Chapter 12

Preclinical Aspects on Magnetic Iron Oxide Nanoparticles and Their Interventions as Anticancer Agents: Enucleation, Apoptosis and Other Mechanism

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Abstract

The broad area of magnetic iron oxide nanoparticle (M-IONP) applications and their exclusive physico-chemical characteristics (superparamagnetic properties *per se*, solubility and stability in aqueous solutions, and high bioavailability *in vivo*) make these nanoparticles suitable candidates for biomedical uses. The most employed magnetic iron oxides in the biomedical field are magnetite and maghemite. Cancer represents a complex pathology that implies multiple mechanisms and signaling pathways, this complexity being responsible for the increased resistance to therapy and the lack of an effective curative treatment. A potential useful alternative was considered to be the use of magnetic iron nanoparticles. The M-IONPs proved to be effective as contrast agents in magnetic resonance imaging, as drug delivery carriers for different therapeutic agents, in magnetic cell separation assays, and are suitable to be engineered in terms of size, targeted delivery and substance release. Moreover, their *in vivo* administration was considered safe, and recent studies indicated their efficiency as anticancer agents. This chapter aims to furnish an overview regarding the physico-chemical properties of M-IONPs (mainly magnetite, maghemite and hematite), the synthesis methods and their *in vitro* biological impact on healthy and cancer cell lines, by describing their potential mechanism of action—enucleation, apoptosis or other mechanisms.

Keywords: magnetic iron oxide nanoparticles (M-IONPs), cancer, enucleation, apoptosis, magnetite, maghemite, combustion method

1. Introduction

Due to the wide potential of applications in various fields, such as biotechnology, biomedicine, magnetic fluids, catalysis, magnetic data recording and storage media, magnetic resonance imaging, magnetic fluid hyperthermia, magnetic drug delivery, cell separation, magnetic paper and more recently in environmental protection, magnetic iron oxide nanoparticles (M-IONPs) are the main components of the modern technology [1–15]. In nature, many forms of iron oxides are found, but the most technologically used are the magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3) and hematite (α-Fe_2O_3) (**Figure 1**).

Magnetite (Fe_3O_4), a natural mineral known as the black iron oxide, is relatively stable at room temperature, very quickly transforms in maghemite and shows the strongest magnetism compared to other transition metal oxides [16]. Fe_3O_4 has an inverse spinel structure with all the Fe^{2+} ions and half of the Fe^{3+} ions distributed in the octahedral sites and the other half of the Fe^{3+} ions distributed in the tetrahedral sites being surrounded by four oxygen atoms [17].

Spin magnetic moments of Fe^{3+} ions distributed in octahedral positions are parallely aligned, as well as those of Fe^{3+} ions distributed in tetrahedral positions but in the opposite direction, leading to an antiparallel coupling. Therefore, the spin moments of all Fe^{3+} ions mutually cancel out and do not contribute to the net magnetization of magnetite (**Figure 2**). All Fe^{2+} ions have magnetic moments aligned in the same direction so that their total magnetic moment is responsible for the net magnetization of magnetite. Therefore, the saturation magnetization of magnetite corresponds to the product between the spin magnetic moment of each Fe^{2+} ion and the number of Fe^{2+} ions, which corresponds to the mutual alignment of all Fe^{2+} ions in magnetite.

Magnetite is oxidized in the presence of air to maghemite, which is also ferrimagnetic, but has a slightly lower magnetic response. This process is called maghemitization and occurs at the surface of the crystals. Crystal centers are also oxidized, and the process is being carried out by diffusion of Fe^{2+} ions from inside to the surface of the crystals, where they are converted to Fe^{3+}. The rate of the oxidation process is determined by the diffusion rate of Fe^{2+} ions and the distance to the surface. Therefore, the particles remain unaffected by the phenomenon of maghemitization, while the small ones are susceptible to oxidation even at room temperature.

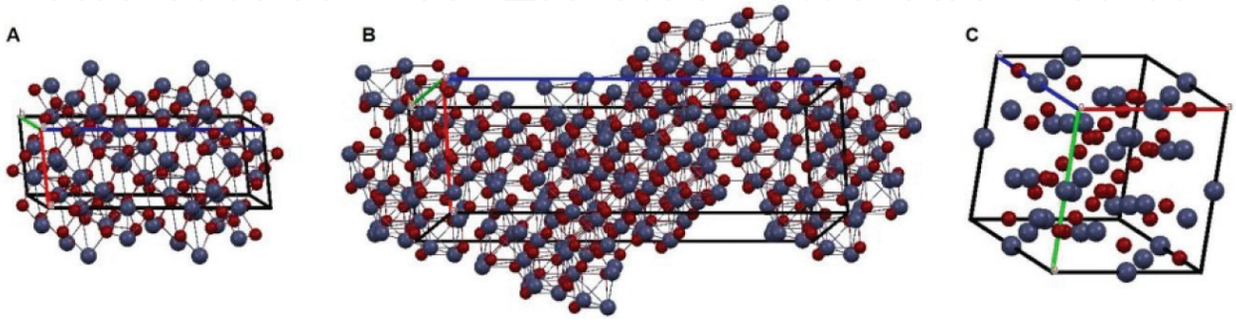

Figure 1. Crystal structure of: A—hematite, B—maghemite and C—magnetite (the blue sphere is Fe^{2+}/Fe^{3+} and the red sphere is O^{2-}). The structures were adapted after the structures found in the Crystallography Open Database (http://www.crystallography.net).

