer chemotherapy or radiation, the sensitivity gets multiplied by the effects of radiation and other therapeutics [31]. Depending upon the amount of temperature rise by magnetic fluid containing magnetic nanoparticles (MNPs), the hyperthermia treatment is classified into three categories: diathermia, moderate hyperthermia, and thermoablation [32]. Diathermia runs at a lower temperature of <41°C and is mainly applied to treat rheumatic diseases in physiotherapy. Moderate hyperthermia generates heat in the range of 41–46°C and is used to stimulate immune responses in the cancer cells, while thermoablation runs at a relatively higher temperature between 46–56°C

[24]. This noxious stimulus induced by heating activates pro-apoptotic proteins to initiate the caspase-8 or caspase-9 mediated pathway, thereby leading to protein denaturation, melting of a lipid bilayer, and mitochondrial membrane destruction [33]. In general, the tissue level effects of hyperthermia are directly associated with the perfusion and oxygenation of the tumor microenvironment, pH changes, amount of heat generated at the tumor sites, AMF exposure time, and some physiological characteristics of cancer cells. Further, the principle of conversion of dissipated magnetic energy into thermal energy by the superparamagnetic NPs was reported to have successfully complemented the available invasive cancer chemotherapies such as radiation, surgery, gene therapy, and immunotherapy. In a normal sense, the conversion of absorbed energy from AMF by the MNPs involves two different phenomena: relaxation and hysteresis loss. The relaxation may be of Néel or Brownian relaxation; the heat generation by Néel relaxation is due to the rapidly occurring changes in the direction of the particle's magnetic moment relative to the crystal lattice (internal dynamics), while the Brownian relaxation causes the physical rotation of the particles itself relative to the dispersion medium (external dynamics). The phenomenon of Néel relaxation is mainly hindered by the energy of anisotropy that tends to orient the magnetic domain in each direction relative to the crystal lattice. The Brownian relaxation is stuck by the phenomena that tend to counter the movement of particles in a viscous medium. These internal (Néel) and external (Brownian) sources of friction lead to a phase lag between the applied magnetic field and the direction of the particle's magnetic moments that further generate thermal losses to the medium in which the particles are dispersed [24]. The efficiency of any MNP towards the heat release is estimated in terms of specific power loss (SPL) or specific absorption rate (SAR). It is influenced by the parameters like MNPs structure (size), magnetic properties (magnetic anisotropy and temperature dependence of magnetizations), amplitude (H), applied frequency (f), and the viscosity of the medium [34].

A similar approach involves the application of NPs induced by external stimuli such as electric current, magnetic fields, temperature, light, and ultrasounds to pulsatile the release of polymer encapsulated drugs [35]. This kind of research accelerated with the development achieved by applying low-frequency oscillatory magnetic fields using a magnetic composite of ethylene-vinyl acetate to externally control the on-demand release of insulin [36]. However, the drawbacks of this study were improved in later years following the modulation of the permeability of polyelectrolyte microcapsule embedded MNPs fabricated using a layer-by-layer self-assembling approach [37]. Further, a similar finding that uses MNPs, such as the direct drug delivery and hyperthermia-based controlled drug delivery technique, has been classified into two types: drug delivery through bond breaking (DBB) and drug delivery through enhanced permeability (DEP). In the DBB method, the MNPs are first bound to the biomolecules through a heat-labile linker and further converted into matrigel before injecting subcutaneously near the posterior mammary fat pad of mice to form model tumors. On the application of the electromotive force (EMF), the fluorescent biomolecules get released into the surrounding tissues near the tumor compared to the unexposed controls, which can be visualized easily using MRI [38-39]. Other researchers demonstrated similar success by the triggered release of fluorophore bimane amine from the surface of SPIONs in the presence of AMF, and the same can be visualized through the MRI technique [40]. But the drug delivery mechanism in DEP involves the release of an encapsulated drug along with MNPs within the polymeric matrices, for instance, the biopolymers, hydrogels, or thermoresponsive materials. A major advantage of this approach is that the drug release from the polymer matrix can effectively be controlled by tuning the external parameters such as the frequency of oscillating magnetic field and the associated applied magnetic field strength.

By using the DEP approach, the magnetic and thermoresponsive properties of individual components are very well demonstrated using the on-demand "on-off" release of sodium fluorescein over multiple magnetic cycles using a prototype nanocomposite membrane with thermosensitive property, poly(*N*-isopropylacrylamide)-based nanogels and SPIONs [41-42]. The other reports describes the concept of DEP using some high density organic and inorganic polymeric particles incorporated with MNPs;

the organic polymers are made from poly-lactic acid (PLA) [43-44], poly(ethylene glycol) ethyl ether methacrylate-copoly(ethylene glycol) methyl ether methacrylate [45], Pluronic F127 (F127) [46], poly(methylmethacrylate) (PMMA) [47-48], poly-n-isopropylacrylamide (PNIPAM) [49-50], poly(ethyleneimine)-modified poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) block copolymer [51] and nanoparticle-assembled capsules (NACs) using poly(allylamine hydrochloride), disodium phosphate, and citrate bound MNPs [52]. The inorganic nanocomposites used for the construction of DEP-type drug delivery systems include the iron oxide core-chitosan shell microspheres [53] and zinc-doped iron oxide nanocrystals encapsulated within mesoporous silica framework [54], and silica [55]. Further, this concept was fully used to demonstrate the efficacy of inductive heating for drug-controlled release by switching the high-frequency magnetic fields on and off from folic acid and β-cyclodextrin (CD)-functionalized SPIONs [56].

