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Superparamagnetic iron oxide nanoparticles (SPIONs): Development, surface modification and applications in chemotherapy

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ABSTRACT

At present, nanoparticles are used for various biomedical applications where they facilitate laboratory diagnostics and therapeutics. More specifically for drug delivery purposes, the use of nanoparticles is attracting increasing attention due to their unique capabilities and their negligible side effects not only in cancer therapy but also in the treatment of other ailments. Among all types of nanoparticles, biocompatible superparamagnetic iron oxide nanoparticles (SPIONs) with proper surface architecture and conjugated targeting ligands/proteins have attracted a great deal of attention for drug delivery applications.
This review covers recent advances in the development of SPIONs together with their possibilities and limitations from fabrication to application in drug delivery. In addition, the state-of-the-art synthetic routes and surface modification of desired SPIONs for drug delivery purposes are described.

Contents

Abbreviations: SPIONs, superparamagnetic iron oxide nanoparticles; NPs, nanoparticles; MRI, magnetic resonance imaging; PVP, polyvinylpyrrolidone; PLGA, polylactic-co-glycolic acid; PEG, polyethylene glycol; PVA, polyvinyl alcohol; ROS, reactive oxygen species; RES, reticuloendothelial system; DLS, dynamic light scattering; TEM, transmission electron microscope; XRD, X-ray diffractogram; EXAFS, extended X-ray absorption fine structure; HUVECs, Human Umbilical Vein Endothelian Cells; FEM, finite element model; PEGF, poly(ethylene glycol)-co-fumarate; DXM, dexamethasone acetate.

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1. Introduction

Nanoscale science and engineering are providing us with unprecedented understanding and control of matter at its most fundamental level: the atomic and molecular scales. In particular, nanoscale particles have attracted much attention due to their unusual electronic [1], optical [2] and magnetic [3] properties. The dimensions of these nanoparticles (NPs) make them ideal candidates for nano-engineering of surfaces and the production of functional nanostructures. Such modifications of NPs facilitate their use in biomedical applications, for example as contrast agents for magnetic resonance imaging (MRI) and for targeted drug delivery in tumour therapy. For instance, the main problems currently associated with systemic drug administration include the general systemic distribution of therapeutic drugs, the lack of drug specificity towards a pathological site, the necessity of a large dose to achieve high local concentration, non-specific toxicity and other adverse side effects. Current attempts to solve these problems are focusing on the use of targeted NPs [4]. If such treatments could be localised, e.g. to the site of a joint, the continuous maintenance of these potent drugs could be possible. Magnetic targeting using superparamagnetic NPs (SPIONs) is one of the principle schemes to achieve this goal.

SPIONs consist of cores made of iron oxides that can be targeted to the required area through external magnets. They show interesting properties such as superparamagnetism, high field irreversibility, high saturation field, extra anisotropy contributions or shifted loops after field cooling [5]. Due to these properties, the particles no longer show magnetic interaction after the external magnetic field is removed. Since the pioneering concept of using an external magnetic field coupled with magnetic carriers was proposed by Freeman et al. [6] in the late 1970s, a variety of magnetic NP and microparticle carriers have been developed to deliver drugs to specific target sites *in vivo* [7–9]. The optimization of these carriers continues today with the objectives (i) to reduce the amount of systemic distribution of the cytotoxic drug, thus reducing the associated side effects, and (ii) to reduce the dosage required by more efficient, localised targeting of the drug. SPIONs typically have two structural configurations: (i) a magnetic particle core (usually magnetite, Fe_3O_4, or maghemite, γ-Fe_2O_3) coated with a biocompatible polymer or (ii) a porous biocompatible polymer in which SPIONs are precipitated inside the pores [10]. The coating acts to shield the magnetic particle from the surrounding environment and can also be functionalised by attaching carboxyl groups, biotin, avidin, carbodiimide and other molecules [11–13] in order to increase the targeting yield. These molecules then act as attachment points for the coupling of cytotoxic drugs or target antibodies to the carrier complex.

From the physical point of view, magnetic targeting is derived from the magnetic force exerted on SPIONs by a magnetic field gradient. The effectiveness of the therapy is dependent on several physical parameters, including the field strength, the gradient and the volumetric and magnetic properties of the particles. The process of drug localization using SPIONs is based on the competition between forces exerted on the particles by the blood compartment and magnetic forces generated from the applied magnetic field. In most cases, the magnetic field gradient is generated by a strong permanent magnet, such as Nd–Fe–B, fixed outside the body over the target site. The drug/carrier complexes, usually in the form of a biocompatible ferrofluid, are injected into the patient via the circulatory system. When the magnetic forces exceed the linear blood flow rates in arteries ($10\ cm\ s^{-1}$) or capillaries ($0.05\ cm\ s^{-1}$), the magnetic particles are retained at the target site by external, high-gradient magnetic fields. Once the drug/carrier is concentrated at the target, the drug can be released either via enzymatic activity or changes in physiological conditions such as pH, osmolality, or temperature [7], and may be internalized by the endothelial cells of the target tissue or be taken up by the tumour cells [14]. This system, in theory, has major advantages over the normal, non-targeted methods of cytotoxic drug therapy.

Biocompatible SPIONs such as magnetite have been widely used for *in vivo* biomedical applications including magnetic resonance imaging (MRI) contrast enhancement [15,16], tissue specific release of therapeutic agents [17], hyperthermia, and magnetic field assisted radionuclide therapy [18]. Their slower renal clearance and higher relaxation values compared to the gadolinium-based contrast agents make them more attractive for imaging purposes [19]. Some SPIONs with core sizes of 3–6 nm and dextran coating (with 20–150 nm hydrodynamic sizes) such as Feridex, Endorem, Combidex and Sinerem are approved for MRI in patients [20,21]. Similarly, drug-loaded SPIONs can be guided to the desired target area using an external magnetic field while simultaneously tracking the biodistribution of the particles [22]. This approach truly makes them *theragnositc* (therapeutic and diagnostic). Table 1 illustrates some of the commercialized SPIONs which are used for different diagnostic and therapeutic applications.

With extensive research in stem cell engineering, this nanotechnology era is also marked with efforts to personalize treatments using either stem cells or even genetically modified/engineered cells. In this scenario, it is extremely important to track the transplanted/injected cells and to evaluate their engrafting efficiency and functional ability. SPIONs are also being explored as potential candidates for these applications [23].

Their application in biology, medical diagnosis and therapy require that the SPIONs be stable in water at neutral pH and physiological salinity. Such colloidal stability depends on the dimensions of the particles, which should be sufficiently small so that precipitation due to gravitation forces can be avoided. Another important factor is the charge and surface chemistry, which give rise to both steric and coulombic repulsions. To control the surface properties of SPIONs, they are coated with a biocompatible polymer during or after the synthesis process in order to prevent the formation of large aggregates, changes from the original structure and biodegradation when exposed to the biological system. In addition, polymer coating can also allow binding of drugs by covalent attachment, adsorption or entrapment on the particles [24].

The biocompatibility and toxicity of SPIONs are other important criteria to take into account for their biomedical applications. Parameters determining biocompatibility and toxicity are the nature of the magnetically responsive component (for instance, magnetite, iron, nickel, cobalt, neodimium–iron–boron or samarium–cobalt), the final size of the particles including their core and the coatings (shell). Ideally, they must also have a high magnetization so that their movement in the blood can be controlled with a magnetic field and so that they can be immobilized close to the targeted pathologic tissue [24]. However, highly magnetic materials such as cobalt and nickel are

toxic and susceptible to oxidation. Hence, they are of little interest. Iron oxide particles such as magnetite (Fe_3O_4) or its oxidised form maghemite (γ-Fe_2O_3) are by far the most commonly employed for biomedical applications.

Another important aspect of SPIONs delivery is their internalization into specific cells. It is severely limited by three factors: (a) short blood half-life of the particles, (b) non-specific targeting, and (c) low internalization efficiency of surface grafted ligands [25]. Various efforts are directed towards improving these SPION properties and modifying their synthesis methods to better control their size and surface. A number of recently published reviews address these approaches collectively or separately as well as focus on specific applications of SPIONs [22,26–28]. In this comprehensive review, we discuss efforts in the field to improve the properties of SPIONs using various synthetic routes, and we examine the issues of their colloidal stability and toxicity. Finally, we discuss various *in vitro* and *in vivo* studies using SPIONs as drug delivery vehicles.

2. SPIONs synthesis

Magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3) and hematite (α-Fe_2O_3) are three main iron oxides that fall under the category of SPIONs. Ferrites, which are mixed oxides of iron and other transition metal ions (e.g. Cu, Co, Mn, and Ni), have also been reported to be superparamagnetic [29,30]. However, this review article focuses mostly on pure iron oxide NPs with superparamagnetic properties.

Fig. 1 presents the three most important published routes for the synthesis of SPIONs, on which we will focus here. However, other chemical routes that have been reported will also be mentioned. Extensive research on the synthesis and magnetic properties of iron oxides NPs as ferrofluids was published during the 1970s [2,31]. A well established method was published by Sugimoto et al. [32], where well-defined spherical magnetite NPs were prepared using ferrous salt in the presence of potassium nitrate and potassium hydroxide by a *co-precipitation* method. Recently, the same authors [33] reported well-defined size, shape (spherical/ellipsoid), structure and magnetic properties of magnetite, maghemite and hematite by a *sol–gel* method. *Precipitation* from the solution is a fundamental method of crystallisation where nucleation and crystal growth are the principle pathways for the formation of solids [34–36]. With this method, the nuclei can grow uniformly by diffusion from the solution to their surfaces. Ideally, monodispersed NPs can be formed by uniform nucleation followed by crystal growth without further nucleation. However, multiple nucleations can also result in uniform NPs by Oswald ripening [37], where large uniform crystals form by crystal growth through the dissolution of small crystallites. Uniform particles of larger sizes can also be obtained by aggregation of small crystallites through coalescence [32,38,39]. Crystal growth in solution is interface-controlled up to a certain critical size and beyond that size, the growth is diffusion controlled [40].

The most common method of SPIONs synthesis was reported by Massart [41], in which the addition of base to an aqueous solution of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions in a 1:2 stoichiometry produced a black precipitate of spherical magnetite NPs of uniform sizes in an oxygen free environment. It is important to have an oxygen free environment during the synthesis otherwise, magnetite can be further oxidised to ferric hydroxide in the reaction medium. Recently, Sen et al. [42] compared Sugimoto's and Massart's methods for magnetite synthesis. They found that Sugimoto's method produced larger particles (30 to 200 nm) of rhombic (stirred condition) and spherical (static condition) morphologies whereas Massart's method produced smaller (<20 nm) spherical particles (Fig. 2). The size and morphology of magnetite NPs could be controlled by varying the ratio of ferrous and hydroxide ions in Sugimoto's method (Fig. 3, unpublished data).

Controlling the crystal growth step in the co-precipitation route is the key step to producing sub nm size SPIONs. The *microemulsion* (water in oil: W/O) method, using water droplets as nanoreactors in a continuous phase (oil) in the presence of surfactant molecules [43–45], is reported to be an alternative and more controlled method. In this method, iron precursors can be precipitated as iron oxide in the water phase specifically located in the centre of the micelles. Iron oxides do not precipitate in organic phase as the iron precursors are unreactive in this phase. The size of the NPs can be controlled by controlling the size of the water droplets. Surfactants, which are responsible for micellisation, can be utilised for the dispersion of iron oxide NPs. Zhang et al. [46] have fabricated hollow magnetite NPs of

Table 1
Commercialized SPIONs.