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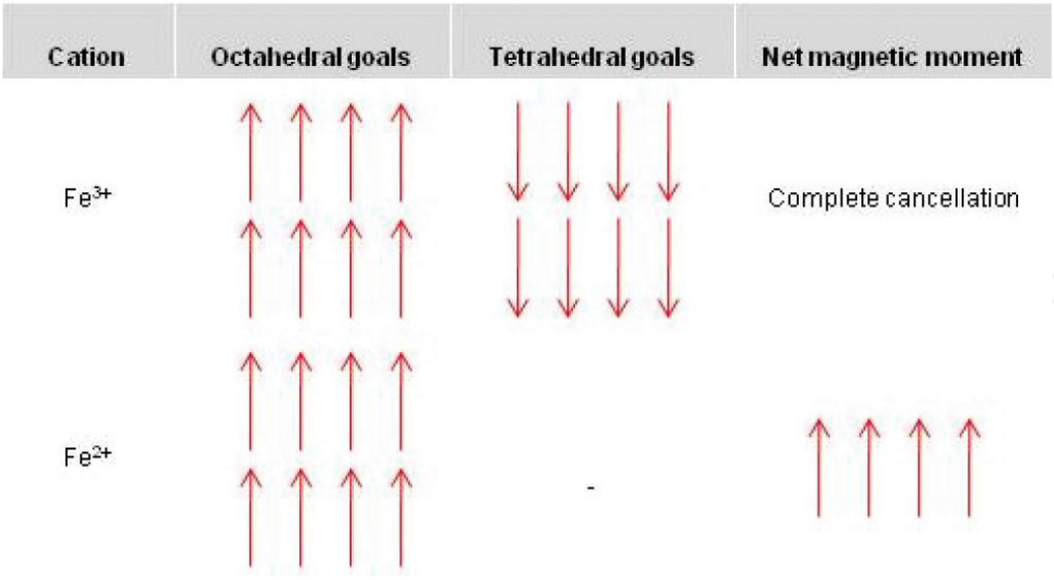

Figure 2. Spin magnetic moment distribution of Fe^{2+} and Fe^{3+} ions in the elemental cell of magnetite.

As magnetite, maghemite (γ-Fe_2O_3) has a spinel structure with the oxygen ions disposed in a closely packed cubic lattice and the iron ions located at interstices. In γ-Fe_2O_3 not all the sites

Property	**Oxide**		
	Magnetite	**Maghemite**	**Hematite**
Molecular formula	Fe_3O_4	γ-Fe_2O_3	α-Fe_2O_3
Color	Black	Reddish-brown	Red
Density (g/cm^3)	5.18	4.87	5.26
Melting temperature (°C)	1583–1597	—	1350
Hardness	5.5	5	6.5
Type of magnetism	Ferrimagnetic	Ferrimagnetic	Weakly ferromagnetic/ anti-ferromagnetic
Curie temperature (K)	858	820–986	956
Saturation magnetization (M_s) at 300 K [A·m^2/kg]	92–100	60–80	0.3
Standard Gibbs free energy of formation (ΔG_f^0) [kJ/mol]	−1012.6	−711.1	−742.7
Crystallographic system	Cubic	Cubic or tetrahedral	Rhombohedral, hexagonal
Structure type	Inverse spinel	Defect spinel	Corundum
Lattice parameter (nm)	α = 0.8396	α = 0.83474 (cubic); α = 0.8347; c = 2.501 (tetragonal)	α = 0.5034; c = 1.375 (hexagonal); α_{Rh} = 0.5427; α = 55.3° (rhombohedral)

Table 1. Physical and magnetic properties of iron oxides [22].

are occupied, Fe^{3+} ions are regularly distributed in only two-thirds of the sites and the rest of the sites remain vacant. After two sites filled with Fe^{3+} ions follows one vacant site [18, 19]. Maghemite is a metastable oxide, product of magnetite oxidation or a product resulting from the heating of other iron oxides.

At temperatures over 300°C, magnetite is oxidized to hematite (α-Fe_2O_3)—an anti-ferromagnetic iron oxide. Hematite (α-Fe_2O_3) has a corundum crystal structure with Fe^{3+} ions distributed in octahedral sites and oxygen ions in hexagonal close-packed arrangement. α-Fe_2O_3, the final product of the transformation of other iron oxides, is a red powder when it is finely divided, very stable at room temperature and very widespread in rocks and soils (**Table 1**) [20, 21].

2. Properties of magnetic nanoparticles

2.1. Magnetic behavior

Magnetic iron oxide nanoparticles (M-IONPs), magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) are materials with iron-magnetic properties under their Curie temperatures (858 K and 986 K) (**Table 1**). The ferro- and ferrimagnetic compounds in their raw state present a multidimensional magnetic structure, without a permanent magnetic moment. The magnetic properties of a material depend on following parameters: (i) temperature, (ii) pressure and (iii) applied magnetic field. The properties of iron oxide nanoparticles by their usual sizes are not similar to the properties of larger scale compounds, which explain their use and interest in nanomedicine [23]. In order to define the behavior of the magnetic field, the key lays in the size and distribution of nanoparticles morphology [24]. A spherical, small nanoparticle made of soft materials with a diameter below the domain size shows an expendable magnetic anisotropy, so that their magnetic moment is free to rotate relatively to the particle and is thus superparamagnetic, i.e., paramagnetic under the Curie temperature [25]. The direction of the magnetic moment of the nanoparticles is determined by thermal fluctuation and the magnetic anisotropy, which tend to fixate on the crystalline structure or particle morphology [26].