Several factors are reported to directly influence the efficiency of the hyperthermia-based therapy and hyperthermia-based drug delivery systems, such as the biocompatibility, applied field, exposure time, the viscosity of the medium, stability of the formulation, and tumor-targeting ability. In addition, the challenges primarily associated with the currently available systems include [24, 57-59]: (i) the high frequency of the oscillatory magnetic fields falls typically in the range of several kHz and MHz, (ii) limited targeting ability by the MNPs and hence very difficult to deliver to the targets located deep inside the body, (iii) the efficiency of the MNPs towards heat or drug release did not improve much by tailoring the physical parameters such as size, shape, and composition, and (iv) limited stability of the formulation in the presence of biological media (tendency to undergo faster precipitation). To overcome these limitations, some researchers attempted to enhance SPL values by modifying MNPs composition with those of Mn, Co, Pt, or Ni were considered successful; however, the oxidative instability and free radical-induced toxic mechanisms (apoptosis mostly) with these elements are still a concern [60-63]. All these factors are reported to adversely influence the MNPs biodistribution, imaging characteristics, and drug targeting/releasing efficiency *in vivo* due to the occurrences of the changes in the physical and/or surface features of original particles (zeta potential, hydrodynamic size, charge, stability, and magnetization) as depicted in Fig.2 [64]. Interestingly, it was hypothesized that the magnetic properties and biocompatibility issues could be fine-tuned by forming a core-shell type structure, with the MNPs being the core and a shell of a different metal. Further, it is observed from the studies that the MNPs modified with suitable ligands respond selectively to a range of frequencies of oscillatory magnetic fields to release heat as well as the conjugated drugs temporally to treat various complications.

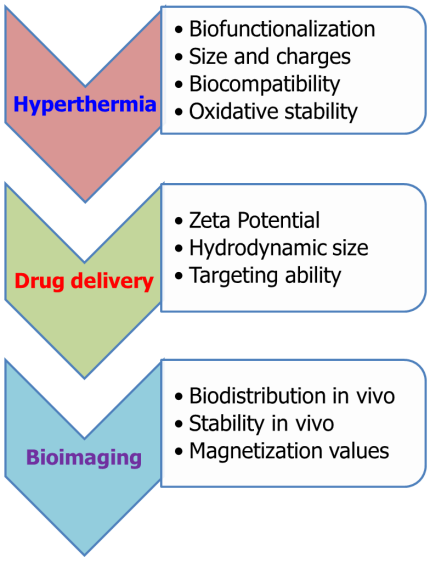


Figure 2. Schematic of various physical factors that can influence the SPIONs applications in hyperthermia, drug delivery, and bioimaging.

Surface-controlled toxicity effects of SPIONs

In general, properties of NPs are influenced mainly by their surface characteristics, and there are several ways such as biomolecule conjugation, stabilization by organic surfactants, polymeric encapsulation, core-shell formation, and biofabrication can be employed to obtain materials with tailored properties. The application of such methods on SPIONs also brings changes in size, shape, or surface chemistry that help manufacture materials with efficient physicochemical and biological properties suitable for *in vivo* conditions. However, for most SPIONs applications *in vivo*, the selection of surface coating or derivatization can be made only based on the nature of end-use, as the properly sequestered SPIONs or their composites only fulfill their role in the biomedical environment. However, the improperly sequestered SPIONs, such as those with an unstable coating or naked, might end up in the lungs, liver, or heart and can be responsible for the initiation of many side effects through cell-mediated toxicity. With that, the presence of iron (Fe) in SPIONs can mediate many toxic reactions through the initiation of free radicals ($O_2^{\bullet-}$, OH•), which further induces significant damage to the cells using inhibiting the mitochondrial oxidoreductase pathway, DNA fragmentation, or strand cleavage [23, 65-66]. Therefore, irrespective of the enormous applications of SPIONs towards disease diagnosis and treatment, their toxicity limits the particle's role in the biomedical sector to some extent.

The different phases of naturally occurring iron oxide with changes in oxidation states and structures include Fe^{o}, FeO (wüstite), Fe_3O_4 (magnetite), γ-Fe_2O_3 (maghemite), α-Fe_2O_3 (hematite), and FeOOH (oxyhydroxide). Among all, the Fe_3O_4 and γ-Fe_2O_3 are the most abundant and stable forms of iron, and they both exhibit the property of superparamagnetism (i.e., magnetization values fall in between ferro- and paramagnetism) when they fall within a nano size-range (<15 nm). The Fe element in Fe_3O_4 maintains two different oxidation states of +2 and +3, while only one stable oxidation state of +3 in γ-Fe_2O_3 [23, 67]. For a majority of biomedical applications where the magnetic properties and also the electron-rich stable surfaces are equally important, magnetite is preferred mainly due to the presence of the +2 oxidation state of Fe. In contrast, the +3-oxidation state of Fe in maghemite is relatively labile. Since the maghemite and hematite forms of iron with their +3-oxidation state has more tendencies to absorb electrons to fulfill the deficiency from the electron-rich sites such as the mitochondria, DNA, and other intracellular proteins, thereby inducing the free radical dependent oxidative stress [68-69]. In general, with Fe^{3+} containing iron oxide NPs, the reactive oxygen species (ROS) of $O_2^{\bullet-}$, OH• in the biological media are mainly released because of the catalytic reactions occurring at the surface of iron oxide NPs, but not due to the dissolved metal ions. In addition, the catalytic centers available at the surface of iron oxide NPs possess at least 50-fold more effective OH• radical producing sites than the corresponding dissolved Fe^{3+} ions [70]. This indicates that the protection of iron oxide NP's surface from mediating any kind of catalytic reactions either directly with the intracellular components of the cells or indirectly through the biological media by oxidizing the dissolved proteins can prevent the cells from undergoing free radical-induced oxidative stress.

To understand the effects of intrinsic toxicity of SPIONs in the biological environment, we have studied the influence of surface ligand with an implication towards the generation or reduction of SPIONs toxicity. For that, four different SPIONs with a change of surface chemistry were selected [23]. We hypothesized that the incorporation of suitable ligands with antioxidant properties onto the surface of iron oxide NPs could protect the iron from mediating catalytic reactions (Fenton), and at the same time, the surface ligand is believed to serve as a free radical scavenger to neutralize the ROS formed by metabolic pathways. In that view, the selected SPIONs with a change of surface chemistry include:

1) SPIONs coated with oleic acid (SPIONs-1),

2) SPIONs without any surface coating (SPIONs-2),

3) SPIONs coated with cysteamine (SPIONs-3), and

4) SPIONs coated with oleic acid and cysteamine (SPIONs-4).

The reason for choosing oleic acid and cysteamine as surface ligands is because they both

possess the antioxidative capacity, such as through the unsaturation in oleic acid and cysteamine's reducing behavior through its –SH (thiol) group [71-72]. The influence of SPIONs surface ligand on the biological media was investigated by making use of the *in vitro* cell culture system of H9c2 cardiomyoblast cell line, where the toxicity was evaluated from the assays of cell viability and proliferation, intracellular ROS formation, DNA damage, mitochondrial transmembrane potential (ΔΨm), and glutathione s-transferase (GSH) release [23].