Company	Reference	Applications
Stemcell Technologies	www.stemcell.com	Automated immunomagnetic cell separation
Micromod	www.micromod.de	Drug delivery, biomagnetic separation, nucleic acid purification and protein separation
Chemicell	www.chemicell.com	Bioseparation- and detection systems
Magnisense	www.magnisense.com	Bioassays for human and animal diagnostics, food safety and environmental protection
Diagnostics Biosensors	www.diagnosticbiosensors.com	Diagnostics biosensors
Dexter Magnetic Technologies	www.dextermag.com	Biomagnetic separation
Ocean Nanotech	www.oceannanotech.com	Synthesis, production as well as R&D of magnetic NPs
Imego	www.imego.com	Medical diagnostics
Integrated Engineering Software	www.integratedsoft.com	Electromagnetic, electromechanical and electrothermal analysis software
EMD chemicals	eshop.emdchemicals.com	Immunoassay and immunoreagent development
Magsense	www.magsenselifesci.com	Bioseparation, diagnostic, immunoassay, and bioanalytical measurements
TurboBeads	www.turbobeads.com	Efficient magnetic separation
European Institute of Science	www.biotechniques.com	Hyperthermia
Magnabeat Inc.	www.magnabeat.com	Separation of many different bio-substances
nanoTherics Ltd.	www.nanotherics.com	Magnetic gene transfection
SEPMAG technologies.	www.sepmag.eu	Large volume magnetic separation systems
Magforce	www.magforce.de	Hyperthermia
AMAG Pharmaceuticals (Advanced Magnetics)	www.amagpharma.com	MRI contrast agents; Diagnosis of cardiovascular disease and cancer
Estapor	www.estapor.com	Application of magnetic microspheres in immunoassays
Miltenyi Biotec	www.miltenyibiotec.com	Reagents and instruments development for use in immunology, cell and molecular biology, bioinformatics, and stem cell technologies
Invitrogen and Dynal Biotech (bought by Invitrogen in 2005)	www.invitrogen.com	In vitro diagnostics, protein, cell and biomolecular purification and separation

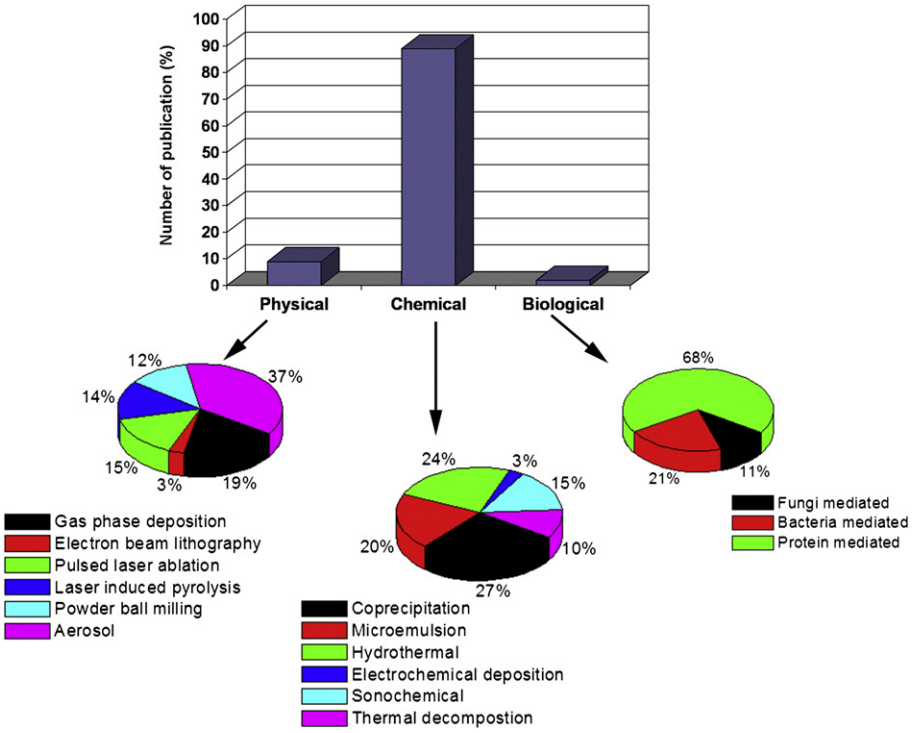


Fig. 1. A comparison of published work (up to date) on the synthesis of SPIONs by three different routes. Sources: Institute of Scientific Information.

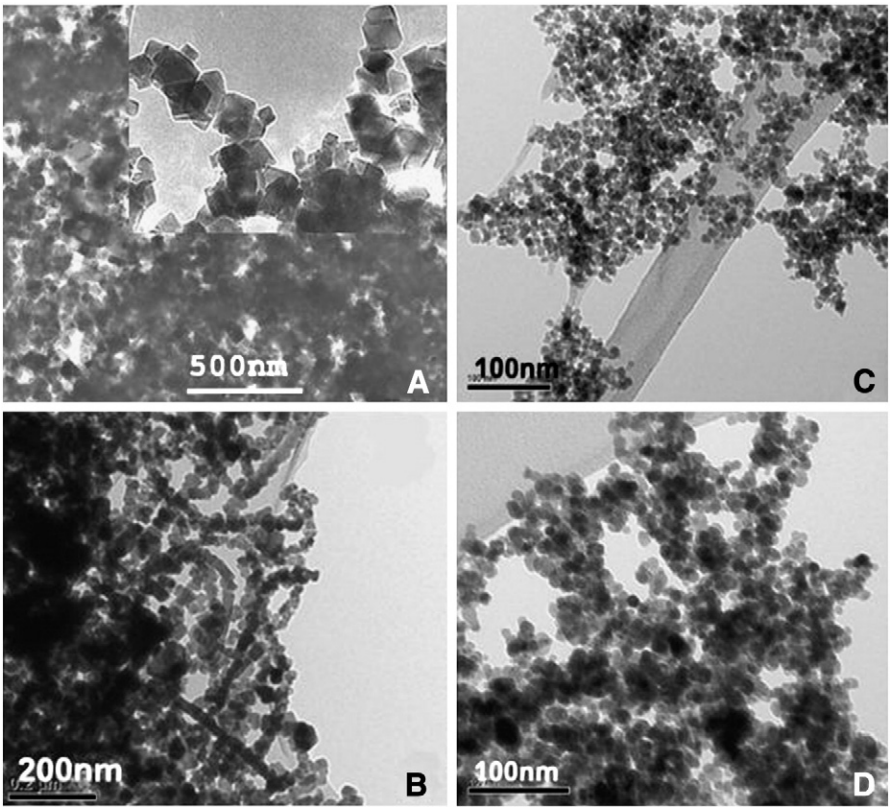


Fig. 2. TEM micrographs of magnetite nanoparticles: A. Prepared by Type 1 reaction in the presence of excess [OH^-]; B. Prepared by Type 1 reaction without stirring and without excess of [OH^-] or [Fe^{2+}]; C. Prepared by Type 2 reaction in the presence of NH_4OH; D. Prepared by Type 2 reaction in the presence of dispersing agent TMAOH and without NH_4OH. Type 1: Sugimoto method; Type 2: Massart method (Reproduced from reference [42] with permission, copyright 2006, Institute of Engineering and Technology, IET Digital Library).

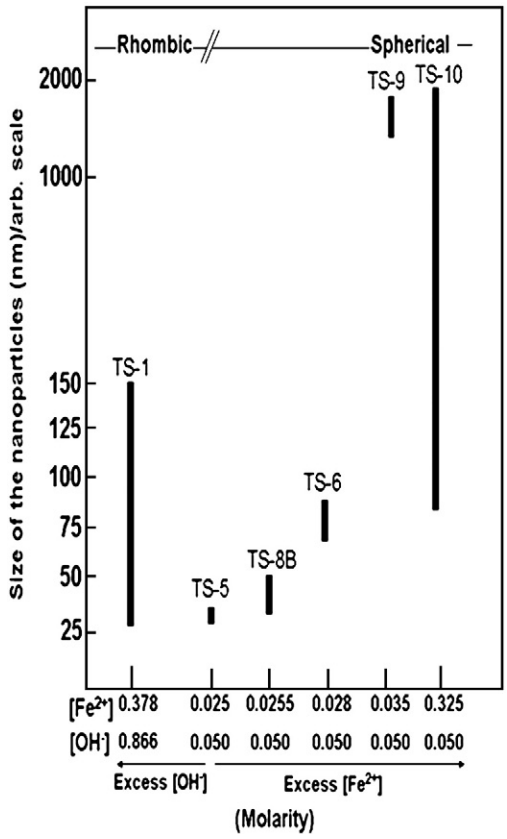


Fig. 3. Effect of particle sizes and their size distribution in the presence of either excess $[Fe^{2+}]$ or $[OH^-]$ ions by Sugimoto method (unpublished results by Sen T and Bruce I J).

200 to 400 nm in diameter by the *microemulsion* route, however these NPs may not be useful for drug delivery purposes. This method suffers from the preparation of adequate crystalline SPIONs on a large scale as the temperature used for such synthesis is low. Hyeon et al. [47] reported highly crystalline maghemite particles with well-defined nanometer sizes by the *microemulsion* method at high temperature using iron (III) acetyl acetonate as an iron precursor. Sun et al. [48] later reported the size controlled synthesis of ultra-small magnetite (12 and 16 nm) NPs using $Fe(acac)_3$ as an iron source by the microemulsion route.

A recent development in the synthesis of SPIONs is the use of *sonochemical* routes [49–51]. In this process, a high energy ultrasonication creates acoustic cavitations that can provide localised heat with a temperature of about 5000 K. At high temperature, the formation and growth of nuclei and the implosive collapse of bubbles can take place. Monodisperse NPs of a variety of shapes can be prepared by this method; however, it lacks large scale synthesis. *Electrochemical deposition under oxidised conditions* (*EDOC*) has also been used to synthesize maghemite and magnetite NPs [52]. In this method, the anode can be oxidised to metal ion species in solution and the metal ion is later reduced to metal by the cathode in the presence of stabilisers. This method, however, also lacks a large scale synthesis. The *hydrothermal method* is reported to be the oldest method of synthesis of magnetite where iron precursors in aqueous medium can be heated at high temperature at autogenous pressure. Recently, this method has been extended using microwave for the synthesis of SPIONs [53]. This method produces SPIONs of uniform sizes and can easily be scaled up. Grzeta et al. [54] reported the synthesis of nanocrystalline magnetite by *thermal decomposition* of iron choline citrate. A similar method was also reported using iron carbonate [55] and iron carboxylate [56]. Recently, Liu et al. [57] prepared magnetic platelets using ethylene diamine as a solvent/reducing agent by the *solvothermal* route. The solvent free thermal decomposition route was also used recently for the preparation of SPIONs [58].

Biomimetic synthesis of SPIONs by magnetotactic bacteria has been known for a long time and various research groups have used bacteria, fungi, Mms6 protein, or globular protein for this purpose [59–64]. For instance, Coker et al. [64] exploited Fe(III)-reducing bacterium *Geobacter sulfurreducens* to synthesize magnetic iron oxide NPs. However, the disadvantage of this method is the lack of large scale synthesis with well-defined sizes and shapes.

Although most of the above synthesis protocols resulted in either spherical or rhombic morphologies, there are few reports of synthesizing SPIONs with other geometries such as ellipsoid [33] and cubic [65]. Such unique geometries can offer well-defined and reproducible magnetic fields. On the other hand, Peng et al. [66] prepared magnetite nanorods through reduction of beta-FeOOH, whereas large scale synthesis of single-crystal magnetic (maghemite, magnetite and hematite) nanorings was achieved by Jia et al. [67] using the hydrothermal route.

3. Characteristics of SPIONs

3.1. Colloidal stability of SPIONs

The stability of SPIONs in suspension is controlled by three principal forces: (a) hydrophobic–hydrophilic, (b) magnetic and (c) van der Waals. SPIONs tend to aggregate to micron size clusters in suspension due to the hydrophobic interactions between the sub nm size particles (large surface area to volume ratio). Micron size clusters further aggregate due to the magnetic dipole–dipole interactions and become magnetized by neighbouring clusters. In the presence of an external magnetic field, further magnetization of these clusters can occur increasing their aggregation [68]. In general, nanometer size particles aggregate in suspension due to the attractive van der Waals forces in order to minimise the total surface or interfacial energy. Consequently, such aggregation can hamper the efficacy of SPIONs in drug delivery (less drug loading) due to their low surface area and larger sizes. Hence, the stabilisation of SPIONs in suspension by modifying their surface is an important issue in the context of drug delivery.

Surface modification of SPIONs can be carried out either during their synthesis or in a post-synthesis process. Table 2 presents a list of materials which have been used as stabilising agents during the synthesis of SPIONs.

The ideal molecules used for stabilization of SPIONs should be biocompatible and biodegradable. However, the most common molecules used are surfactants such as oleic acid, lauric acid, alkane sulphonic acids, and alkane phosphonic acids [115]. Surfactant molecules are amphiphilic in nature and they play their role at the interface of SPIONs and the solvent. Most of the surfactant mediated syntheses are reported in organic solvents, i.e. hexadecane, toluene, n-hexane etc., as the hydrophobic tail groups (hydrocarbon chain) of the surfactant molecules lay as a shell around SPIONs. However, SPIONs in organic suspension cannot be used for biological purposes, especially, for drug delivery.

Wang et al. [116] reported a method where the hydrophobic surface of coated SPIONs was inverted to a hydrophilic surface using alpha-cyclodextrin by host–guest interactions and the NPs were able to disperse from organic to aqueous solution. Such inversion of hydrophobic surfaces to hydrophilic surfaces was also achieved by using an amphiphilic polymer shell [117]. Various polymers such as poly (ethylene-co-vinyl acetate), polyvinylpyrrolidone (PVP), poly-lactic-co-glycolic acid (PLGA), polyethylene glycol (PEG) or polyvinyl alcohol (PVA) have also been used as coating materials in aqueous suspension [118]. Natural dispersants including gelatin, dextran, polylactic acids, starch, albumin, liposomes, chitosan, ethyl cellulose have been extensively used for dispersion of SPIONs in aqueous medium (Table 2) for the purpose of drug delivery. The synthesis of

Table 2
Materials used for coating or encapsulating SPIONs and their applications.