The interaction between an external magnetic field and the magnetic field of a nanoparticle determines: (i) the orientation of the magnetic moment of the particle as to become parallel with the magnetic field applied to minimize energy and bipolar interaction and (ii) the transition of the particle in the direction of the gradient, as in magnetophoresis [26]. Many applications of the magnetic nanoparticles are based on their ability to be manipulated using magnetic fields. This capability depends on the effectiveness of the magnetophoretic force, determined by the time of the particle and the magnetic field gradient, to fasten or to move the particle [25]. The magnetophoretic force exercised over superparamagnetic nanoparticles with a single core is less effective due to their small diameter and magnetic moment, but in the case of multicore particles, the magnetic momentum induced in the field is strong enough to allow magnetic targeting to moderate values of the magnetic field intensity and field gradient. Therefore, in order to assess the applicability of magnetic particles or magnetic fixing, the magnetic momentum of the particles is more relevant than mass magnetization [25, 27].

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2.2. Size

The size and the size distribution of superparamagnetic iron oxide nanoparticles are important parameters for their biological application. Also, their magnetic properties are in close touch with their size. It has been demonstrated that the magnetic dipole-dipole interactions are significantly reduced in superparamagnetic iron oxide nanoparticles due to their scale of r6, r being the radius of the particle [28]. The advantages of using magnetic nanoparticles with sizes smaller than 100 nm are due to their surface efficiency to easily attach ligands and small settling velocities which give a high stability in suspension and improve tissue diffusion. Particles should be small enough to bypass the endothelial reticule system. They are supposed to remain in circulation after injection and be able to pass through the capital systems, organs and tissues, avoiding the embolus. Particle size is also important for getting an effect of improved permeability and retention. For example, particles larger than 10 nm may not penetrate the endothelium in physiological conditions, but can enter in pathological conditions, such as inflammations or tumors [28].

When magnetic nanoparticles loaded with medicinal substances are injected into the systemic circulation, size, morphology and surface charge are the three important parameters for their behavior in the bloodstream. Kupffer cells in the liver are very sensitive to both microorganisms and nanoparticles. Plasma proteins can easily adsorb onto their surface nanoparticles, depending on their size, surface charging and their morphology. Particles with sizes larger than 200 nm or below 10 nm are not suitable due to their absorption by the endoplasmic reticulum system [28].

2.3. Charge

Loading surface and biodistribution of superparamagnetic iron oxide nanoparticles play an important role in the colloidal stability. Surface charging can be described qualitatively by the nature and behavior of surface groups in the solution at a given pH and in the presence of an electrolyte. In terms of quantity, it can be measured as an electric potential in the double layer of the interfacial surface of the nanoparticles found in a suspension state. A high value of zeta potential is an indication of stability in dispersion of superparamagnetic iron oxide nanoparticles due to electrostatic interaction. Composition and structure of nanoparticles are very important for their interaction with biological fluids. In a known environment, superparamagnetic nanoparticle characteristics, such as the chemical composition, both core and neural crest cells, its size and size distribution, shape and angles of curvature, its crystalline structure, smoothness or surface roughness and hydrophobic or hydrophilic levels, are important for their *in vivo* applications. These features can determine their stationary time in the circulatory system [28].

Osaka and his colleagues [29] have reported a correlation between surface charge of magnetite nanoparticles and their cellular absorption efficiency on different cell lines. For example, a superparamagnetic particle with positive charge showed a greater internalization in human breast cancer cells in comparison with those charged negatively, while there was no difference

in the degree of internalization in endothelial cells of human umbilical bladder. Thus, the superparamagnetic nanoparticles absorption depends not only on their surface properties but also on cell type.

2.4. Surface functionality and colloidal stability

Both the surface chemistry of magnetite particles and its properties are particularly important in various applications. Iron atoms at the surface of the magnetite particle that are not bound to oxygen atoms act as Lewis acids and coordinate the molecules that can give a pair of electrons. In aqueous systems, these atoms coordinate water molecules that rapidly dissociate resulting magnetite with functionalized surface with Fe-OH hydroxyl groups. So, the chemistry of the surface of magnetite particles is strongly dependent on the pH value; at low pH values, the surface of the magnetite particles is protonated (positively charged), and at high pH values, it is negatively charged (**Figure 3**). The preformed hydroxyl groups on the surface of magnetite have amphoteric character; therefore, they can react either as acids or bases [30].

Another problem that arises after obtaining the magnetic iron oxide nanoparticles (M-IONPs) is their agglomeration that is installed due to the van der Waals forces and the magnetic forces. Nanoparticles without coatings (naked nanoparticles) are not stable in aqueous environments, easily aggregating and precipitating. After application *in vivo*, nanoparticles often form aggregates in the bloodstream and are retained by the macrophages. Therefore, they must be covered with a variety of fragments which have the property to eliminate or minimize their aggregation in physiological conditions [31]. The magnetic nanoparticles are coated with an impervious wrapper so that oxygen does not reach at the surface of the magnetic nanoparticles in order to ensure an effective stabilization of iron oxide nanoparticles. Some stabilizers, such as a surfactant or a polymer, usually are added during preparation to prevent the aggregation of nanosized particles. Most of these polymers stick to the nanoparticles surface in a specific substrate manner. Nanoparticle surfaces can be composed of several organic and inorganic materials, including polymer. Also, polymer coating materials can be classified in turn into synthetic and natural. Polymers such as poly-ethylene-co-vinyl acetate, poly-vinylpyrrolidone,