In the SPIONs-induced cell viability and proliferation studies of H9c2 cells, the concentration-dependent and time-dependent (Figures 3a and b) were tested against various concentrations and time intervals. From the concentration-dependent cell viability studies, the SPIONs-2 particles exhibited a significant reduction (40%) in the percentage viability of cells at a concentration of 200 µg/mL compared to other SPIONs having different surface ligands. This primarily indicates that the presence of biomolecules of oleic acid and cysteamine surface ligands influences the reduction of toxicity originating from the SPIONs surface. Further from the time-dependent cytotoxicity studies for a 200 µg/mL concentration of all the SPIONs, the toxicity effects seem to be prominent (45% loss) only after 16 h of exposure, meaning that sufficient interaction of SPIONs surface with cells occurs after the said time.

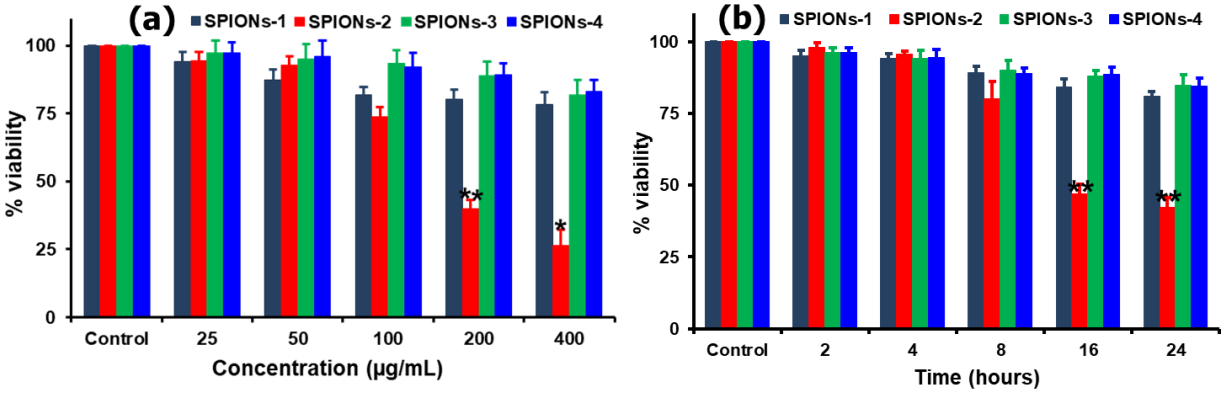


*Figure 3. MTT assay provided cell viability studies of SPIONs (1-4) treated H9c2 cells (a) concentration-dependent (0–400 µg/mL; 24 h) and (b) time-dependent (0–24 h; 200 µg/mL). * and ** indicates the significance at $p < 0.05$ and $p < 0.01$ versus untreated controls. SPIONs-1 refers to iron oxide particles with oleic acid, SPIONs-2 refers to iron oxide particles without any surface coating, SPIONs-3 refers to iron oxide particles coated with cysteamine, and SPIONs-4 refers to iron oxide particles with both oleic acid and cysteamine ligands.*

The mechanism of toxicity by naked SPIONs and the protective action of surface-coated SPIONs were further explained through the intracellular ROS formation (Figure 4a) and the DNA fragmentation (Fig. 4b) for the cells treated with 200 µg/mL concentrations over a 16 h exposure period. As compared to the positive reference (menadione, 100 µM) and untreated control measurements, the SPIONs-2 treated cells exhibited a significant amount of ROS production (76%) and DNA cleave (about 5-fold). However, no such ROS production (Fig. 4a) or DNA damage (Fig. 4b) was witnessed when the same SPIONs-2 particles co-incubated with the well-known antioxidant, Trolox (a water-soluble analog of α-tocopherol). Also, a dose-dependent reduction in the amount of ROS and DNA cleavage on the co-incubation of SPIONs-2 with trolox can be seen due to the direct protecting effects of the trolox groups [73]. Moreover, the surface-coated SPIONs such as SPIONs-1, -3 & -4 did not seem to form much ROS production or DNA fragmentation as compared to untreated control and the reference. This further gives evidence for the influence of oleic acid or cysteamine biomolecule groups at the surface of SPIONs towards either directly controlling the ROS formation from the Fe-induced toxicity or indirectly neutralizing the formed ROS as soon as they are generated. The same groups are expected to be responsible for the DNA protection from undergoing cleavage by the Fe-inducing reactions.

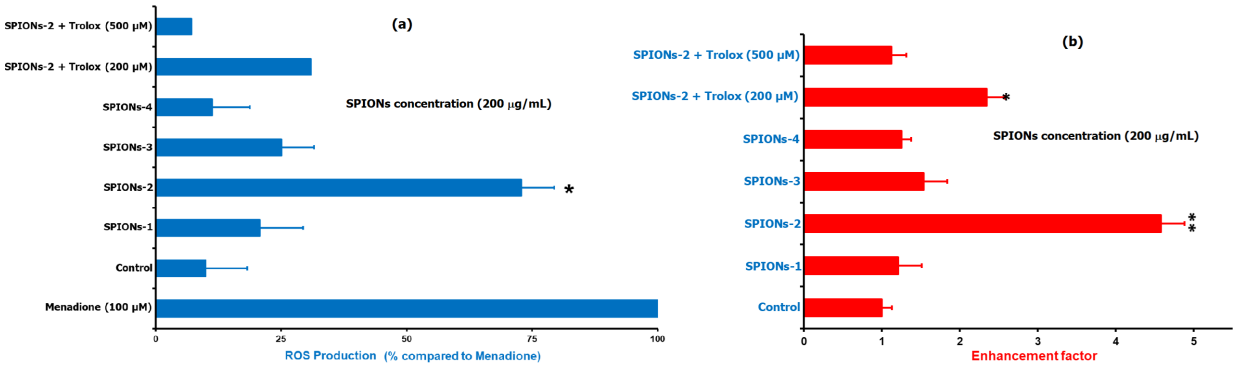


*Figure 4. (a) Comparison of % intracellular ROS formation (a) and DNA fragmentation (b) in cardiac cells on the treatment of SPIONs-1 through SPIONs-4 (200 µg/mL, 16 h) and the effect of supplementation of trolox. Positive control of Menadione (100 µM) was used for ROS assay, and the cells without any treatment were used as a negative control. * and ** indicates the significance at $p < 0.05$ and $p < 0.01$ versus untreated controls.*