Materials used	Size and size distribution	Applications	Advantages	Ref.
Amorphous silica	20–200 nm, broad	Isolation of biomolecules, e.g. genomic and plasmid DNA, extraction of nucleic acids from soil, drug delivery, extraction of phenolic compounds from environmental water	Does not require any organic solvents and eliminates the need for the repeated centrifugation, vacuum filtration or column separation	[69–75]
Mesoporous silica	10–300 nm, broad	Controlled drug delivery, removal of mercury from industrial effluent, support for enzyme immobilisation for bio-catalysis, fluorescence, isolation of genomic and plasmid DNA	Uniform pore size, large surface area, and high accessible pore volume	[76–81]
Polyethylene glycol (PEG)	10–50 nm, narrow	*In vivo* NMR imaging, *in vivo* contrasting	Improves the biocompatibility, blood circulation time and internalization efficiency of the NPs, easy to functionalise	[82–84]
Polyvinyl alcohol (PVA)	10–50 nm, narrow	*In vivo* imaging and drug delivery	Prevents coagulation of particles, giving rise to monodisperse particles	[85,86]
Polyvinyl pyrrolidine (PVP)	10–20 nm, narrow	Contrasting and drug delivery	Enhances the blood circulation time and stabilises the colloidal solution	[87,88]
Polyacrylic acid	~250 nm, narrow	Target thrombolysis with recombinant tissue plasminogen activator	Increases the stability and biocompatibility of the particles and also helps in bioadhesion	[89]
Polystyrene	10–20 nm, narrow	Cellular imaging and DNA hybridisation	Stable and uniform size particles in suspension	[90,91]
Polymethyl methaacrylate	10–50 nm, narrow	DNA separation and amplification	Novel, simple and labour-saving; can be applied in automation system(s) to achieve high throughput detection of single nucleotide polymorphisms	[92]
Polydipyrrole/dicarbazole	20–100 nm, broad	DNA hybridization	Molecular diversity for engineering functional polyDPyr-/polyDCbz-shell outer layers of magnetic nanocomposites	[91]
Ethyl cellulose	20–50 nm, broad	Extraction of pharmaceutical chemicals	Enhanced the drug absorption into the surrounding tissues for a prolonged period of time	[93,94]
Chitosan	20–100 nm, broad	Tissue engineering, hyperthermia	A natural cationic linear polymer that is widely used as non-viral gene delivery system, biocompatible, hydrophilic, used in agriculture, food, medicine, biotechnology, textiles, polymers, and water treatment	[95–98]
Dextran	10–200 nm, narrow	Isolation of *E. coli*, drug delivery, imaging	Enhances the blood circulation time, stabilises the colloidal solution	[99–101]
Starch	10–20 nm, narrow	Contrasting and imaging	Natural polymers, biocompatible	[102,103]
Liposome	50–200 nm, broad	Imaging, drug delivery, hyperthermia, contrasting	Long-circulating time	[104–108]
Albumin	100–200 nm, broad	Cell separation	Magnetic tagging and separation, does not affect cell viability and proliferation	[109,110]
Erythrocytes	10–100 nm, broad	MRI imaging, drug delivery	Avoids the rapid clearance by the reticuloendothelial system (RES) and permits a long half-life in blood circulation	[111,112]
Gelatin	50–100 nm	Isolation of genomic DNA, drug delivery	Hydrophilic, biocompatible, natural polymer. Improves the efficiency of drug loading and is a rapid, simple, and a well-suited method for DNA extraction	[113,114]

hydrophilic magnetic NPs has also been reported *via* a reverse emulsion approach using PEG [45]. Other chemicals used for surface stabilisation of SPIONs include Disperbyk 120 [42] and tetramethyl ammonium hydroxide [119]. Furthermore, Park et al. [120] reported graphitic carbon coated magnetite and maghemite NPs using ferrocene in supercritical water.

The post-synthesis modification of SPIONs, known as core–shell NPs, has also been widely studied. Materials used for core–shell SPIONs are mostly polymers, silica, metal (e.g. gold and cadmium/selenium) and organic dye molecules. The coating materials protect the core against oxidation and, hence, maintain the magnetic property of SPIONs. Iron oxide particles are known to be non-toxic; however, some of the coating materials could be toxic. Silica is known to be biocompatible but not biodegradable. Amorphous silica coating on magnetite NPs was first reported by Philipse et al. [121] with a sol–gel approach. As silica is hydrophilic in nature, the silica-coated core–shell particles were reported to be well dispersed in aqueous suspensions. The silica-coated SPIONs can be negatively charged above the isoelectric point of silica (pH ~2); hence, they have been used for separation of biomolecules through electrostatic interactions. In addition, silica has hydroxyl groups (silanol group: Si–OH) useful for the attachment of further functionalities through covalent bonding with organosilanes. Bruce et al. [122] reported large scale synthesis and modification of magnetite NPs by amorphous silica. In this method, silicic acid (ion exchanged from sodium silicate) was added slowly to diluted suspension of magnetite at pH 13. The suspension's pH was adjusted from 13 to 10 by slow addition of diluted hydrochloric acid. Core–shell NPs of magnetite core coated with a thin silica shell were prepared and the percentage of coating was characterised by DNA binding and elution behaviour. The reverse microemulsion method [45] has also been used for silica coating using non-ionic surfactants (Triton X100, Brij-97 and Igepal CO-520) and the silica coating was reported to be as thin as 1 nm. To gain advantage of surface area of silica-coated SPIONs, various authors have used a templating route for the preparation of microporous (zeolites) and mesoporous silica coating for biological applications. Sen et al. [81] used a surfactant templating route for the fabrication of high surface area mesoporous silica-magnetite core–shell NPs of spherical and tubular morphologies for the extraction of nucleic acid from cells. Recently, Zhao et al. [123] prepared SPIONs with magnetite core and microporous zeolite shell for the immobilisation of trypsin.

Atomic transfer radical polymerisation (ATRP) is another common method developed by Wang et al. [124] for coating SPIONs. Li et al. [125] used the ATRP method for coating iron oxide with polystyrene

using divinylbenzene as a crosslinker. Several other polymers, i.e. PVA, poly(glycerol monoacrylate), poly(glycerol monomethyl acrylate) and tri-block copolymers, have also been used as coating materials [126,127]. Inverse emulsion is another way of coating iron oxide NPs with PEG [45]. However, it should be noted that the use of non-magnetic materials for coating SPIONs may result in a decrease in saturation magnetization [128]. Indeed, Voit et al. [129] found a similar effect when polymers (i.e. PVA and starch) were used as coating materials. Sen et al. [78] also reported a decrease in the saturation magnetisation value from 90 emu/g to 15 emu/g due to the presence of amorphous silica in a biphasic mixture of silica and magnetite NPs.

Due to the interaction of SPIONs with biological fluids, the formation of free hydroxyl radicals and reactive oxygen species (ROS) can be significantly increased. In order to protect the *in vivo* environment from these toxic by-products, biocompatible and rigid coatings such as gold (Au) has been employed. For instance, Kim et al. [130] reported the reverse micelle method for Au coating on SPIONs. Recently, Goon et al. [131] reported an aqueous synthesis of Au coated SPIONs using polyethyleneimine for the dual functions of attaching 2 nm gold NPs onto magnetite particles and preventing the formation of large aggregates. On the other hand, quantum dots (CdSe/ZnS) can be used as coating materials for the fluorescence marker [87,88]. An organic fluorescence molecule (pyrene) has also been used recently as a coating material for multifunctional SPIONs [132].

3.2. Shape, size and size distribution

The size and size distribution of SPIONs are also important parameters related to their biological applications. The magnetic property of SPIONs is size dependent. Mahmoudi et al. [133] employed a multiphysics finite element model in order to study the effects of an applied magnetic field on SPIONs with various sizes and shapes in a simulated blood vessel. For this purpose, a 2D model triangular mesh was used, consisting of a blood vessel 1 cm in width, tissue 1 cm in width, an external magnet, and the surrounding environment (Fig. 4A); fluid flow is from left to right. It is notable that various synthesis parameters (e.g. stirring rates and base molarity) were used for preparation of SPIONs with different sizes and shapes. The FEM model iteratively solved coupled Maxwell and Navier–Stokes equations to predict both the induced magnetic flux density and fluid velocity fields (see Fig. 4). Simulation results suggest that both the strength of the applied magnetic field and the magnetic properties of SPIONs (which obtained by their various size and shapes) affect the velocity field fluctuations (flow turbulence) and amplitude (peak velocity). These parameters showed some interactions in defining the shape and amplitude of the velocity field. The authors hypothesized that the velocity field with less fluctuation and higher amplitude, especially in the direction normal to tissue, is believed to be preferable to facilitate drug delivery.

It has been shown that the magnetic dipole–dipole interactions are significantly reduced in SPIONs because they scale as r^6 (r is the particle radius). Varanda et al. [134] reported that the saturation magnetisation of SPIONs and their sizes are linearly correlated as the surface curvature changes with size. Another main advantage of using particles of sizes smaller than 100 nm is their higher effective surface areas (easier attachment of ligands), lower sedimentation rates (i.e. high stability in suspension) and improved tissular diffusion. Particles should be small enough (<100 nm) to escape from the reticuloendothelial system (RES). They should remain in the circulation after injection and be capable of passing through the capillary systems of organs and tissues avoiding vessel embolism. Similarly, the particle size is important for achieving enhanced permeability and retention (EPR) effect. For instance, particles larger than 10 nm cannot penetrate the endothelium at physiological conditions [135], but they can penetrate it in pathological conditions such as inflammation or tumour infiltration [136].

Various methods are available to measure the sizes of SPIONs either in suspension or in dry state. However, it is the size in suspension which is important for their applications, since the size in dry state does not include the solvation shell around the SPIONs. In addition, the drying step can have an effect on aggregation behaviour. The techniques used in order to determine particle size include dynamic light scattering (DLS), transmission electron microscope (TEM), Scherrer analysis of X-ray diffractograms (XRD) and extended X-ray absorption fine structure (EXAFS). The DLS technique determines the particle size in suspension whereas the other techniques determine the crystallite size in its dry state. The DLS technique provides information of mean sizes based on volume, intensity and number distribution. Scherrer analysis of XRD determines size based on the broadening of the diffraction peak and can be calculated by the equation $\tau = (K\lambda)(\beta\cos\theta)^{-1}$, where K is the shape constant (usually 0.89 but it varies with the actual shape of the crystallite), λ is the wavelength of the X-ray used, β is the width of the peak at half height in radians and θ is the Bragg angle. TEM is the most powerful technique to determine the crystallite and particle sizes and their morphology. This technique gives information on the size distribution as well as details of the core–shell structure due to the difference in electron density of core and shell materials.