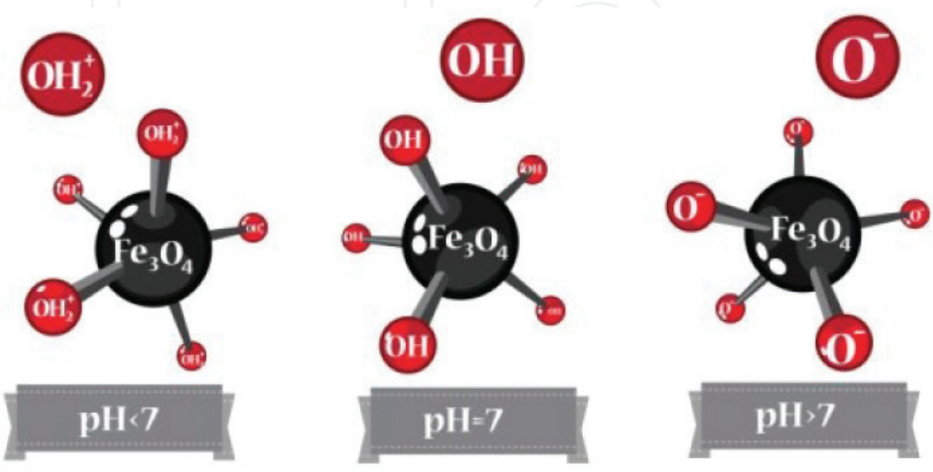

Figure 3. The behavior of Fe_3O_4 nanoparticles depending on pH.

poly-acid-lactic-co-glycolic, polyethylene glycol, etc. are typical examples of synthetic polymeric systems. Natural polymer coatings include gelatin, dextran, chitosan, etc. The molecules used for stabilization of magnetic nanoparticles must be biocompatible and biodegradable. The most common surfactant molecules are oleic acid, lauric acid, acids, sulfonic acids, alkanes and alkane phosphonates. The surfactants are amphiphilic compounds and they manifest their role at the interface between nanoparticles and solvent. However, magnetic nanoparticles covered with organic compounds, in suspension cannot be used for biological purposes, especially in the delivery of medicines. Changing the surface of nanoparticles post-synthesis is known as core-shell nanoparticles, also used widely. The most commonly used materials are polymers, silica or metals (e.g. gold, cadmium, selenium, silver). Coating materials protect the core against oxidation and therefore keep the magnetic property of nanoparticles. It is known that the iron oxide nanoparticles are non-toxic, but some coating materials may be toxic. For example, silicon dioxide is biocompatible, but is not biodegradable [28].

Many researchers have prepared magnetic nanoparticles covered with various surfactants or biomolecules that have been introduced directly in the synthesis process. For example, Salavati-Niasari et al. [32] have synthesized Fe_3O_4 nanoparticles covered with octanoic acid using a facile chemical precipitation method. The surfactant was present in the reaction system to improve dispersity. The authors have obtained magnetic nanoparticles with a size range of 25 nm. Liu et al. prepared magnetic nanoparticles coated with chitosan, for the immobilized lipase, using the co-precipitation method. They replaced water with 2% chitosan in acetic acid solution during the reaction process [33].

Atomic transfer radical polymerization (ATRP) is another common way to cover magnetic nanoparticles, developed by Wang et al. [34]. Due to the magnetic interaction of the iron oxide nanoparticles with biological fluids, the process of formation of free radicals of oxygen reactive species may be increased. To protect the environment *in vivo* from these toxic by-products, some materials have been used for biocompatible and rigid coatings, such as gold [28].

3. M-IONPs synthesis methods

In the past decade, numerous synthesis methods have been developed to obtain M-IONPs. On the basis that the method of preparation plays an essential role in obtaining nanoparticles with tailored properties, the research work regarding the development of new synthesis methods to control the size, shape, morphology and magnetic properties of these nanoparticles is a permanent challenge. In the same time, the synthesis method has to be environmentally friendly, simple, inexpensive and reproducible. Many scientific publications have described efficient synthesis methods, which allow the obtaining of monodisperse magnetic nanoparticles, stable for a long time with controlled shape.

The synthesis method has to ensure the obtaining of magnetic nanoparticles with specific properties to their application domain by changing the experimental reaction conditions. For biomedical applications, superparamagnetic iron oxide nanoparticles with a specific surface chemistry (for *in vivo* applications), high magnetization values and a narrow size distribution

of the particles with size below 100 nm are needed. Magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) have attracted particular attention because, under certain synthesis conditions, they are superparamagnetic, being also biocompatible, thus becoming the magnetic nanomaterials most commonly used in medical applications.

Hereinafter, the most popular synthesis methods used for obtaining M-IONPs will be described. After the chemical surface modification of magnetic nanoparticles by binding drugs, proteins, enzymes, antibodies, etc., they can be directed to an organ, tissue or tumor with the help of an external magnetic field. The methods described below allow to obtain magnetic nanoparticles with narrow size dimensions, desired shape and morphology, by changing the conditions and/or parameters of the synthesis. The most used and popular method for the synthesis of magnetite, being in the same time simple and efficient, is the chemical precipitation of iron salts [35–39].