The naked SPIONs containing Fe-mediated toxicity and the protective action of ligand groups on surface-covered SPIONs were further explained using the GSH assay (Figure 5a) and the mitochondrial transmembrane potential assay (Figure 5b). It was observed from the analysis that the SPIONs-2 treated cells exhibited a significant reduction (42%) in the total GSH (%) release and the reduction (63%) in membrane potential when compared to the untreated control measurements. These effects seemed to be restored on supplementation of the SPIONs-2 with the antioxidant trolox (500 µM) and the other ligand containing SPIONs. However, does not seem to be involved in the loss of total GSH release (Figure 5a) or transmembrane potential (Figure 5b).

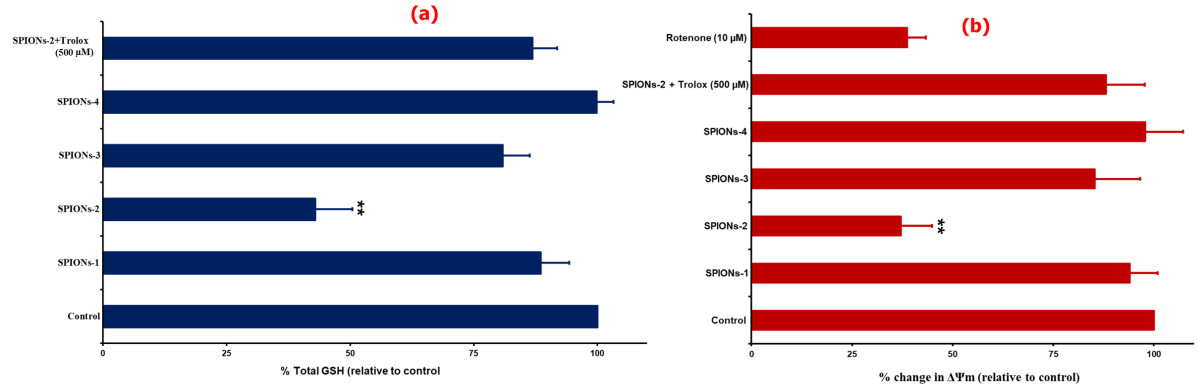


Figure 5. Comparison of % change in the total GSH release (a) and % change in the mitochondrial transmembrane potential (b) of SPIONs-1 through SPIONs-4 treated H9c2 cells (200 µg/mL concentration, 16 h).

Such an observation of results is ratifying the apoptotic pathway of cell death mediated by ROS. The glutathione synthesis is generally blocked for the cells undergoing apoptosis at a certain point. Still, the observation of a slightly higher level of glutathione for the SPIONs-4 treated cells can be due to the sparing effects from the two coated ligands on the newly synthesized GSH by the cells. The two coated ligands with an unsaturation and thiol functionality contribute to an enhanced GSH production by diminishing the free radical effects and thus supporting cell proliferation. Similarly, the observation of DNA fragments and the physical damage of mitochondrial membrane are due to the localization of SPIONs-2 (without any coating) into the mitochondria and the corresponding oxidative damage of organelle, disrupting its oxidoreductase reactions and moving towards the loss of cell viability. Since the cell's organelle is more susceptible to free radical-induced oxidative stress as its inner membrane consists of unsaturated fatty acids, densely packed proteins, and DNA molecules that are

essential for the effective functioning of mitochondria [23, 74].

By the comparison of all the molecular biology results, it is confirmed that there exists a difference between the naked SPIONs and the surface-covered SPIONs using the protective action against the ROS provided and is from the effects of oleic acid and cysteamine ligands. For those ligands, the C=C bond in oleic acid and the –SH group in cysteamine reduces the Fe^{3+} in iron oxide by oxidizing. In general, the Fe present in iron oxide possesses some pro-oxidant properties due to the electron-deficient Fe^{3+} state, and the extent of the toxicity is decided by the total Fe^{3+} content, as the Fe in its isomeric forms has two oxidation states of Fe^{2+} and Fe^{3+}. Since the Fe^{3+} form is more electron-deficient, it can absorb electrons from intracellular components and is responsible for ROS generation. The uncontrolled effects of the formed ROS can finally result in cell death, and in our case, ROS's loss of cardiac function was observed by exposure to naked SPIONs. However, an effective surface coating on iron oxide NPs can protect the intracellular components from undergoing Fe-induced oxidative stress. In addition to controlling the toxicity, the properly sequestered iron oxide NPs can also be used to destroy the free radicals produced by other chemotherapeutic agents. In a study, for example, the ability of Fe to catalyze red-ox reactions was used for scavenging the generated ROS in cardiac cells by the anthracycline antibiotics during cancer treatment [75]. Also, for most applications in the biomedical sector, the iron oxide NPs should possess sufficient hydrophilicity, which is mainly decided by the type of surface ligand. For example, the SPIONs-1 in our case is not hydrophilic due to the coverage of hydrophobic oleic acid molecules, while the SPIONs-3 and SPIONs-4 are more hydrophilic due to the existence of cysteamine molecules on their surfaces. Therefore, it can be expected naturally that the aqueous dispersion of these particles and associated parameters like magnetization values, zeta potential, and surface charges are different.