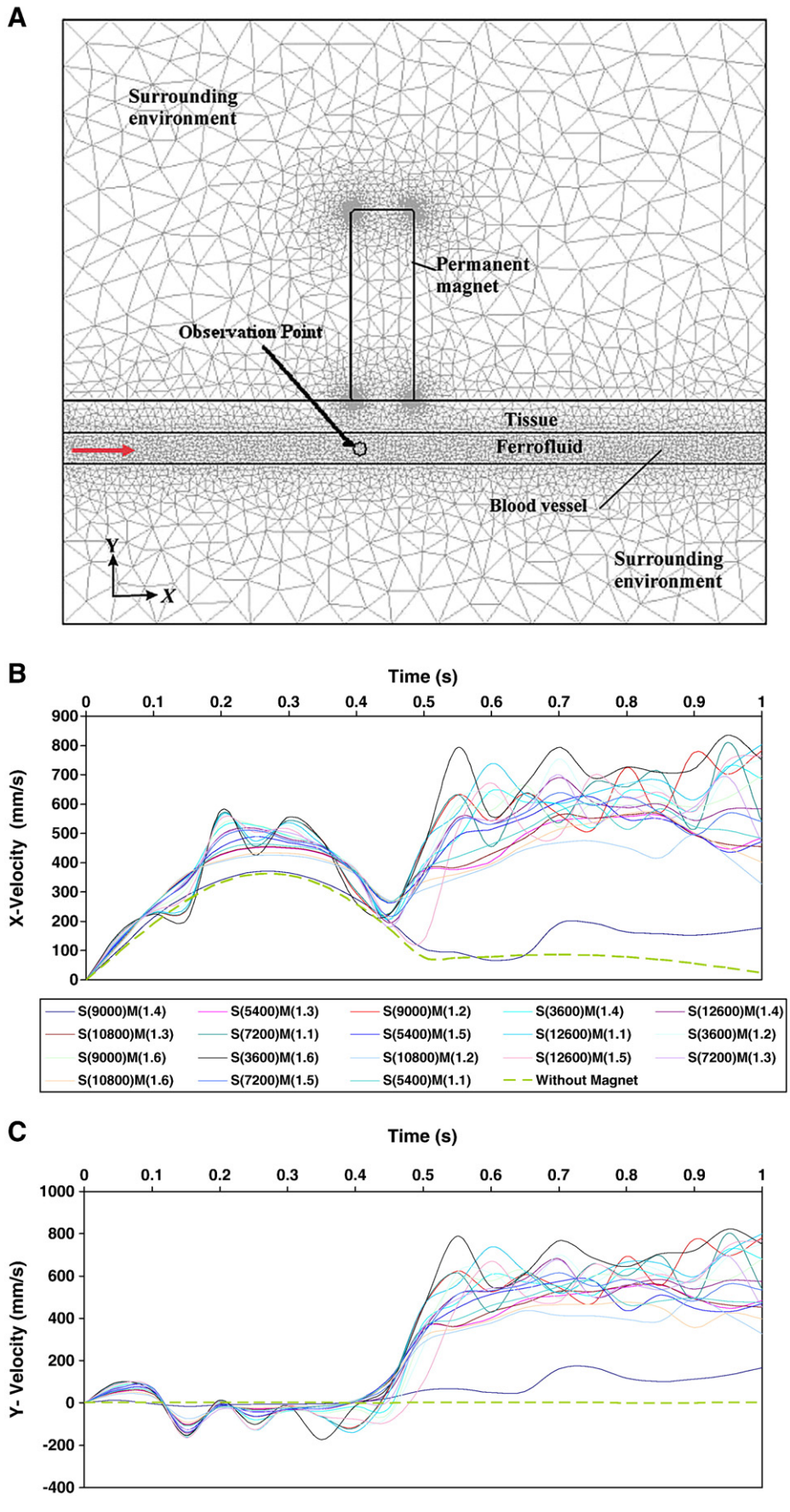
When the drug-loaded NPs are injected systemically into the bloodstream, the size, morphology and surface charge are the three important parameters for their behaviour in the blood stream. Tissue macrophages (i.e. Kupffer cells in the liver) are highly sensitive to invading micro-organism and NPs [136]. The plasma proteins (opsonins) can easily adsorb on the surface of invading NPs depending on their sizes, surface charge and morphology. Particles with sizes above 200 nm or below 10 nm are not suitable due to their uptake by the RES. Gupta et al. [137] reported that the sizes between 10 and 100 nm are most effective for drug delivery purposes because they can evade the RES.

3.3. Surface charge

The surface charge and the biodistribution of SPIONs play a significant role in colloidal stability. It can be qualitatively described as the nature and behaviour of the surface groups in solution at a certain pH in the presence of an electrolyte. Quantitatively, it can be measured as an electrical potential in the interfacial double layer on the surface of NPs in suspension. A high zeta potential (+ or −) value is an indication of the dispersion stability of SPIONs due to the electrostatic interaction. Sun et al. [138] studied the effect of excess surface concentration of Fe^{2+} or Fe^{3+} ions on the zeta potential of SPIONs. According to their results, zeta potential of magnetite had positive and negative values in the absence of multivalent cations in acidic and basic solutions, respectively. Furthermore, in the presence of excess iron cations, specific adsorption took place at the surface of magnetite, considerably affecting its zeta potential.

The composition and structure of NPs are very important in their interactions with biological fluids. Magnetite is reported to be an inverse spinel structure with oxygen forming a face-centred cubic (FCC) closely packed arrangement and Fe cations occupying the interstitial tetrahedral and octahedral sites [139]. The structure of maghemite is similar to that of magnetite except that all Fe ions are in a trivalent state (Fe^{3+}). Coey et al. [140] found that the surface charge of SPIONs also determines their cellular interaction, especially during endocytosis and phagocytosis.

In a known medium, the characteristics of SPIONs such as chemical composition of both core and shell, size and its distribution, shape and angle of curvature, crystallinity, smoothness/roughness, hydrophobicity/hydrophilicity are important for their *in vivo* applications; these characteristics could determine the residence time of SPIONs in the circulatory system [141]. Recently, Osaka et al. [142] reported a correlation between the surface charge of magnetite NPs and their


A
Surrounding environment
Permanent magnet
Observation Point
Tissue
Ferrofluid
Blood vessel
Surrounding environment
Y
X
B
Time (s)
X-Velocity (mm/s)
S(9000)M(1.4)
S(5400)M(1.3)
S(9000)M(1.2)
S(3600)M(1.4)
S(12600)M(1.4)
S(10800)M(1.3)
S(7200)M(1.1)
S(5400)M(1.5)
S(12600)M(1.1)
S(3600)M(1.2)
S(9000)M(1.6)
S(3600)M(1.6)
S(10800)M(1.2)
S(12600)M(1.5)
S(7200)M(1.3)
S(10800)M(1.6)
S(7200)M(1.5)
S(5400)M(1.1)
Without Magnet
C
Time (s)
Y- Velocity (mm/s)

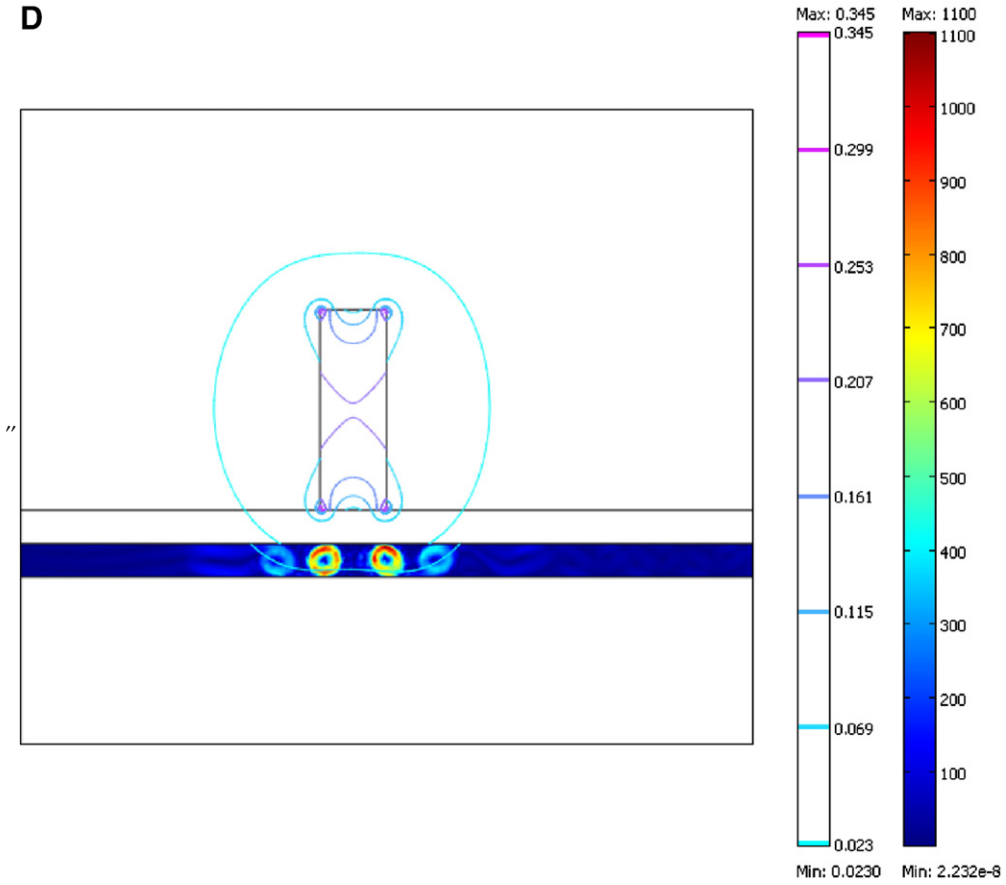


Fig. 4. (A) 2-D geometry and finite element mesh model of a blood vessel under a permanent (static) magnetic field for drug delivery. The (B) *x* and (C) *y* velocities of the ferrofluid containing SPIONs with various sizes. Results are presented for the observation point shown in (A). Samples are herein referred to as $S(x)M(x)$, where *S* is the stirring rate and *M* is the NaOH molarity. For instance, $S(3600)M(1.2)$ indicates a sample prepared with a stirring rate of 3600 rpm and a molarity of 1.2. (D) contour lines of magnetic flux density and ferrofluid velocity surface contours in the blood vessel model (note: results are shown for $t = 1$ s for $S(12{,}600)M(1.1)$; at the observation point marked in (A), the value of magnetic flux density is 27.7 mT. (Reproduced from reference [133] with permission.)

cellular uptake efficiency into different cell lines. SPIONs with a positive charge showed higher internalization into human breast cancer cells compared to negatively charged SPIONs; whereas there was no difference in the degree of internalization into Human Umbilical Vein Endothelian Cells (HUVECs). Thus, the uptake pattern of SPIONs depends not only on their surface properties but also on the cell type.

Various authors have reported the protein adsorption on NPs either *in vitro* or *in vivo* [143,144]. Moghimi et al. [136] performed an extensive study of the opsonisation process (adsorption of plasma protein) based on the NP's surface charge, size and hydrophilicity/hydrophobicity. They concluded that the smaller the size and the higher the hydrophilicity of the NPs, the less efficient is the opsonisation process. In this context, PEG and folic acid coating have been found to be efficient for inhibiting the protein adsorption *in vivo* [145]. Recently, Lee et al. [146] reported the use of polymer, *Poly{3-(trimethoxy silyl propyl) methacrylate-r-polyethylene glycol methacrylate}*, coating on magnetite to generate a stable, protein-resistant magnetic resonance imaging probe. Their results suggested that the polymer-coated SPIONs can possess long-circulating properties in plasma by escaping their uptake by the RES, e.g. by macrophages.

3.4. Toxicity of SPIONs

Biomedical applications such as drug delivery, cellular labelling/cell separation, tissue repair, magnetic resonance imaging, magnetic hyperthermia and magnetofection are well known examples of the use of SPIONs and have been extensively reported. Since these applications involve use of humans or other animals, it is important to study their toxicity in appropriate animal models. Despite potential applications of SPIONs, very few reports are available on their toxicological effects. Some of them are discussed below.

3.4.1. Composition toxicity

The cytotoxicity of PVA-coated SPIONs with different shapes and morphologies (e.g. nanospheres, nanorods, nanoworms, magnetite colloidal nanocrystal clusters and nanobeads) has been comprehensively explored by Mahmoudi et al. [133,147] in mouse fibroblast cells and human leukaemia cells. According to their results, SPIONs showed no or little toxicity. On the other hand, Karlsson et al. [148] studied the toxicity of SPIONs (20 and 40 μg/ml) in human lung cancer cell lines. Neither DNA damage nor intracellular ROS (i.e. production of reactive oxygen species) toxic effects were observed; however, small oxidative DNA lesions were detected. In another study, the effect of SPIONs on the cell cycle confirmed the arrest in the G_0G_1 cell-life gap [149]. Very recently, Mahmoudi et al. [150,151] observed the existence of gas vesicles in SPIONs-treated cells (by staining with the crystal violet dye) with increased granularity of the cells (Fig. 5). It was suggested that autophagy may be the possible cause of the cytotoxicity. It should be noted that the *in vitro* cytotoxicity studies can encounter a great deal of error in the obtained results due to the large amount of protein absorption on the surface of SPIONs (Section 3.4). In order to obtain reliable and exact cytotoxicity results, a modified method has been reported [152], in which the iron oxide NPs were introduced to the cell medium and the solution was kept in contact for a period of 24 h in order to create a stable protein corona on the surface of the SPIONs. The medium was then replaced with a fresh one and the obtained