3.1. Precipitation method

The first synthesis of superparamagnetic iron oxide nanoparticles was reported by Massart, and the method consists in mixing two salts of Fe^{3+} and Fe^{2+} in a molar ratio of 2:1 in aqueous medium followed by precipitation of these salts using a precipitating agent (a base – NH_3) under inert atmosphere or at elevated temperature, resulting a black magnetic precipitate [40]. The equation of the chemical reaction which underlies the formation of magnetite may be written as Eq. (1):

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe(OH)_2 + 2Fe(OH)_3 \rightarrow Fe_3O_4\downarrow + 4H_2O \quad (1)$$

Magnetite is not stable at room temperature, being sensitive to oxidation in contact with air, easily transforming into maghemite, according to Eq. (2):

$$2Fe_3O_4 + \tfrac{1}{2}O_2 \rightarrow 3\gamma - Fe_2O_3 \quad (2)$$

The precipitation process is based on two steps regarding the formation of the solids [41, 42]: (i) nucleation—a very short period, occurs only when the concentration of the constituent species reaches suprasaturation and (ii) slow controlled growth of the preformed nuclei, by diffusion from the solutions to the surfaces of the crystal. To avoid the formation of polydispersed nanoparticles, it is necessary that the two stages to be separated, i.e., nucleation does not take place simultaneously with crystal growth. By controlling the two processes, monodispersed magnetic particles can be obtained. If the nuclei start to form in the same time, the growth of these nuclei leads to particles with very narrow size distribution. Therefore, the size of the obtained particles can be controlled but only in the nucleation step because the size of the particles does not change during the growth process.

It has been shown that by controlling both the pH of the reaction medium and the ionic strength, it is possible to control the mean size of the particles. Jiang et al. have demonstrated that the size of the particles has an inverse proportionality with the pH and the ionic strength of the precipitation medium [43]. These two parameters (pH and the ionic strength) also affect the chemical surface of the crystals and the electrostatic surface charge [44].

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Other parameters that can influence the size, shape and composition of the magnetic iron oxide nanoparticles are the nature of iron salts (chlorides, perchlorates, nitrates, sulfates, etc.) and the molar ratio Fe^{3+}/Fe^{2+}. Roth and co-workers published a good analysis regarding the influence of the reaction conditions on the formation of superparamagnetic iron oxide nanoparticles. The authors demonstrated that for obtaining particles with a size between 3 and 17 nm with high saturation magnetization a higher reaction temperature, higher iron salt concentration, Fe^{3+}/Fe^{2+} molar ratio below 2 and a molar ratio of hydroxide ions/iron ions of 1.4:1 are needed [45].

Wu et al. investigated the effect of the vacuum drying method on the change of the morphology and magnetic properties of magnetic iron oxide nanoparticles (M-IONPs). They revealed that the obtained nanoparticles tend to agglomerate more easily when their average diameter decreased, but the structure and morphology are maintained better by ambient air drying. They also obtained magnetic nanoparticles with high saturation magnetization after drying the obtained nanoparticles in a vacuum at 70°C [36].

The same group of researchers in another study has synthesized Fe_3O_4 nanoparticles by utilizing ultrasonic-assisted chemical co-precipitation. They used high purity iron separated from iron ore tailings by an acidic leaching method and obtained superparamagnetic iron oxide nanoparticles without a protecting gas [46]. Pereira and co-workers have synthesized superparamagnetic Fe_3O_4 nanoparticles with small particle size (4.9–6.3 nm) and improved magnetic properties by one-step aqueous precipitation route based on the use of a new type of alkaline agents [47]. The alkaline agents that they have used include alkanolamines, isopropanol amine and diisopropanolamine. The base that they have used, instead of the most used—NaOH, leads to smaller particle sizes (up to 6 times) and enhanced saturation magnetization (up to 1.3 times). Generally, the size of the particles is proportionally with the magnetization saturation, but the above results showed improved magnetic properties while keeping their small size.

Besides the many advantages of the precipitation method (high saturation magnetization, rapid synthesis with high yield, versatility, nanoparticles with the desired morphology and characteristics), it shows several disadvantages, like oxidation, magnetic nanoparticles with particle size distribution that cannot be controlled, polydispersion and weak crystallization which leads to nanoparticles with low saturation magnetization.

3.2. Thermal decomposition

Thermal decomposition of organometallic compounds in high boiling organic solutions in the presence of stabilizers is also a popular method for the synthesis of the spinel structured Fe_3O_4 and a very promising technique for obtaining high-quality superparamagnetic iron oxide nanoparticles. The magnetic nanoparticles obtained by this method proved to be superior to those obtained by precipitation, because the nucleation process can be separated by the growth process and the hydrolysis reaction is avoided [48].

The method is based on the decomposition of an iron precursor at high temperature in the presence of solvents which contain stabilizing surfactants (such as oleic acid or oleylamine) [49–51]. By varying the reaction mixtures and modifying the synthesis condition, it can be

obtained M-IONPs with controlled size, size distribution and composition. The most commonly used precursors employed to prepare monodispersed M-IONPs with diameter ranging from 3 to 50 nm are of the form: (I) metal acetylacetonate—$[M(acac)_n]$ (where M = Fe, Co, Ni, Mn, Cr; n = 2 or 3) [52]; (II) metal cupferronates—$[M^x(cup)_x]$ (where cup = N-nitrosophen ylhydroxylamine); (III) metal oxalate—$[M(C_2O_4)_n \bullet 2H_2O]$; (IV) metal carbonyl—$Fe_3(CO)_{12}$ [53] or $Fe(CO)_5$ [50]; (V) metal acetate—$[M(CH_3COO)_n]$; (VI) metal carboxylate, (VII) metal-urea complex—$[Fe(CON_2H_4)_6](NO_3)_3$ [54]; (VIII) Prussian Blue—$Fe_4[Fe(CN)_6 \bullet 14H_2O]$ [55, 56]; (IX) metal chloride and (X) ferrocene—$Fe(C_2H_5)_2$ [57].