Biomolecule-influenced magnetization of SPIONs

In general, the bulk gold atoms maintain the diamagnetic behavior while the single atoms of the same gold element are paramagnetic. Such an altered behavior of gold element's magnetic property, such as the diamagnetism of bulk gold, is due to the counteraction of paramagnetic behavior of conduction electrons by the orbital and ionic core diamagnetism. At the same time, the paramagnetic nature of single gold atoms is from the unpaired 6s electrons [76]. Based on this phenomenon, distinct changes can be noted in the magnetic behavior of gold with changes in its arrangement and structure, such as from gold atoms to nanogold to bulk. Also, a chemically induced ferromagnetism was observed for the gold NPs capped with thiol group-containing ligands. This point is of the highest importance in the biomedical sector due to the alteration of the magnetic behavior of gold atoms using only surface coatings [77]. Like Au and Cu, the chemically induced permanent magnetism in Au NPs under the chemisorption conditions is from the interaction of *s* and *p* orbitals of adsorbates such as thiolates with that of metal atoms 5d orbitals. This interaction further leads to the emergence of holes in 5d-localized orbitals because of the significant charge transfer from the metal atoms to sulfur atoms in the coated ligand molecules. Understanding the concept of magnetism by chemisorption and other influencing parameters is of particular significance from both fundamental and application points of view, as biomagnetic materials will play a critical role in achieving the concept of theranostic devices. The magnetic materials in theranostic devices are designed for dual application sites of disease diagnosis-based therapy; the diagnosis is based on magnetic or fluorescence imaging, while the therapy utilizes hyperthermia and magnetically controlled drug delivery. Therefore, in-depth analysis to fully understand this concept of magnetism by chemisorption in thiol-containing ligands and the magnetism associated with S-Au bonding in amino acid capped Au clusters having a superparamagnetic core of magnetic studies was performed. For that, SPIONs of different structures and surface ligands were formed by starting with SPIONs and then coated with a uniform layer of gold by thermal decomposition resulting in the core-shell particles (SPIONs@Au) of spherical shape, monodispersed nature, and size of around 6.4 nm. The aqueous soluble and thiol-containing ligands were obtained by the bond-

ing of SPIONs@Au NPs with that of cysteamine (Cyst) using a surface exchange reaction. Further, to test the magnetic behavior of SPIONs@Au in the presence of thiolated amino acids, peptides, or nucleic acids, the SPIONs@Au-Cyst particles were coated with LHRH (luteinizing hormone-releasing hormone). Since LHRH is an endocrine protein overexpressed by the cancers of the breast, ovary, and prostate and its bonding with SPIONs helps to target specific tumor cells without going for any non-target tissues [78].

The extensive analysis of changes in the morphology, size, and shape of all the four SPIONs with differences in surface functionality is shown in Figure 6. It can be observed that the surface functionalization did not bring any changes in the morphology of particles, as the particles seem to be spherical for all. Further, the analysis of magnetization data indicated distinct changes in terms of the total magnetization values about the surface functional changes of SPIONs. Starting with SPIONs, the saturation magnetization (M_s) and magnetic remanence (M_r) values were slightly increased for SPIONs@Au, significantly for SPIONs@Au-Cyst, and further decreased for SPIONs@Au-Cyst-LHRH; however, one of the properties of superparamagnetism, such loss of hysteresis at room temperature (300 K) happened only to the first three SPIONs.

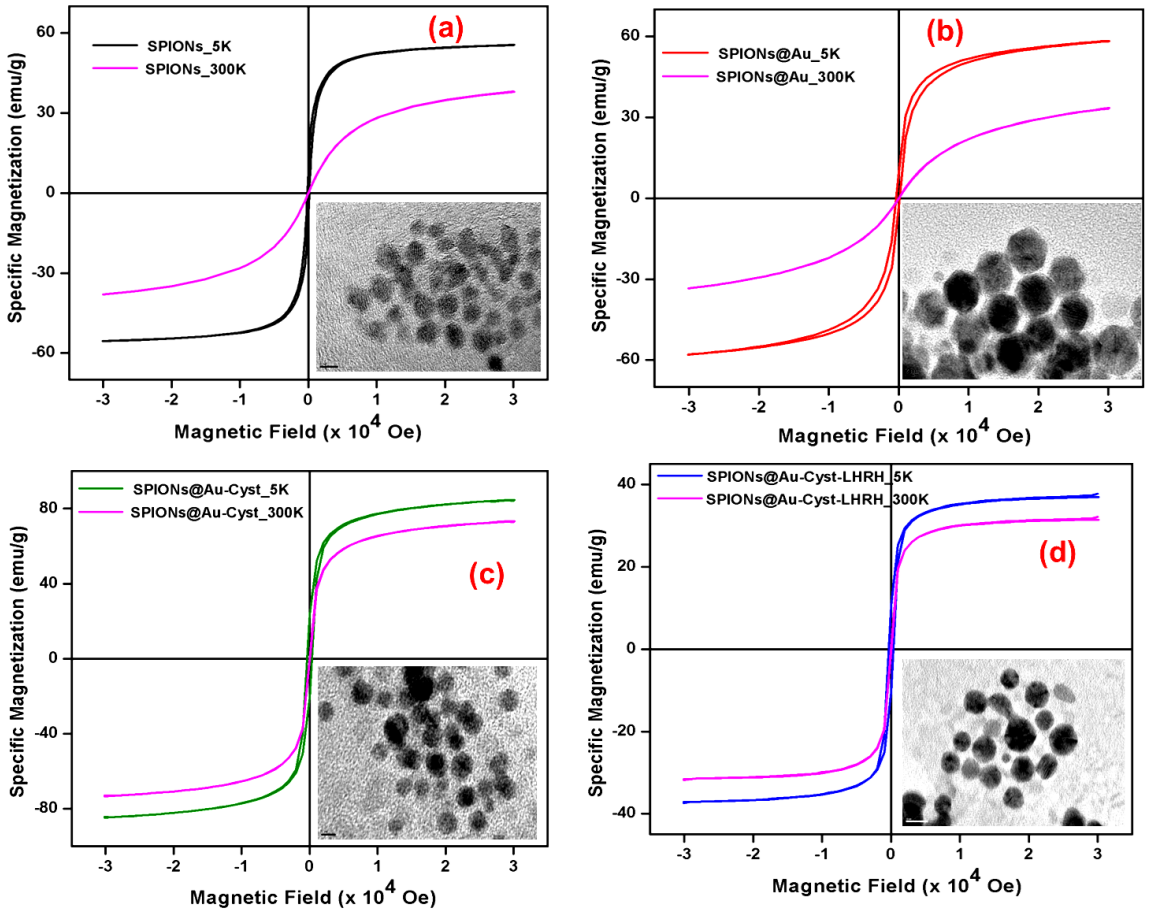
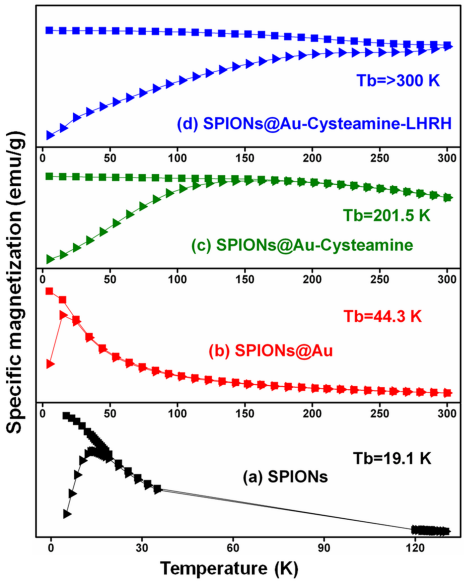