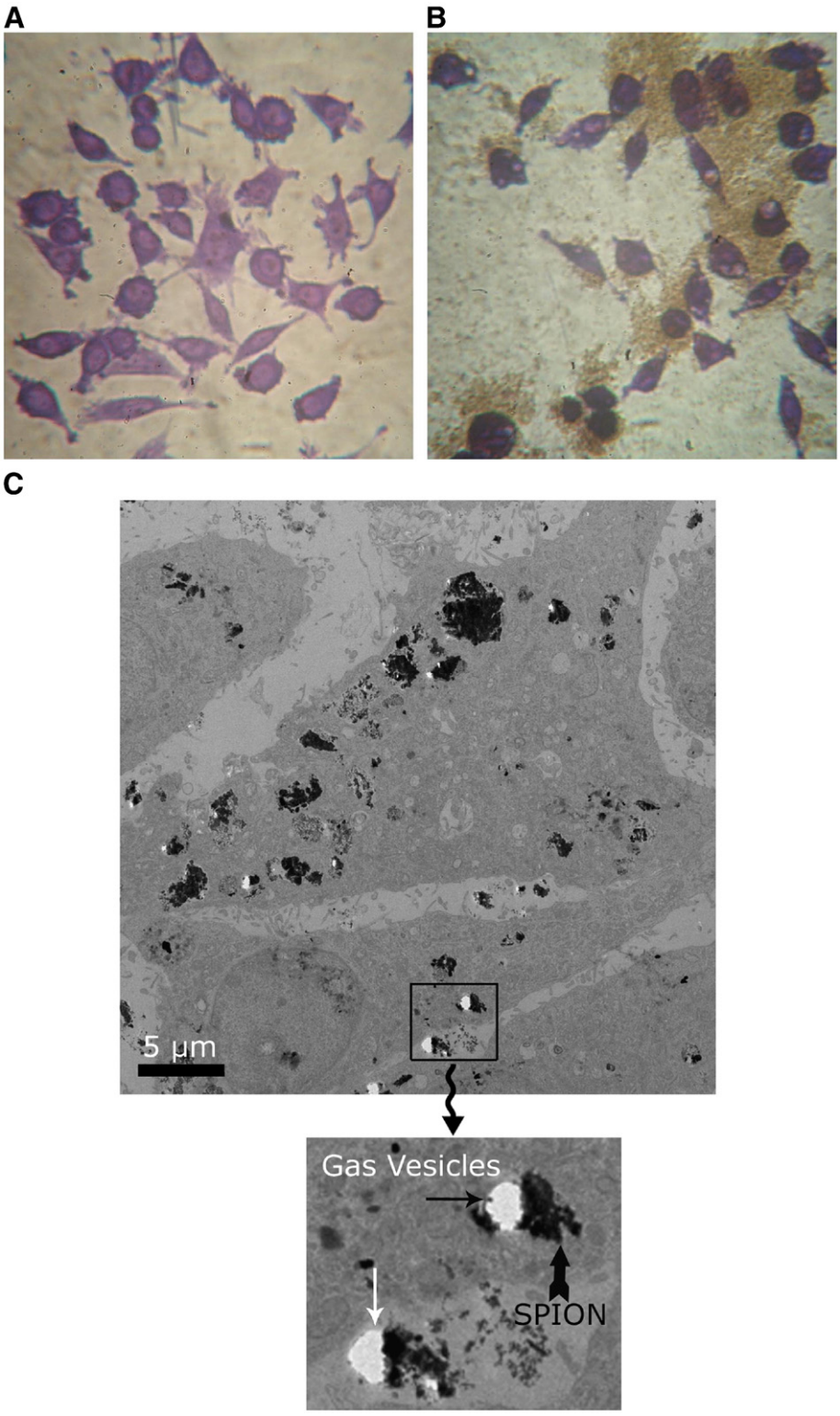


Fig. 5. Optical microscopy (800×) of dyed L929 cells for (A) control, (B) cells containing 800 mM uncoated SPIONs after a 72 h interaction with cells showing the existence of gas vesicles. (C) TEM images of SPIONs-treated cells showing gas vesicles. (Reproduced from reference [151] with permission.)

SPIONs were employed for toxicity assays. The conventional *in vitro* examination methods (such as MTT) contained large errors as compared to the modified method due to the fact that NPs can cause significant changes in the cell medium, such as denaturation of proteins, which in turn can cause cytotoxicity. Using this modified approach, the toxicity of NPs is found to decrease significantly.

3.4.2. Protein–nanoparticle interactions

The use of NPs in medicine has been intensively developed in the past decade. The understanding the interactions between these nanomaterials and proteins is very important. In a biological fluid, proteins can be adsorbed or associated on NPs. This adsorption can have significant impacts on biological, biochemical and cellular behaviour

[153,154]. More specifically, it is now well recognized that the nanoparticle–protein interaction is a key issue for defining the toxicity of NPs [153,154]. The unfavourable changes in the protein configurations, due to the SPIONs-protein interactions, should be firmly probed in order to predict biological injuries resulting from possible changes such as fibrillation, exposure to new antigenic epitopes and loss of function [153,155,156]. More specifically, the denaturation of the proteins after interaction with SPIONs could cause the exposure of new antigenic sites which may commence a new immune response [156]. Cedervall et al. has studied the specific binding rates and affinities of different plasma proteins to NPs [157]. Three methods were proposed to study these interactions: size-exclusion chromatography (SEC), surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). ITC and SEC allow studying the affinity and stoichiometry of protein bonded to particles, SPR gives the rates of protein association and dissociation. Lynch et al. [158] also tried to identify and quantify the proteins associated to different sized nanosystems.

What the biological cell, organ, or barrier actually "sees" when interacting with a NPs is highly related to the protein corona (see Fig. 6) [159]. Since there are numerous potential proteins for interaction with SPIONs in the biological environment, the major problem is finding a method that has the capability to measure such a large number of interactions. To overcome this problem, Gerber et al. [160] introduced an *in vitro* protein expression and interaction analysis platform based on a highly parallel and sensitive microfluidic affinity assay and used it for 14,792 on-chip experiments, which exhaustively measured the protein–protein interactions of 43 *Streptococcus pneumoniae* proteins in quadruplicate.

3.5. Protein and peptide functionalized SPIONs

In order to increase the targeting capability of NPs, their surfaces can be conjugated by targeting species including low-molecular weight ligands (folic acid, thiamine, and sugars), peptides (RGD, LHRD), proteins (transferrin, antibodies, and lectins), polysaccharides

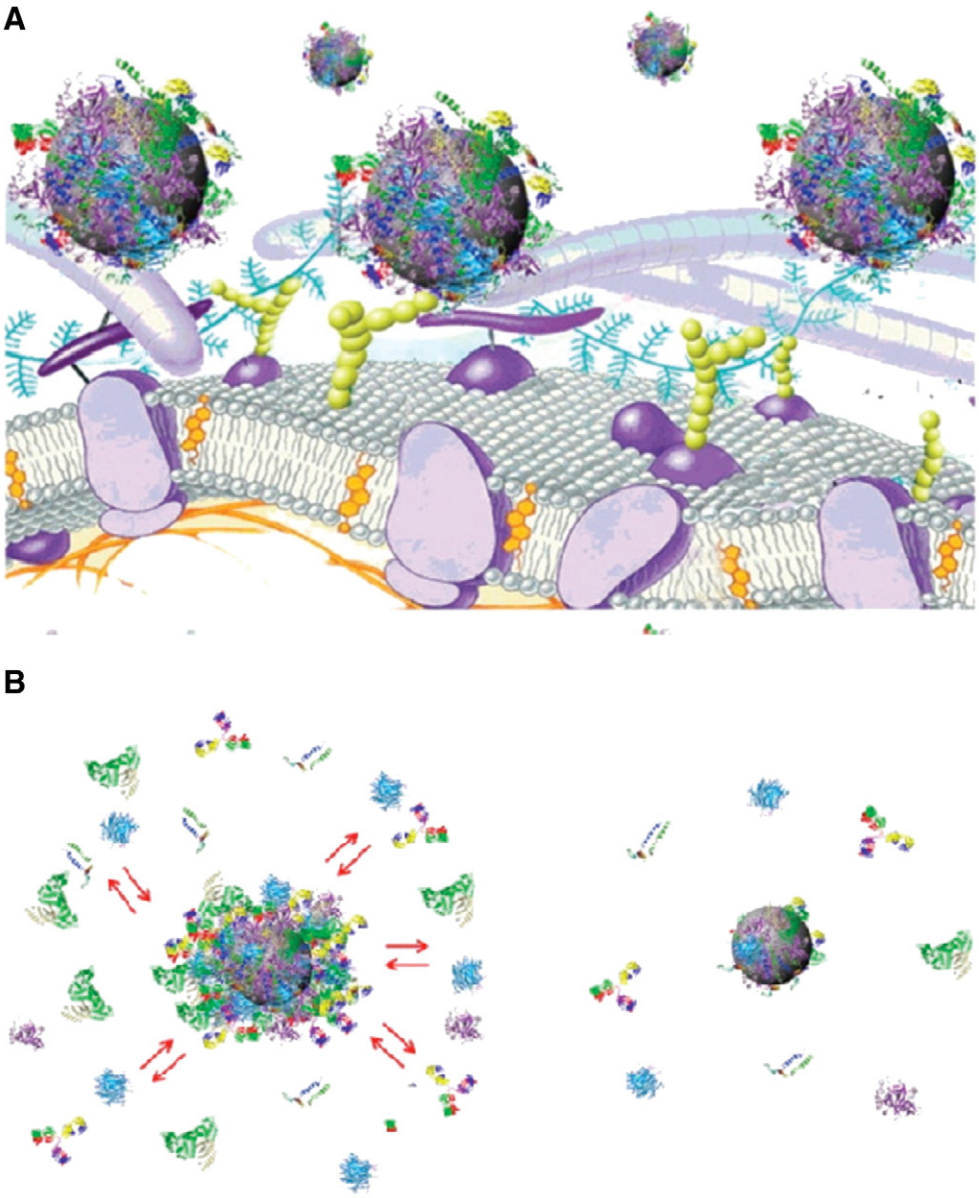


Fig. 6. (A) Schematic representation of the possible exchange/interaction scenarios at the bionanointerface at the cellular level. (B) Schematic drawing of the structure of protein–nanoparticle in blood plasma confirming the existence of various protein binding (e.g. an outer weakly interacting layer of protein (full red arrows) and a hard slowly exchanging corona of proteins (right)). (Reproduced from reference [159] with permission.)

(hyaluronic acid), polyunsaturated fatty acids, peptides, DNA, etc. [161,162] It is notable that most of the current clinically approved nanotechnology products relatively experience the passive targeting approach (i.e. lack of active targeting) according to their biophysiochemical properties (see Fig. 7) [163]. Interestingly, various molecules (e.g. peptides, peptidomimetics, proteins, and antibodies) have been investigated for *in vivo* targeting of SPIONs [164,165]. Some antibodies or fragments directed to several types of receptors (HER2/Neu, myosine, lymphocyte, selectin, V-CAM1, etc.) have been coupled to SPIONs and have been tested either *in vitro* or *in vivo* [166,167]. The development of phage display technique allowed the selection of peptides for targeting a specific target. A modified cellular ELISA (enzyme-linked immunosorbent assay), has been developed as an application of MRI for *in vitro* clinical diagnosis [168]. To validate the method, three contrast agents targeted to integrins were synthesized by grafting to ultrasmall particles of iron oxide (USPIO): (a) the CS1 (connecting segment-1) fragment of fibronectin (FN) (USPIO-g-FN); (b) the peptide GRGD (USPIO-g-GRGD); and (c) a non-peptidic RGD mimetic (USPIO-g-mimRGD). The apparent dissociation constants ($K(d)^*$) of the three contrast agents were estimated based on the MRI measurement.

Targeting of the endothelial inflammatory adhesion molecule E-selectin by MRI was successfully performed in the context of *in vitro* and *in vivo* models of inflammation [169]. The specific contrast agent was obtained by grafting a mimetic of sialyl Lewis^x (sLe^x), a natural ligand of E-selectin expressed in leukocytes, on the SPIONs. This new contrast agent, SPIONs-g-sLe^x, was tested *in vitro* on cultured HUVECs stimulated to express inflammatory adhesion molecules, and *in vivo* in a mouse model of hepatitis. USPIO-g-sLe^x is thus well suited for the MRI diagnosis of inflammation and for the *in vitro* evaluation of endothelial cell activation.

Quinti et al. conjugated synthetic phosphatidylserine binding ligands in a multivalent fashion onto magnetofluorescent nanoparticles [170]. Their results showed that the synthetic NPs bind to apoptotic cells, that there is an excellent correlation with annexin V staining by microscopy, and that FACS analysis with NPs allows the measurement of therapeutic apoptosis induction. Table 3 gives some examples of vectorized SPIONs for targeted molecular or cellular imaging.

4. SPIONs in drug delivery

The regularly employed SPIONs in drug delivery consist of NPs, nanospheres, liposomes and microspheres. In these systems, the drugs are bound to the SPIONs' surface (especially for NPs) or encapsulated in magnetic liposomes and microspheres. The recent applications of SPIONs in diagnosis and therapy are presented in Fig. 8. More specifically, SPIONs-assisted drug delivery systems have been designed to deliver peptides, DNA molecules, and chemotherapeutic, radioactive and hyperthermic drugs. The most recent delivery systems are focused on anti-infective, blood clot dissolving, anti-inflammatory, anti-arthritic, photodynamic therapy, and paralysis-inducing drugs as well as on stem cell differentiating/tracking [208,209].