Using the thermal decomposition method, it can be easy to control the size and morphology of magnetic nanoparticles by controlling the ratio of the starting reagents, i.e. the ratio between the organometallic compounds, surfactant and solvent. Reaction time, temperature and aging period are equally important for the control of size and morphology. Hyeon obtained monodispersed iron oxide nanoparticles with size range from 4 to 20 nm by thermal decomposition of $Fe(CO)_5$ in the presence of oleic acid at 100°C. Initially, he obtained an iron-oleic acid complex, which was leaved to aging at high temperature (300°C) [50].

Pérez-Mirabet et al. used oleylamine both as stabilization agent (for the stabilization of the particles in solution) and as capping ligand (for the control of particles size), respectively, by one-pot thermal decomposition of $Fe(acac)_3$ and $M(acac)_2$ (M = Co, Mn, Cu and Zn) in oleylamine. They obtained magnetic spinel ferrite nanoparticles with average size of 12 nm and a saturation magnetization Ms = 76 emu/g, very close to the bulk magnetite (92 emu/g) [58].

This method is also suitable for synthesis of nanocubes and nanospheres, which are magnetic nanoparticles as well. Amara et al. synthesized Fe_3O_4 nanocubes and nanospheres by a new simple and single-step process [59]. They used various mixtures of ferrocene and polyvinylpyrrolidone (PVP) by solventless thermal decomposition. Lynch et al. obtained magnetic colloidal iron oxide nanoparticles by thermal decomposition. They generated gas bubbles (Ar) by boiling solvents. Their results illustrated that the argon bubbles had a stronger effect on the nucleation process of magnetic iron oxide nanoparticles than on their growth process [60]. Due to the nucleation process that involves boiling solvents, most often the accurate shape of the magnetic iron oxide nanoparticles is not fully reproducible using the thermal decomposition method.

3.3. Microemulsion method

A microemulsion is formed when a colloidal substance is dispersed in a solvent, that is not compatible with the substance (e.g. water and oil), through a surfactant. Finally, a microemulsion must be clear and stable, as long as it is an isotropic mixture of oil, water and surfactant. The surfactant forms a monolayer film at the oil/water interface, in which the hydrophilic head groups of the surfactant are dissolved in oil phase (consisting of a mixture of hydrocarbons and olefins) and the hydrophobic tail of the surfactant in the aqueous phase (consisting of metal salts) and vice versa, depending on the used surfactant. There are known two types of microemulsion: direct microemulsion, when the oil is dispersed in water and reversed microemulsion, when the water is dispersed in oil. Both have been used to synthesize the magnetic iron oxide nanoparticles with tailored size and shape. The most common surfactants that are widely used in the fabrication of M-IONPs by microemulsion method are bis(2-ethylhexyl) sulfosuccinate (AOT), sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB)

and poly-vinylpyrrolidone (PVP). Throughout time, the microemulsion method proved to be a simple and versatile method for fabrication of nanosized magnetic nanoparticles [61–65].

According to the literature, the size of the resulted nanoparticles can be controlled if the surfactant is proper chose and also by varying the ratio of water/oil/surfactant, the initial concentration of the reactants and the droplet size and by controlling the reaction temperature and time [66, 67]. The size of the synthesized nanoparticles can also be controlled in suitable narrow range by carrying out the reaction in nanoreactor [62, 64, 67].

Lu et al. demonstrated that the surfactant nature has an important role on the final properties of the nanoparticles [64]. The authors have investigated the effect of SDS (anionic surfactant), DTAB and CTAB (cationic surfactants) and non-ionic surfactant on the preformed crystal, on stoichiometric situations and on the magnetic properties of the resulted Fe_3O_4 nanoparticles. In all the cases, the authors have obtained Fe_3O_4 nanoparticles with size less than 16 nm, but in the case of using the cationic surfactants also obtained a good saturation magnetization, which is an essential parameter for biological applications.

Okoli et al. have prepared M-IONPs by the two types of microemulsion (water/oil and oil/water), to be used in binding and separation of proteins. The authors demonstrated that by using a water/oil microemulsion, it can obtain magnetic iron oxide nanoparticles with a surface area of 147 m^2/g compared to 304 m^2/g for the magnetic nanoparticles obtained by oil/water microemulsion [68, 69]. The M-IONPs specific surface area is inversely proportional with the size of nanoparticles, the higher is the specific surface area the smaller nanoparticles size is obtained.

The advantage of this method is the fact that it can be obtain magnetic nanoparticles with uniform morphology and controllable size; but the major drawbacks are the requirements of a large amount of solvent and the excess of surfactant that has to be eliminated.

3.4. Hydrothermal and solvothermal methods

In case of these methods, the reaction takes place in aqueous medium (the hydrothermal method) or in organic medium (solvothermal method), in reactors or autoclaves, at temperatures between 130 and 250°C under high vapor pressure, in the range 0.3–4 MPa [70–72]. Using this method, it can be obtained magnetic iron oxide nanoparticles with tailored properties (size and shape) by tuning the reaction conditions. The hydrothermal method is known as an environment-friendly process for the obtaining of M-IONPs, due to the raw materials used such as sulfates and chlorides—as cation source, dissolved in water [73].