Figure 6. Comparison of the magnetic hysteresis curves of (a) SPIONs, (b) SPIONs@Au, (c) SPIONs@Au-Cyst, and (d) SPIONs@Au-Cyst-LHRH. The insets show the corresponding transmission electron microscopy images where all the particles appear to be spherical, and no significant changes to the surface and morphological properties due to the surface biofunctionalization. Reproduced with permission from [76].

We observed ferromagnetic behavior for SPION@Au-Cyst-LHRH through a residual magnetic moment, non-zero coercivity, and a non-zero remanence ratio (M_r/M_s = 0.012) at room temperature. Like the magnetization values, the blocking temperature (T_B) values also seem to be significantly influenced by the surface functionalization, as shown in Figure 7.

From the comparison of blocking temperature data in Figure 7, SPIONs started to achieve higher T_B values concerning an increase in the weight on their surface (T_B values are in the order of SPIONs < SPIONs@Au < SPIONs@Au-Cyst < SPIONs@Au-Cyst-LHRH). The increase in Ms and T_B values from SPIONs to SPIONs@Au can be due to a rise in the total metallic content (Fe and Au), while Cyst coating is from the chemically induced magnetism in the Au shell utilizing the thiol-Au bonding. Further decrease of Ms values in SPIONs@Au-Cyst-LHRH can be due to the long-chain carbon and high molecular weight of LHRH, thus contributing to a change in the electron/hole density at the d band of Au clusters by the nature of the capping system. With that, we have demonstrated a stepwise change in the magnetic behavior of iron oxide NPs concerning surface functionalization [76]. These results are significant from the perspective of biomedical applications of hyperthermia, drug delivery, and biomagnetic protein separations, as the magnetic properties are greatly influenced by the nature of surface ligands. Since biofunctionalization is required to have the SPIONs to protect particles from the induction of direct toxicity and for the loading of various drugs and in that way, the total magnetization values of the final SPIONs get changed.



	SPIONs	SPION@Au	SPION@Au-Cyst	SPIONs@Au-Cyst-LHRH
Ms @ 5K (emu/g)	55.4	58	84.5	37.7
Ms@300K (emu/g)	38	33.2	72.7	32.1
H_C @ 5K (Oe)	133	115	334	325
H_R @5K (emu/g)	4.8	8.6	21.3	9.6
H_C @ 300K (Oe)	0.0016	0.0019	20	32
H_R @ 300K (emu/g)	0.007	0.045	0.02	0.4
T_B (K)	19.1	44.3	201.5	300

Figure 7. Zero field cooled and field cooled curves of (a) SPIONs, (b) SPIONs@Au, (c) SPIONs@Au-Cys, and (d) SPIONs@Au-Cys-LHRH. From the figure, the increase of TB values with an increase in the molecular weight of the surface molecule is getting observed. Reproduced with permission from [76].

Hyperthermia-medicated cancer cell death through SPIONs

Magnetic fluid hyperthermia involves the generation of heat by the effects of MNPs when they are exposed to oscillatory magnetic fields. Hyperthermia using SPIONs has opened up many investigations in cancer therapy due to their superparamagnetic, multifunctional, biocompatible, biosensing, and non-toxic (some isotopes only) behavior, in addition to their ease of synthesis/availability. It has also been observed that the unique magnetic properties of SPIONs can further be fine-tuned for any application model by simply modifying its shape, size, or chemical composition. In one such example, we found that the heat releasing capacity of SPIONs was found to be improved significantly (about 5 times) when they were coated with Au nanoshell, and the observation of such results can be attributed to an increase in the magnetization values due to the changes in the metal content and associated morphological/geometrical changes (Figure 8a and b). Further, it was also noted that the heat releasing properties (measured by SPL) due to the changes in the size and surface morphology seem to be different when tested in other solvents like water, ethanol, and toluene. Observing such changed SPLs for different solvents with the SPIONs@Au is directly linked to the surface-controlled interaction of particles with respective solvents and viscosity behavior.

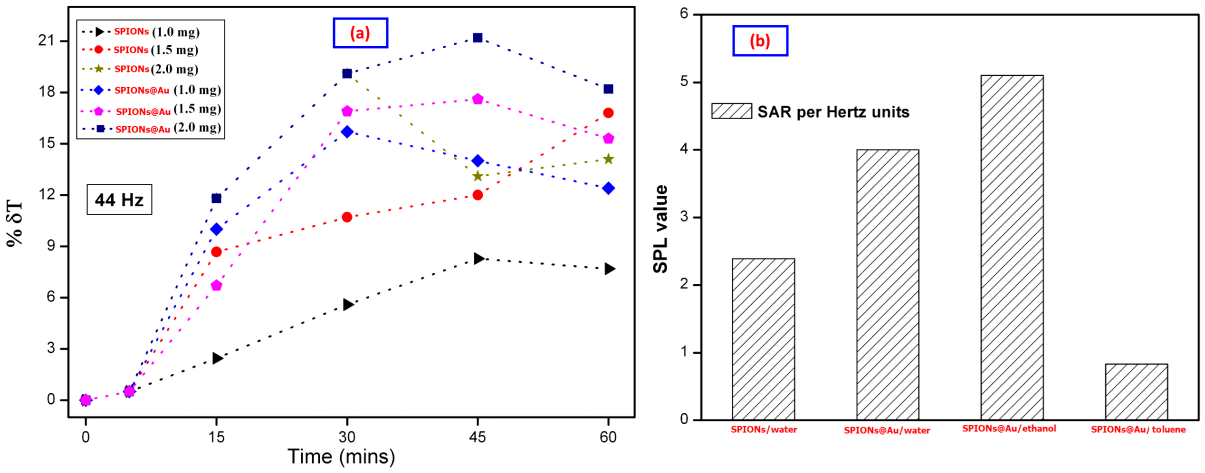


Figure 8. (a) Comparison of the heat release studies between SPIONs and SPIONs@Au with a change of amount at the magnetic field frequency of 44 Hz. It can be noted from the figure that there is a significant increase in the heat release by the SPIONs@Au particles as compared to the naked SPIONs. (b) Comparison of the SPL values of SPIONs@Au in three different solvents of water, ethanol, and toluene when operated at the same 44 Hz magnetic field frequencies.