The surface engineered SPIONs (e.g. with targeting ligand/molecules attached to their surfaces) used together with the aid of an external magnetic field is recognized as a modern technology to introduce particles to the desired site where the drug is released locally. Such a system has the potential to minimise the side effects and the required dosage of the drugs [161,210,211]. However, once the surface-derivatized NPs are inside the cells, the coating is likely digested leaving the bare particles exposed to other cellular components and organelles thereby potentially influencing the overall integrity of the cells [212,213]. It is hypothesized that rigid coatings such as crosslinked PEGF could postpone this shortcoming [212].

4.1. Important considerations for SPIONs in drug delivery applications

For drug delivery applications, the surface engineered iron oxide NPs are required to have superparamagnetic properties together with a specific size, which should be suitable for its delivery place and system, and a very narrow size distribution in order to have very uniform biophysicochemical properties. A recent report showed that

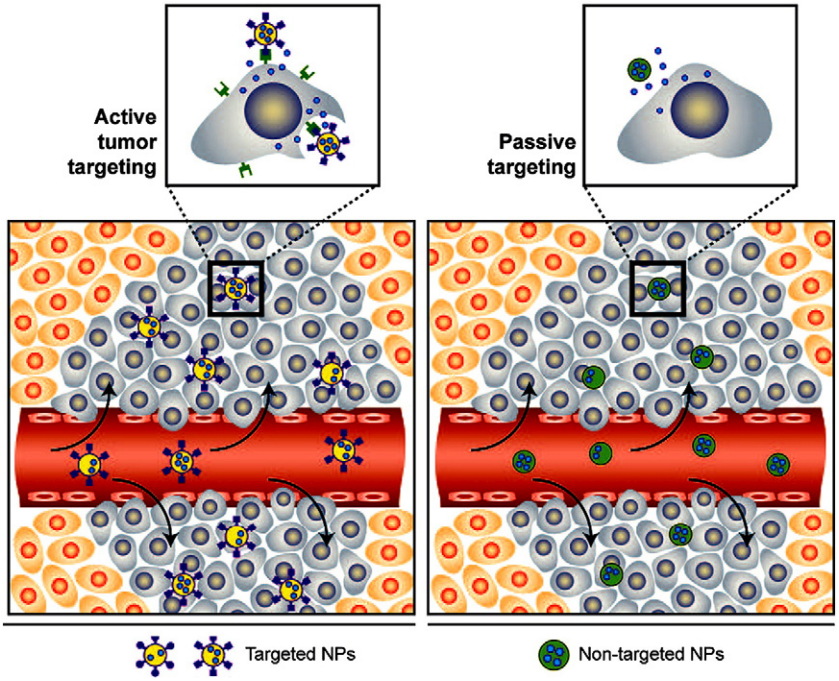


Fig. 7. Passive vs active targeting. (Right) Nanoparticles tend to passively (by their biophysiochemical properties) extravasate through the inflammated vasculature; (Left) Once nanoparticles have extravasated in the target tissue, the presence of targeting ligands (e.g. proteins) on the nanoparticle surface can result in active targeting of nanoparticles to receptors that are present on target cell or tissue resulting in enhanced accumulation and cell uptake through receptor-mediated endocytosis. (Reproduced from reference [163] with permission.)

Table 3
Targeted iron oxide nanoparticles for molecular and cellular imaging.

Targeted contrast media	Biological Target	Ref.
MION-Wheat Germ Agglutinin	Axon terminals	[171–174]
MION-mAb(L6)	Surface Antigen on human carcinoma	[175–177]
SPIONs-transferrin protein	Transferrin receptors	[178]
MION-mAb(antimyosin)	Myocardial infarction	[179]
MION-Aβ1-40	Aα-amyloid plaque	[180]
Tf-MION	Transferrin receptors	[181]
MION-Ab(anti Her2/neu)	Her2/neu receptor on tumour cells	[182]
MION succinylated polylysine	Lymph nodes	[183]
SPIONs-antibody	Neuroblastoma cancer	[184]
SPIONs-PEG-antibody	Human colon carcinoma	[185]
MION-20 CKK	Pancreatic cholecystokinin receptor	[186]
SPIONs-mAb-610	Surface Antigen on colon carcinoma cell line	[187]
SPIONs-mAb(antiEGFR)	Epidermal Growth Factor Receptor (EGFR) in esophageal squamous cell carcinoma	[188]
SPIONs-PEG-anti-CEA	Carcino embryonic antigen	[189]
SPIONs-$\alpha_v\beta_3$	Tumoral angiogenesis	[190]
SPIONs-mAb(anti-lymphocyte)	Lymphocyte	[191,192]
Tf-SS-CLIO	Transferrin Receptors	[181,193]
CLIO-F(ab′)$_2$ (anti E Selectin)	Endothelial cells	[194]
CLIO-Annexin V-Cy5.5	Apoptotic cells	[195–197]
CLIO-Cy5.5-mAb(antiVCAM-1)	Vascular cell adhesion molecule 1 VCAM-1	[198]
CLIO-Cy5.5-bombesin	Pancreatic ductal adenocarcinoma	[199]
CLIO-Cy5.5	Delineation of brain tumour	[200]
SPIONs-fibronectin fragment	Integrin	[168]
Anti Her2 Neu-WSIO	Her2/neu receptors on tumour cells	[201,202]
SPIONs annexin V	Apoptotic red blood cells	[203–206]
SPIONs herceptin	Her2neu positive cancer cells	[207]

the particle size distribution can have a considerable effect on the hysteresis losses of the magnetic field amplitude [214]. Furthermore, a wide particle size distribution would result in heterogeneous colloidal properties due to the wide range of blocking temperatures [215].

In addition to particle size and its distribution, the magnetic properties are strongly related to impurity content or structural imperfections of the particles, the polymer type and the length of adsorbed/expressed polymeric shell. Furthermore, the concentrations

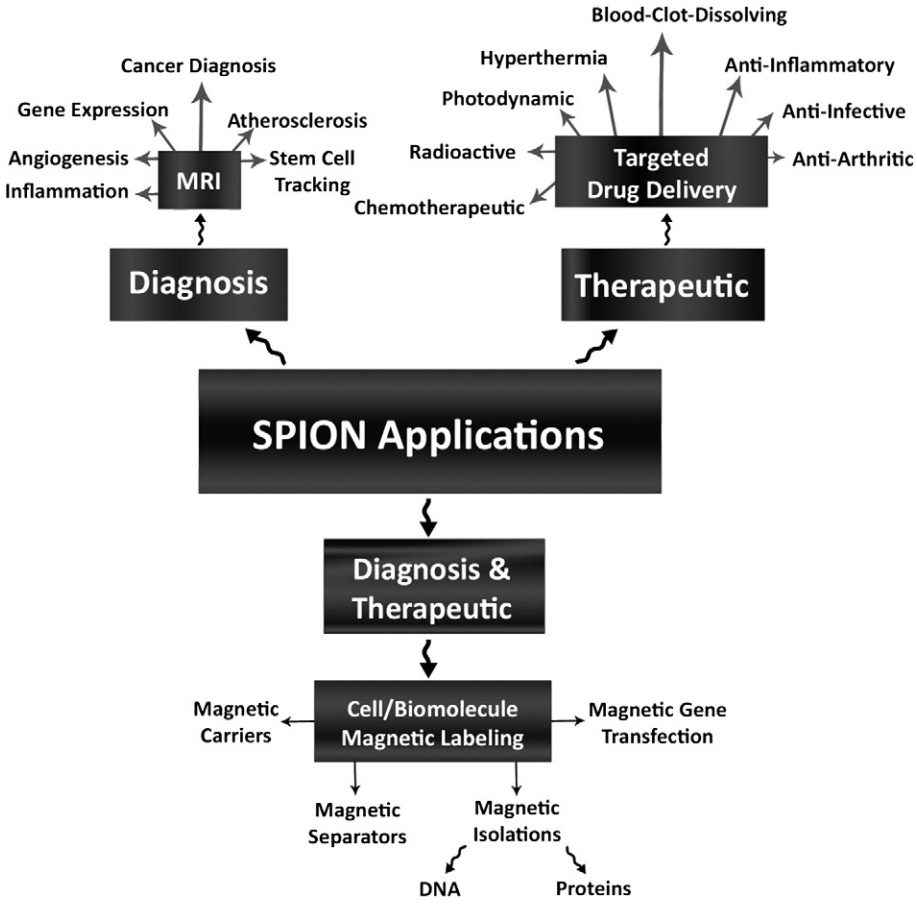


Fig. 8. Recent diagnostic and therapeutic applications of SPIONs.

of SPIONs in colloids are being recognized as having crucial importance. By increasing the concentration of magnetic NPs, a clustering of the particles may occur, leading to magnetic interactions and having a significant effect on the net magnetization.

Similarly, careful consideration should be given to SPIONs with different sizes and shapes as they will experience different fluid environments during their movement through the systemic circulation and will behave differently due to the effect of viscous force rather than inertial force [216]. More particularly, as they move through narrow capillaries, the agglomeration of some particles may occur leading to clogging (embolizations) of small blood vessels [217].

The stabilisation of SPIONs in biological suspension is a critical matter in order to improve their function as drug carriers. The isoelectric point of SPIONs is around pH 7 [218,219], which is same as that for biological fluids; hence, the colloidal stability of SPIONs in such surrounding environments is recognized as a major shortcoming [220,221]. The coatings (Table 2) could change the isoelectric points, and consequently the blood circulation half-life of SPIONs could increase significantly. In addition to the isoelectric point, there are many other crucial parameters that affect the colloidal stability and magnetic field of drug-loaded SPIONs. The mentioned parameters are particle size, size distribution, shape, surface characteristics of the particle, concentration and volume of the SPIONs, reversibility and strength of the drug/ferrofluid binding (desorption characteristics), access to the organism (infusion route), duration/rate of the injection/infusion, geometry and strength of the magnetic field and duration of magnetic field application [222]. In addition, patient-related physiological parameters such as size, weight, body surface, blood volume, cardiac output and systemic vascular resistance, as well as tumour-related parameters such as circulation time, tumour volume and location, vascular content of tumour and blood flow in tumour are also important [222] in anticancer therapy.

It has been recognized that the internalization of particles as well as uptake by specific cells is necessary for their targeting. This depends strongly on the size, shape and surface properties of the magnetic NPs [45,137,213,223]. A multi-physics finite element model (FEM) has been employed to study the effects of an applied external magnetic field on PVA-coated SPIONs (with different purity, shapes and sizes [85]) in a simulated large blood vessel (1 cm in diameter) [133]. The FEM model iteratively solved coupled Maxwell and Navier–Stokes equations to predict both the induced magnetic flux density and the fluid velocity fields. The characteristics of SPIONs (i.e. size, size distribution, purity and shape) showed some interactions in defining the shape and amplitude of the velocity field. Furthermore, it was hypothesized that increased external magnetic field gives rise to a more turbulent flow. The results also confirmed that a velocity field with less fluctuation and higher amplitude, especially in the direction normal to tissue, could be recognized as a preferable vector to facilitate drug delivery. By increasing the magnetic properties of the drug carriers, the yield of the targeting could be significantly increased.

Engineered fluorescent SPIONs have shown great potential for drug delivery and imaging of brain-derived structures. Cengelli et al. [224] synthesized fluorescent SPIONs coated with PVA functionalised with a fluorescent reporter molecule and administered to a microglial cell culture containing immune cells of the nervous system. The results confirmed good biocompatibility and strong intracellular uptake of the engineered SPIONs. Consequently, the mentioned SPIONs have been envisaged as potential vector systems for drug delivery to the brain, which may be combined with MRI detection of active lesions in neurodegenerative diseases.

4.2. Drug release properties of SPIONs

Once accumulated inside the required tissue/cells, SPIONs, as a drug delivery system, should be able to release their drug payload at an optimal rate. However, it is observed that a majority of the drug payload is quickly released upon injection into the *in vivo* environment (i.e. burst effect), since the drug is loaded on the surface of SPIONs. Consequently, very small (inadequate) amounts of the drug reach the specific site after, for example, magnetic drug targeting. In order to reduce the burst effect, Mahmoudi et al. [212] prepared iron oxide NPs with a crosslinked poly (ethylene glycol)-co-fumarate (PEGF) coating. To investigate if the coating could reduce the burst effect, tamoxifen (i.e. an anti-oestrogen drug used to treat breast cancer) was loaded onto the surface of coated NPs (*via* hydrogel properties of PEGF). The results confirmed that the cross-linked PEGF coating reduced the burst release by 21% in comparison with the non cross-linked tamoxifen loaded particles. In another study, monodisperse SPIONs with a mesoporous structure were prepared via simple solvothermal method by Guo et al. [225]. A typical anticancer drug, (Doxorubicin, Dox), was used for drug loading. The release behaviours of Dox indicated that these SPIONs had a high drug loading capacity and favourable release kinetics for this drug.