Lin et al. [74] has used hydrothermal and solvothermal methods to obtain hollow M-IONPs. Briefly, they used $FeCl_3$ (as source of iron), ethylene glycol (as reducing agent), ammonium acetate and urea were used to guide the formation of hollow magnetite spheres. After homogeneous dispersion, the mixture is transferred to a Teflon-lined stainless steel autoclave and sealed to heat at about 200°C for 8–24 h. The authors demonstrated that the Fe^{3+} ions on the surface of the hollow spheres exist in the form of Fe_3O_4, and the results are confirmed by the Mössbauer measurements. Tian et al. synthesized ultra-small monodisperse Fe_3O_4 nanoparticles, with precise size control of 1 nm, by solvothermal method [75]. They used $Fe(acac)_3$ as iron source, n-octanol as a solvent and n-octylamine as a reductant. The authors obtained Fe_3O_4 nanoparticles with a size range from 4 to 6 nm, by varying the volume ratios

of n-octylamine and n-octanol, without the need of a gas (N_2) bubbling or reflux conditions. By comparing this method with the thermal decomposition methods for obtaining Fe_3O_4 nanoparticles, this solvothermal process was more convenient.

Stoia et al. [14] synthesized Fe_xO_y and Fe_xO_y/C nanocomposite by solvothermal method, with the purpose of using these nanocomposites as adsorbents for methylene blue removal from aqueous solutions. The authors used $FeCl_3$ as iron source, 1,2-propanediol as solvent and diethylamine as precipitating agent. The activated carbon was introduced into system in order to obtain homogenous Fe_xO_y/C composites with high specific surface area and magnetic properties. Some researchers have attempted to modify the hydrothermal process. Ahmadi et al. obtained Fe_3O_4 nanoparticles at low temperature (140°C) without having to autoclave. They have studied kinetics of the reaction, but the magnetic properties of the resulted nanoparticles are inadequate in short reaction time (below 2 hours) [76].

As advantages, the hydrothermal and solvothermal methods are suitable for obtaining shape-controlled M-IONPs. As a disadvantage, in the case of hydrothermal technique, the reaction takes place for a long time and the amounts of resulting products are low [76].

3.5. Combustion method

The combustion method is an alternative to the currently used methods, being barely mentioned in the literature for the synthesis of M-IONPs. The combustion method have a lot of advantages due to the simplicity of the working technique, short reaction time and low energy consumption, being in the same time environmentally friendly.

The combustion method involves the strong exothermic redox reaction between an oxidizing agent (iron nitrate) and various reducing agents (fuels) of organic nature. The initiation of the combustion process takes place by rapidly heating the mixture of raw materials at relatively low temperatures below 500°C (**Figure 4**). The reaction stoichiometry has a decisive role in the characteristics of the reaction product, especially in the granule size, since a combustion reaction does not occur for any molar fuel/oxidizing agent ratio.

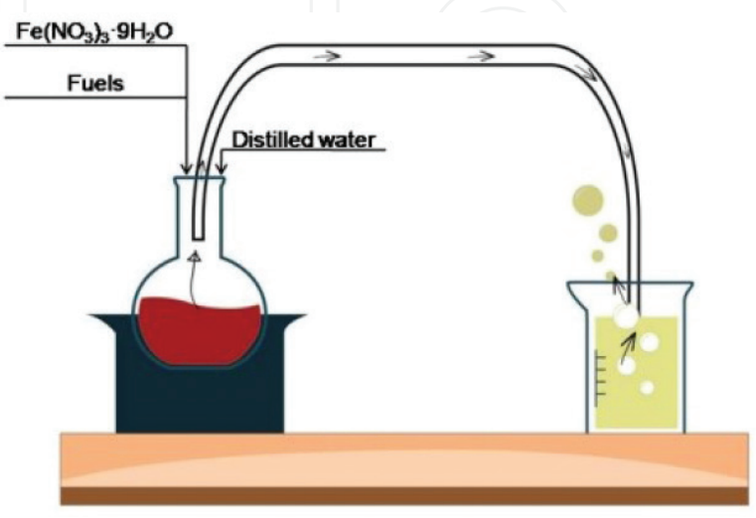

Figure 4. The general scheme for obtaining iron oxide magnetic powders using the combustion method.

The use of metallic nitrates in a mixture with a suitable fuel has the great advantage that, following the combustion reaction, the gases are released without a high risk of toxicity: CO_2, N_2 and H_2O [77]. By using a proper fuel, proper auxiliary additives, as well as an appropriate oxidizing agent/fuel ratio, it can be tailored the size of the particles, the specific surface area and the crystallinity degree of the obtained material [78, 79].

Ianoş et al. reported a new combustion synthesis technique for the preparation of nanosized Fe_3O_4 nanoparticles [80]. The authors developed a new, facile and cheap scheme of installation for combustion synthesis of Fe_3O_4 nanoparticles in the absence of air. They also investigated the effect of both the reaction atmosphere (in the presence or in the absence of air) and the fuels nature on the properties of the resulted nanoparticles. Using sucrose, citric acid and glucose as fuels, the authors demonstrated that the reaction atmosphere is very important for obtaining Fe_3O_4 nanoparticles as a single crystalline phase. There were obtained Fe_3O_4 nanoparticles in the size range of 10 (when glucose was used as fuel) to 18 nm (when citric acid was used as fuel).

Mihoc et al. also investigated the effect of both the fuels nature and the reaction atmosphere for obtaining magnetic nanoparticles used as adsorbent for the removal of phenol and p-chlorophenol from wastewater [81]. The authors revealed that the working atmosphere influences the phase composition of the combustion reaction product. Using urea with ammonium chloride as fuels, the final product of reaction was α-Fe_2O_3 (when the reaction took place in air). Working in the absence of air, using oxalic, tartaric and citric acid as fuel, the single phase resulted in combustion reaction was Fe_3O_4, irrespective of the nature of the fuel.