The changes in the SPL values of the particles concerning the solvent can particularly be useful when these particles are used for treating cancer cells by taking advantage of the amount of production of intracellular proteins among cancer and healthy normal cells. Notably, the non-cancer cells are getting protected from the number of intracellular proteins produced or maintained by the healthy normal cells is far higher than the cancer cells due to the changed morphology and mutation of DNA-containing cells. Due to these changes in the number of proteins, the amounts of heat stress influenced by various cancer and non-cancer cells are different, so the cellular effects are compared following the magnetic fluid hyperthermia. The study results indicated that the non-cancer cells are way more resistant to heat-induced stress through the expression of heat shock proteins (HSPs) better than corresponding cancer cells (Figure 9). On the other hand, the cancer cells that produce HSPs are not good enough to control the heat shock offered by the MNPs oscillation in response to the external magnetic field. In this way, the sustainable therapeutic approaches for cancer treatment are designed where the healthy normal cells are cleverly protected from the highly aggressive anticancer drugs.

The induction of cytotoxicity to the cells not using the NP's intrinsic surface properties, such as in an indirect way for killing diseased cells, is called therapeutic toxicity. The probe does not hold any toxicity effects in a therapeutic toxicity approach. However, it can potentially generate toxic responses that can be controlled externally and are lethal to the cells on which they get tested. Two ways of inducing therapeutic toxicity involve the SPIONs direct hyperthermia and the delivery of loaded drugs. The MNP's hyperthermia-induced toxicity when we engaged the SPIONs bonded with the cancer cell-specific targeted ligand, LHRH provided the results that the approach is quite potential for treating cancer cells and leaving the non-cancer to their normal state. We found from the mechanistic study that the cell death occurrence is through caspase-3 dependent pathway for the LnCap cancer cells. However, the testing of the healthy and non-cancer GT1-7 neurons under similar conditions indicated that the cells are resistant enough to fight against the targeted SPIONs-induced damage (heat stress) and other associated mechanisms such as the mitochondrial oxido-reductive changes, DNA damage, and the ROS generation, due to the increased secretion of many intracellular proteins. Since the cancer cells subjected to the same heat stress could not resist the heat-induced damage and were involved majorly in the imbalance of mitochondrial red-ox transfers, DNA cleavages and ROS-mediated oxidative stress finally led to the loss of cell viability. Based on this study, it can be inferred that the SPION's physical properties are directly linked to the therapeutic toxicity. The SPIONs influencing physical properties such as the size, surface ligand, agglomeration capacity, chain length of surface ligand, and its molecular weight strongly influence the magnetic properties, which are directly

associated with the heat generation at the targeted site. Since the amount of heat generated in an externally controllable manner is a strong deciding factor in knowing whether the heat stress experienced by the cells is sufficient to be lethal or not. Moreover, the toxicity-induced mechanisms followed by the cells before undergoing cell death, in this case, are almost like the case discussed earlier (natural toxicity), and the good thing here is that the extent of toxicity can be controlled externally.

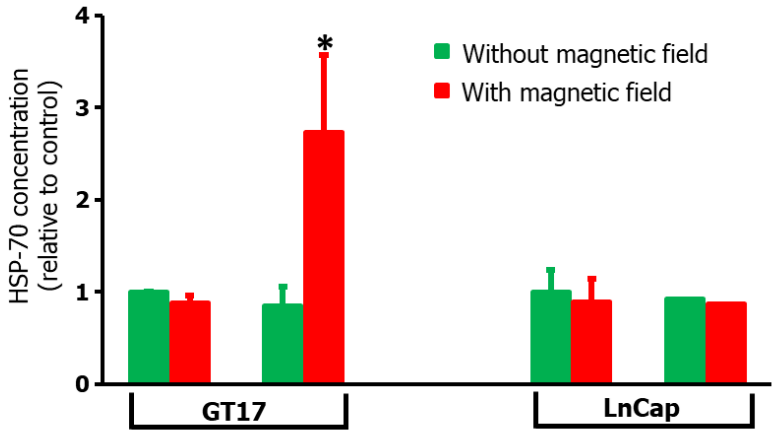


Figure 9. Comparison of HSP release among the two cell lines of non-cancer GT17 and cancer cell of LnCap following the treatment of SPIONs@Au-Cys-LHRH cells and on exposure to the external magnetic field. The GT17 cells could withstand heat stress through the expression of HSP-70 protein compared to the LnCap cells.

Externally controlled drug delivery through SPIONs

Similar to hyperthermia-mediated toxicity, the induction of toxicity to the diseased cells by the drug delivery approach also falls under the category of indirect toxicity, such as therapeutic toxicity. In this approach, the toxicity predominantly originates from the therapeutic drug loaded onto the surface/conjugated with the MNPs, where the nanoparticulate systems are free from the toxicity. This approach also works similarly to the MNP's hyperthermia-induced toxicity, where the amount of toxicity to the exposed cells can be controlled externally by using a magnetic field or pH levels. The nanoparticulate system is provided with polymers that directly affect the pH, temperature, or other environmental conditions. For example, trimethyl chitosan polymer is susceptible to pH, where the pH changes are associated with the polymer's swelling and solubility properties. In that way, by linking the chitosan polymer's solubility properties under strongly acidic or basic conditions, the drug release can be controlled by maintaining the pH, meaning that the amount of therapeutic drug to be released at the targeted site can be controlled externally. Also, in some cases, the drugs are loaded onto thermo-responsive polymers for which slight changes in the temperature of the medium force them to release the loaded drug [79-80]. Thus, by taking advantage of the externally controlled property for the release of loaded drugs, we tested the drug-releasing effects from the surface of SPIONs using two different cancer therapeutic drugs, doxorubicin [81] and 5-fluorouracil [82] (Figure 10). From the analysis of results, it can be confirmed that the drug release effects can be controlled externally, and this particularly be more useful in cancer treatment where the present treatment methodologies are associated with so many side effects of non-targeted attacking of highly toxic anticancer drugs. The current methodologies of cancer treatment suffer from major side effects where the therapeutics cannot differentiate between cancer and non-cancer cells, and sometimes, drug overdose can be a major issue. However, the targeted MNP-based drug delivery can deliver the anticancer drugs specific to the cancer cells in a controllable way, where the cancer cell death mechanism is majorly linked to the loaded drug only, but not from the nanoparticulate system. It has been found that the particles which maintain monodisperse properties have higher drug loading capacity and more efficient release due to an increased surface area and