In another report, phospholipid vesicles incorporating magnetic NPs (magnetic liposomes) were filled with drugs and used for targeted delivery applications [226,227]. The magnetic microspheres, which are formed from encapsulation of SPIONs (with core size of 5–15 nm) in biocompatible, non-toxic (FDA approved) and biodegradable polymeric microspheres, such as PLGA and poly(L- or DL-lactide) (PLA), are recognized as another promising drug carrier. The major advantage of magnetic microcarriers as compared with nanocarriers is their lower burst effect. It is noteworthy that the first magnetic microspheres were designed for localised radiation therapy [228] as well as antiangiogenic therapy [229,230]. The homogeneity of SPIONs throughout the particle matrix is a distinguished feature of the application of microspheres for intravascular administration [123]. The achieved microspheres must be smaller than red blood cells and delivered through blood vessels without clogging the smallest capillaries of 7–8 μm in diameter. In order to increase the chance of targeting, the microparticles should also have a very narrow size distribution.

It is worth noting that the mentioned magnetic drug delivery systems follow similar rules as other pharmaceutical drug delivery strategies. More specifically, the NPs or magnetic microspheres, magnetically driven to the desired site, not only should release a chemotherapeutic drug in order to eradicate the tumour, but should also undergo the same firm rules with respect to sterility, non-immunogenicity, and non-toxicity as any other enveloping drug delivery systems.

4.3. Examples of SPIONs for chemotherapy

The first application of magnetic drug delivery systems was developed by Widder et al. [231]. According to their report, doxorubicin (Dox) was used as drug and encapsulated in albumin magnetic NPs. In another study, Dox was loaded to the magnetite NPs which were embedded in PLGA through hydrophobic interaction [232]. An antibody was conjugated to the drug and the obtained SPIONs were used for simultaneous diagnosis and treatment of cancer. Results showed that Dox encapsulated in polymeric NPs released sustainably without any inhibition due to the presence of magnetic nanocapsules. The SPIONs have been injected not only in mice and rats, but also in 14 patients (through intravenous injection very close to the tumour site) for targeting an anticancer drug to locally advanced tumours [233,234]. Nine patients received two treatment courses, three patients received one course, and the two received three courses of drug-loaded magnetic NPs. It is noteworthy that the second planned treatment was inhibited for one case; in contrast a third treatment was used for two cases due to an episode of chills 130 min after infusion of the magnetic drug, in one case, and good responses after the first two therapies, in the other. Epirubicin, which was chemically attached to the surface of magnetic NPs, was used as

drug with a dose of 5–100 mg/m^2. The same dose of epirubicin, which was not bonded to magnetic fluid, was injected systemically 3 weeks after drug targeting for intra-individual comparisons. Results confirmed that epirubicin-loaded magnetic NPs were well tolerated. According to the results obtained by magnetic resonance tomographic techniques, pharmacokinetics and the histological detection of magnetites, the authors claimed that the ferrofluid could be successfully directed to the tumours in about one-half of the patients. Furthermore, organ toxicity did not increase with the treatment; however, epirubicin-associated toxicity appeared at doses greater than 50 mg/m^2.

In order to increase the yield of magnetic targeting, Widder et al. [235,236] employed an intra-arterial injection proximal to the tumour site (Dox filled magnetic particles). The results confirmed the 200 times more targeting yield in comparison with an intravenous injection [237]. Since this study was published, success in cytotoxic drug delivery and tumour remission has been reported by several groups using animal models including swine [238,239], rabbits [7] and rats [240–242]. Kubo et al. [243] recently offered a variation on these techniques. They implanted permanent magnets at solid osteosarcoma sites in hamsters and delivered the cytotoxic compounds via magnetic liposomes. This method resulted in a four-fold increase in cytotoxic drug delivery to the osteosarcoma sites when compared with normal intravenous (non-magnetic) delivery [243]. Results also showed a significant increase in anti-tumour activity and the elimination of weight-loss as a side effect [226]. This technique has been employed also to target cytotoxic drugs to brain tumours. These tumours are particularly difficult targets due to the fact that the drug must cross the blood-brain barrier. Pulfer and Gallo [242] demonstrated that particles as large as 1–2 μm could be concentrated at the site of intracerebral rat glioma-2 (RG-2) tumours. Though the concentration of the particles in the tumour was low, it was significantly higher than that of non-magnetic particles. In a later study, the group demonstrated that 10–20 nm magnetic particles were even more effective at targeting these tumours in rats [242]. Electron microscopic analysis of brain tissue samples revealed the presence of magnetic carriers in the interstitial space in tumours, but in normal brain tissue they were only found in the vasculature. Mykhaylyk et al. [175] recently had less success using magnetite-dextran NPs but were able to target rat glial tumours by disrupting the blood-brain barrier immediately prior to particle injection.

Preliminary successful animal trials, where for the first time documented tolerance and efficacy was observed in mice and rats and in which no LD50 could be found for the ferrofluids, has lead to human trials. The treatment protocol consisted of an intravenous infusion of the chemically bound drug and one course of conventional chemotherapy. During infusion, and for 45 min after, a magnetic field was built up as close as possible to the advanced and unsuccessfully pretreated tumour (distance assured to be less than 0.5 cm). It was shown that the ferrofluid could be successfully directed to the tumours in about half of the patients. However, it was also concluded, based on MRI techniques, pharmacokinetics and clinical detection that although the treatment seemed safe, improvements were needed to make it more effective [210]. FeRx Inc. introduced magnetic NPs (metallic Fe coated with activated carbon) which carried doxorubicin as drug [239] and was later granted fast-track status to proceed with multi-centre Phases I and II clinical trials of their magnetic targeting system for hepatocellular carcinomas (a type of liver tumour). However, in April 2004, FeRx halted its clinical trial, putting into doubt its ability to continue as a going concern.

In order to probe the yield of a magnetically controlled drug targeting mechanism on the tissue at microcirculatory level, the biological effects, bioavailability and the *in vivo* desorption time of the anticancer drug epirubicin were studied for starch derivates at the surface of SPIONs [222]. It was found that microcirculatory techniques can help in identifying the efficacy of magnetically controlled drug targeting [222]. The technique allowed the precise quantification of the SPIONs in the microcirculation of the target tissue upon intravenous injection. Magnetic particles could be retained within the microvessels of normal tissue (skeletal muscle), while there was extravasation of the SPIONs into the tumour's interstitial space. Alexiou et al. [7] treated squamous cell carcinoma in rabbits with SPIONs bound to mitoxantrone that was concentrated with a magnetic field. Experimental VX-2 squamous cell carcinoma was implanted in the median portion of the hind limb of New Zealand White rabbits. When the tumour had reached a volume of ~3500 mm^3, the SPIONs–mitoxantrone was injected intra-arterially from the femoral artery or ear vein. Furthermore, an external magnetic field was focused on the tumour. The intratumoural accumulation of SPIONs was visualized both histologically and by magnetic resonance imaging. The cross-section of the tumour was prepared just after treatment with magnetic NPs. Results confirmed that the intraluminal SPIONs were accumulated and deposited on the endothelium nearest to the magnetic field and were separated from the erythrocyte pool; however, SPIONs were also detected in the tumour interstitium as well as in the adjacent surrounding tissues. The same histological analyses were performed after 3 months. According to the results, only fibrosis was seen at the tumour implantation site. No metastases were found in the regional lymph nodes or in any other organs. Traces of SPIONs were detected in the spleen of the animals, but none were found in the liver, lungs, or brain or at the implantation site and surrounding musculature and skin. Furthermore, investigations of other organs confirmed that there were no histological or macroscopic pathological changes. The authors proposed that the application of SPIONs–mitoxantrone was successful in treating experimental squamous cell carcinoma and offered a unique opportunity to treat malignant tumours locally without systemic toxicity. In another study, lactide-co-glycolide ethylene oxide fumarate was used as a new coating material for SPIONs in order to either decrease the side effects of paclitaxel (a mitotic inhibitor used in cancer chemotherapy) and/or to increase the circulation time of SPIONs and target the drug to tumour vasculature [62]. Khurshid et al. [244] designed a potential drug delivery system by combining low-molecular-weight heparin to poly-L-lysine-coated iron oxide magnetic NPs with an average size of 20 nm. The data provided direct evidence that the heparin was immobilized on the surface of poly-L-lysine-coated iron oxide NPs. However, this study did not further investigate the effect of heparin coatings on drug release.

Butoescu et al. [245] synthesized magnetic biodegradable microparticles (10 μm in size) containing dexamethasone acetate (DXM) for intra-articular administration. The SPIONs in the microparticles had a homogeneous distribution, which is an important factor for preserving superparamagnetic properties. The results showed that DXM release profiles were quite similar *in vitro* (ca. 6 days) and *in vivo*, using a mouse dorsal air pouch model (ca. 5 days). Moreover, the anti-inflammatory effect of DXM-containing microparticles was more important than that of blank microparticles or microparticles containing only SPIONs [245]. The presence of a magnet did not induce a greater inflammatory reaction in comparison with a no-magnet situation.

Since most of polymeric coatings in SPIONs are selected from hydrogel categories, the drug release from these hydrogel shells could be engineered by controlling their physical and chemical properties. Permeability, temperature sensitivity, pH sensitivity, osmolarity sensitivity, surface functionality, swelling, biodegradability, and surface biorecognition sites are recognized as major mechanisms for controlled drug release applications of hydrogels [246]. For instance, by using the thermal sensitivity of hydrogels on the surface of multifunctional SPIONs (e.g. for simultaneous imaging, hyperthermia and drug delivery applications), the release of chemotherapeutic drugs (examined in both *in vitro* and *in vivo*) can be controlled by local heating using an alternating current magnetic or electromagnetic field with an approximate frequency of 1 MHz [247,248]. Magnetoliposomes, which were made by magnetic oxide NPs with a diameter of 8 nm, released the drug upon magnetic field irradiation [247]. The

Dox released were strongly dependent on the colloidal concentration of NPs in the magnetoliposomes and the length of the exposure to the alternating magnetic field. Since the magnetoliposomal membrane has a phase transition temperature of 42 °C, the authors claimed that the release of drug is related to the local heating of the liposomal membrane.

Table 4 gives some examples of various SPIONs containing drug delivery systems that are currently being investigated.

4.4. Radio-labelled magnetic drug delivery systems

The attachment of radiotracers to SPIONs has been recognized as a promising approach to probe the fate of SPIONs *in vivo*. Another advantage of this method is its high local dose to enhance tumour cell eradication, in contrast to external radiotherapy covering the general body, and thus an exposure limited to a low dosage requiring a longer treatment with duration of six weeks. Gallium-67 labelled SPIONs were prepared in order to track the biodistribution (1 and 24 h after injection) of NPs with a size of 5 nm in normal rats [18]. Results confirmed that the labelled uncoated SPIONs were accumulated mostly in the RES, especially in liver, lung and spleen. There are other reports on nanoradiotracers used in order to track the fate of SPIONs in the body and for treatment purposes, such as ^{64}Cu-radiolabelled folate-NPs [259], ^{99m}Tc-nanoliposomes [260] and ^{99m}Tc-SPIONs [260]. Hafeli [261] employed various radioisotopes to study different treatment ranges. According to their results, the β emitter ^{90}Y has the ability to irradiate up to a range of 12 mm in tissue whereas ^{131}I can irradiate only up to 2.4 mm.

To track the labelling efficiency, microspheres can be conjugated with radiotracers. PLA microspheres (coated magnetites that are hemocompatible and produce no hemolysis [123]) tailored with a tridentate chelating group were radiolabelled with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ [262]. Labelling efficiencies over 95% were achieved in a 5 min reaction using 100% of the ligand–polymer or within 15 min using a 5% ligand–polymer blend. The addition of 1.5% of PEGylated co-polymer to the blend did not affect the labelling efficiency; however, a significant change in its *in vivo* behaviour was detected. MicroSPECT/CT imaging proved the uptake of non-PEGylated microspheres by the murine lung, but only the liver and spleen took up PEGylated microspheres. The authors proposed that ^{99m}Tc radiolabelled biodegradable microspheres could be useful diagnostic imaging agents for visualisation of the functioning RES system. Furthermore, other sizes of the same microspheres can allow imaging of lung perfusion and bone marrow, as well as lymph and inflammation scintigraphy and radioembolization therapy.

4.5. Limitations of SPIONs for drug delivery

4.5.1. Restriction and progress in magnets

The major limitation of SPIONs for drug delivery applications is the inadequate magnetic gradient (due to the distance between magnet and targeted site) in order to control the residency time of NPs at the targeted site. Preliminary investigations of the hydrodynamics of drug targeting suggest that for most magnetite-based carriers, flux densities at the target site must be of the order of 0.2 T with field gradients of approximately 8 T m^{-1} for femoral arteries and greater than 100 T m^{-1} for carotid arteries [263]. This suggests that targeting is likely to be most effective in regions of slower blood flow, particularly if the target site is closer to the magnet source. Richardson et al. [264] have recently developed mathematical models to determine particle trajectories for a variety of field/particle configurations in two dimensions, including consideration of their motion as they approach the vessel wall. It should be indicated that the particle's motion is no longer governed by Stoke's law for the drag force when it comes near the blood wall, and the hydrodynamic parameters are modified. Using a simple network model which can describe the deposition of magnetic particles in a hierarchy of vessels, it was observed that the orientation of the vessels with respect to the magnetic force crucially affects particle deposition rates leading to heterogeneous particle distributions. Two-dimensional computational simulations of magnetic particle motion in the carotid artery bifurcation have previously been performed [265,266]. They showed that it was not possible to obtain a maximum magnetic force (on a magnetic particle) inside the body using an externally applied magnetic field. Since drug targeting is effected by pulling magnetic particles to the edge of vessels, this suggests that it will not be possible to target interior regions of the body without targeting some of the surrounding regions of the body more strongly. This leads to

Table 4
Various SPIONs-assisted drug delivery systems.

Coating	Delivery system	Drug tested	Examination route	Ref.
Carbon	Aqueous media	Carminomycin and rubomycin	*In vivo* (Rat) Injected into the tail vein	[161]
Anhydroglucose	Aqueous media	Epirubicin	*In vivo* (Human) Intravenous injection	[234]
Phosphated starch	Aqueous media	Mitoxantrone	*In vivo* (Mice) The magnetic NPs were injected either into the femoral artery close to the tumour or intravenously	[245]
Poly(lactic-*co*-glycolic acid)	Microspheres and microcapsules	Dexamethasone acetate	*In vitro* and *In vivo* (intra-articular)	[249]
Amphiphilic block copolymers of maleimide-terminated poly(ethyleneglycol)-block-poly(D,L-lactide) and methoxy-terminated poly-(ethylene glycol)-block-poly(D,L-lactide)	Multifunctional polymeric micelles	Doxorubicin	*In vitro*	[250]
Albumin	Microspheres	Doxorubicin	*In vitro*	[231]
Polyethylene glycol fumarate	Aqueous media	Tammoxifen	*In vitro*	[212]
Oleic acid	Aqueous media	Doxorubicin and paclitaxel	*In vitro*	[251]
Oleic acid–Pluronic	Aqueous media	Doxorubicin	*In vitro*	[252]
Poly(acrylic acid)	Aqueous media	Taxol	*In vitro*	[253]
None	Porous hollow nps	Cisplatin	*In vitro*	[254]
Chitosan, O-carboxymethylchitosan and (N-succinyl-O-carboxymethylchitosan	Aqueous media	Camptothecin	*In vitro*	[255]
Poly(lactic acid) and poly(lactic-*co*-glycolic acid)	Microspheres	Interferon alpha-2b	–	[256]
Silica	Hollow mesoporous sphere	Ibuprofen	*In vitro*	[257]
Cross-linked chitosan	Microspheres	Aspirin	*In vitro*	[258]

conjecture that the use of magnetically targeted drug delivery with an externally applied field is appropriate only for targets close to the surface of the body [267].

Recently, the use of superconducting magnets has been considered for magnetic drug targeting [268–271]. A novel navigation system has been made by applying a strong external (magnetic) field through SmBaCuO and YBaCuO bulk superconductors. The results demonstrated that with this system ferromagnetic particles can accumulate inside a flow system up to at least 20 mm from the magnet [271]. Some investigations have used permanent Nd–Fe–B (neodymium–iron–boron) magnets in combination with SPIONs and found excellent magnetic properties and increased depth of the magnetic field up to 10–15 cm [161,162].

With the aim of increasing the yield of magnetic drug delivery, the use of the magnetic implants has also been considered in both computational [272,273] and experimental approaches [274,275]. In order to target locations further below the skin, the use of magnetic stents [276,277] and magnetic implants [278–280] have been investigated. This implant-based drug delivery system functions by placement of a weakly magnetisable stent or implant at precise locations in the cardiovascular system, followed by the delivery of magnetically susceptible drug carriers. The stents are capable of applying high local magnetic field gradients within the body, while only exposing the body to a modest external field. The local gradients created within the blood vessel create the forces needed to attract and hold drug-containing magnetic NPs at the implant site [277]. Theoretical simulations and experimental results support the assumption that using magnetic implants in combination with an externally applied magnetic field will optimize the delivery of a magnetic drug to selected sites [274].

4.5.2. Brain delivery

Results have confirmed the low efficacy of SPIONs for brain targeted imaging and drug delivery applications, due to their restrictions in crossing the blood-brain barrier (BBB) [213]. The BBB, which has as a role isolating the brain tissue with special endothelial cells, has the potential to prohibit the entrance of therapeutic compounds (e.g. for treatment of neurological or psychiatric disorders) to the brain [281]. One alternative is the direct injection of SPIONs to the desired part of the brain tissue by disruption of the BBB; however, this method may suffer from unpredictable and high risks for patients [175–177]. In one study, SPIONs with a size of 10–20 nm were taken up by blood tumour barrier and their ability to target rat glioma tumours was analysed [242]. It is worth noting that a magnetic field of 6000 G was applied to the brain in order to increase the targeting efficacy. After desired times, the rats were sacrificed and the brain tissue was analysed for iron contents. The results confirmed significant differences between the SPIONs-targeted tumour tissues and normal ones (i.e. a 2–21 fold increase in concentration 0.5 and 6 h after injection of SPIONs, respectively). It is interesting to note that the total concentration of SPIONs in normal brain tissue was low.

5. Conclusions and future perspective

The major focus of current research on SPIONs' synthesis is finding new methods or improving the conventional ones in order to obtain reliable/reproducible SPIONs with optimum surface charge, shape, size, colloidal stability in a biological environment, biocompatibility and saturation magnetization. An important issue needs to be considered when selecting SPIONs for drug delivery: the fate of the SPIONs after the drug delivery, i.e. elimination route or retention time

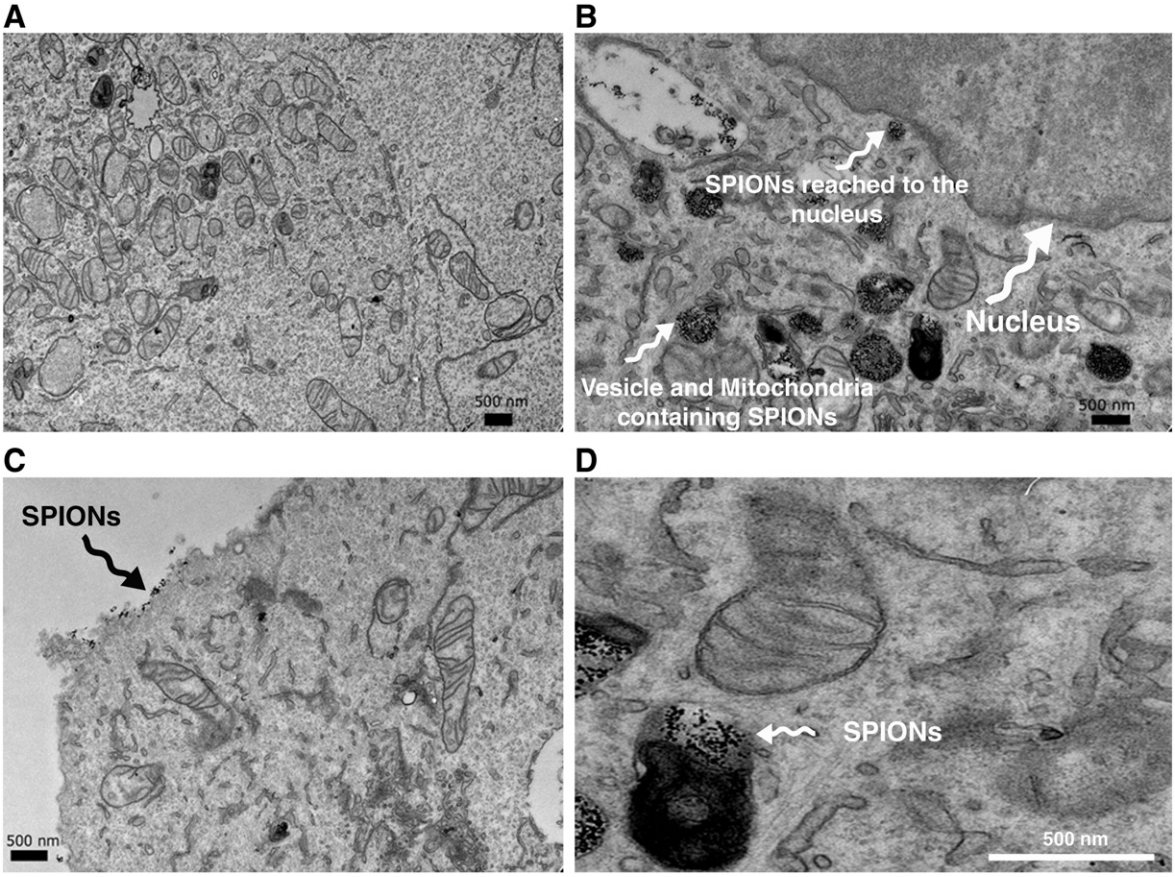


Fig. 9. TEM images of HeLa cells; (A) control and exposed to (B) positively, (C) negatively charged SPIONs and (D) the internalization of magnetite nanoparticles inside of mitochondria (Unpublished work by M. Mahmoudi).

in the body system if they are biodegradable and the relevant side effects. For example, silica-coated SPIONs could be biocompatible; however, if the iron oxide core is exposed, it can cause an oxidative stress which could be associated with neurological disorders. Similarly poly(methyl methacrylate) is biocompatible but its biodegradable products, such as methaacrylate monomer, could be reactive and toxic. The selection of SPIONs for specific drug loading should be carefully judged based on how the drug and shell materials complement each other; otherwise a burst effect could produce toxic chemicals by combination of drug and shell materials.

A comprehensive understanding of the various interactions of SPIONs with biomolecules in the body system (i.e. protein–nanoparticle interactions) would lead to novel SPIONs with optimum surface properties that could overcome the problem of "rejection of magnetic NPs by the human body", "biocompatibility" and "toxicity" in the near future. For example, the efficacy of magnetic NPs as well as their biomedical fate within cells is highly dependent on their surface charges. In this review, we have shown that positively charged SPIONs (e.g. with PVA-NH_2^+ coating) are capable of acting as drug carriers to the nucleus by escaping from lysosomes (see Fig. 9). In contrast, negatively charged SPIONs (e.g. with PVA-COO^- coating) can be attached to cell membranes and, consequently, cell membrane disorders can be targeted for delivery of drugs. Hence, the use of SPIONs with a tuneable surface charge will be of great interest for applications in the human body. For instance, in order to increase the efficacy of magneto-transfections and nuclear drug delivery, the net charge of injected primary NPs should be negative (to facilitate escape from the RES). After reaching the targeted cell membrane, the charge of particles is changed to positive *via* interactions with the membrane and its local environment, so the nucleus can be easily targeted.

An ultimate goal of using SPIONs in biomedicine is to reduce patient suffering by applying selective treatments where efficiency is increased through local concentrations, while general side effects are avoided; in addition, the metastasis of cancer cells will be limited. Although, for the most part, SPIONs are currently researched in an experimental scale (except for FDA-approved products for human injections as MRI contrast agents such as Endorem™, Feridex®, and Resovist®), there are a number of sophisticated technologies developed for synthesis, coating and functionalisation of multifunctional SPIONs. The finding of a trace of SPIONs in mitochondria (Fig. 9D) leads to great hope among researchers in the field for the possibility to treat in the near future mitochondrial disorders and cardiac dysfunctions by using drug-loaded particles. Finally, given their mitochondrial role, it is possible that one day some "intelligent SPIONs" could be designed to control the aging process. In addition to drug delivery, the use of multifunctional SPIONs with the potential for simultaneous imaging, hyperthermia, stem cell tracking and gene delivery is going to dramatically enhance the coincident diagnosis and local therapeutic applications.

In order to increase the targeting efficacy of SPIONs to the brain tissue, it may be useful to examine the use of the promising polymers (e.g. n-hexadecylcyanoacrylate and polyethylene glycol), which showed very good potential for crossing the BBB, as coating materials. This enhancement in efficiency would be very useful for transferring drugs (e.g. anti-depressants) into the deep areas of the brain, such as the striatum, hippocampus, and hypothalamus.

The burst effect is the main problem of employing SPIONs for drug delivery purposes. The use of highly sophisticated surface engineering on stable SPIONs in the physiological environment is needed in order to control this burst effect. Cross-linkable polymers are very promising candidates for these purposes and should be considered for future research.

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