higher oscillations under the oscillatory magnetic fields. Also, the particles with a suitable ligand can protect them from undergoing agglomeration, contributing to higher drug loadings by providing suitable functional groups for the drug conjugation and offering electrostatic interactions towards the drug [82]. Hence, to induce higher toxicity levels in a therapeutic approach, MNPs must maintain the monodispersity, not with very high surface charges, and are suitable ligands.

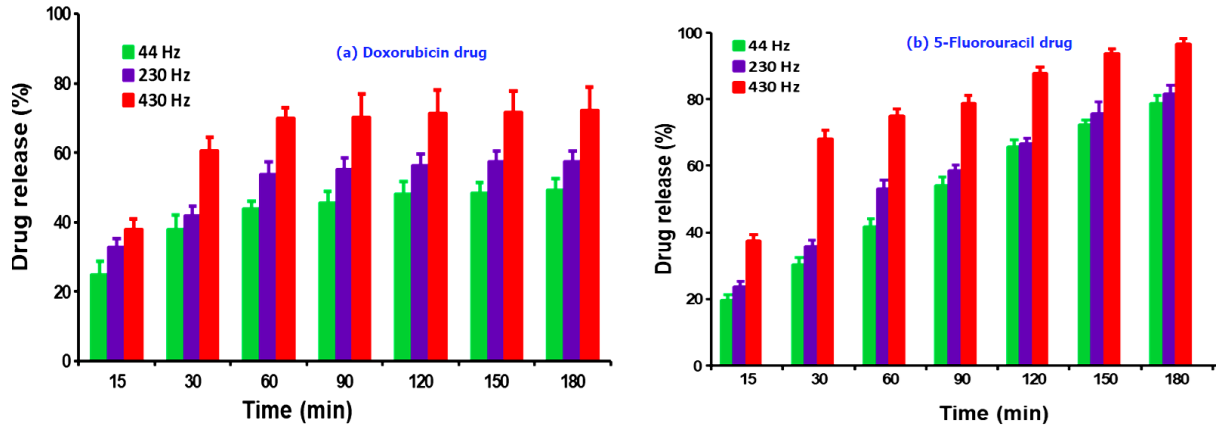


Figure 10. Comparison of the magnetic field-induced drug release effects of (a) doxorubicin drug and (b) 5-fluorouracil drug over 15-180 min and at three different frequencies of 44, 230, and 430 Hz. Control studies correspond to the drug-loaded SPIONs without magnetic field exposure. The analysis of results showed that the amount of drug release increases with that time and applied frequency, meaning that the drug release can be fully controlled at the targeted site.

Summary and future directions

In summary, we present the importance of engineered SPIONs for the induction of therapeutic toxicity directly or indirectly, either through the release of heat or loaded drugs. The MNPs synthesized or obtained in natural ways possess intrinsically low levels of surface-mediated toxicity due to the quenching effects of meal-catalyzed cellular oxidations. Also, the MNPs should be amicable for the bio-functionalization so that they can be used for the theranostic applications of targeted hyperthermia and bioimaging in tumor cells and tissues of reproductive systems (e.g., breast and prostate). Similarly, most studies dealing with the MNPs for hyperthermia, drug delivery, or bioimaging applications have limited or no targeting ability to specifically reach the tumor cells or tissues. For this reason, the MNPs are directly injected into the tumor bed, which cannot possibly be done by a direct injection for tumors located far inside the body. Therefore, the targeting strategies involving the overexpressed proteins such as folic acid and LHRH need to be developed, and cumulative analysis of heat and drug-releasing effects at the targeting organs are to be investigated. Further, the combination treatments, including a combination of hyperthermia with photodynamic therapy (PDT), radiation, and gene therapy, need to be developed as the literature provides the information that the cancer treatment is more effective when applied in conjugation with other treatments than applying alone. When used synergistically with radiation therapy, the released heat first brings some changes to the cancer cell cycle that in lateral stages helps for the faster denaturation of malignant cells through the aggregation of nuclear proteins.

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Authors contribution:

Faruq Mohammad: Conceptualization, funding; Hamad A. Al-Lohedan: Literature review; Payal B. Joshi: Literature review, Writing-Editing; Prasanna Kumar Obulapuram: Statistical analysis and data preparation; Murthy Chavali: Figures, Writing-Reviewing; Aiesha Nawaf Al-Balawi: Proof-reading manuscript and guidance; Maria P. Nikolova: Data analysis, creative ideas, and discussions.

Conflict of Interests

The authors declare no conflicts of interest. For signed statements, please contact the journal office: editor@precisionnanomedicine.com

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Abbreviations:

MNPs-magnetic nanoparticles
SPIONs@Au-coated superparamagnetic iron oxide nanoparticles
DEP-drug delivery through enhanced permeability
SPL-specific power loss
SAR-specific absorption rate
HSPs-heat shock proteins
LHRH-luteinizing hormone-releasing hormone
AMF-alternating magnetic field
PTT-photothermal therapy
NACs-nanoparticle-assembled capsules
T_B-blocking temperature
M_s-saturation magnetization
M_r-magnetic remanence
SPIONs-1: SPIONs coated with oleic acid
SPIONs-2: SPIONs without any surface coating
SPIONs-3: SPIONs coated with cysteamine
SPIONs-4: SPIONs coated with both oleic acid and cysteamine