Using the combustion method, the magnetic oxide nanoparticles are covered with some organic residues resulting from fuel combustion. Mihoc et al. demonstrated that these materials exhibit better adsorption capacity as compared with the naked magnetic oxides [82].

However, if it is desired to remove the residual carbon resulting from the combustion process, Ianos et al. found a method in which the residual carbon was eliminated by washing the magnetic nanoparticles several times with H_2O_2. They revealed that by combustion reaction between $Fe(NO_3)_3 \bullet 9H_2O$ and $C_6H_{12}O_6$ a black magnetic nanoparticle containing γ-Fe_2O_3 and residual carbon was obtained. The authors demonstrated that by H_2O_2 treatment of the resulted magnetic nanoparticles the carbon was removed by chemical oxidation, from 32.7 to 0.4%, and the color of the sample changed from black to reddish brown [83].

M-IONPs proved to be versatile due to the large range board of applications in medicine. Nanomedicine is an emerging field that offers new approaches but especially new solutions for many medical problems. For example, the discovery of antibiotics has been of historic importance, but over time, antibiotic resistance has become an issue and new approaches are therefore needed. Many groups of researchers have already demonstrated that the synergic effects of the antimicrobials agents (not only the antibiotics) with nanoparticles can be promoted as a new method for the severe infection treatments, even with low antimicrobial doses.

For the biomedical uses, only the M-IONPs which fulfill the following requirements are proper: superparamagnetic properties at room temperature, large saturation of magnetizations, biocompatibility and sizes around 20 nm for *in vivo* administration. To convert the pure magnetic nanoparticles in biocompatible colloidal suspensions, many researchers have pro-

posed the use of different polymers like covering agents or surfactants like starch, heparin, chitosan, dextran, oleic acid, polyethylene glycol (PEG), etc.

Polymer coating can be accomplished during or after the synthesis of magnetic nanoparticles. Polyethylene glycol (PEG) is a water-soluble, biocompatible hydrophilic polymer that can be used successfully in the synthesis of biocompatible nanoparticles with increased resistance to blood circulation [84]. Another alternative to covered magnetic nanoparticles is the use of copolymers that produce core-shell nanoparticles with possible applications in drug transport (drug vector) [85].

The use of inorganic compounds such as gold, silver, silica gel and carbon as surfactants not only provides good stability to the nanoparticles but also allows functionalizing their surface by grafting certain biological ligands. Covering of magnetic nanoparticles with gold seems to be ideal because of its low reactivity; however, coating the magnetic nanoparticles directly with gold is very difficult due to the different nature of the two surfaces [86–89]. The silica gel is the most widely used compound in the preparation of functionalized iron oxide nanoparticles surface, because it has several advantages: excellent biocompatibility, hydrophilicity, the feasibility of integrating other functional groups on the surface due to terminal silanol groups that can react with different coupling agents, provides good stabilization of the magnetic iron oxide nanoparticles in the solution, prevents the interaction between the nanoparticles thus preventing the agglomeration of the particles over time and ensures better encapsulation [90]. A very good coating of carbon layers provides an effective barrier against oxidation and acidic erosion of magnetic nanoparticles. It is therefore possible to synthesize carbon-coated magnetic nanoparticles that are thermally stable, biocompatible and also have high oxidation stability, which is crucial for certain applications [89].

4. *In vitro* biological impact

4.1. Magnetic iron oxide nanoparticle effect on normal cells

Iron ions play major biological roles in different physiological processes, including DNA synthesis, oxygen transport, mitochondrial respiration, heme synthesis, and in metabolic functions at central nervous system level (nitric oxide metabolism, oxidative phosphorylation and myelin and neurotransmitter synthesis). Moreover, iron proved to be an essential factor for an appropriate function of neurons by acting as cofactor for tyrosine hydroxylase, an enzyme with a critical role in dopamine synthesis and the viability of neural cells. A dysregulation of the iron homeostasis or transport leads to unbalanced physiological functions and cytotoxic reactions. The free intracellular Fe^{2+} ions react with hydrogen peroxide (H_2O_2) and determine the generation of reactive oxygen species (ROS), process known as Fenton reaction. An increased ROS concentration activates a cascade of events (release of iron ions into the cytosol by inducing an augmented permeability of the outer mitochondrial membrane and detrimental effects on lysosomal membrane; lipid peroxidation, damaged proteins, break of DNA chains and degradation of bases, mutations, deletions or translocations at nuclear level) that has as endpoint cell death. The pathologies that are associated with this type of cellular

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damage are aging, cancer and neurodegenerative diseases [91]. Another mechanism of inducing cell death by the iron ions is the apoptotic pathway via mitochondria, as follows: a high amount of iron ions into the mitochondria determine the opening of the mitochondrial transition pore, release of Ca^{2+} and cytochrome c and activation of apoptotic cascade [91, 92].

Based on these data, concerning the toxicity induced by iron ions, it is imperative to study the possible toxic effects induced by M-IONPs, mainly since these particles present a higher reactivity as compared with the normal sized ones. The magnetic character of iron oxide nanoparticles offers some advantages, including the capacity of this nanosized compounds to be driven to targeted sites by an external magnetic field, even to tissues and organs that are difficult to reach in normal conditions (blood brain barrier and central nervous system). M-IONPs penetrate into the cells via receptor-mediated endocytosis and settle into the lysosomes, organelles characterized by the presence of an acidic medium, where it takes place the metabolization of the nanoparticles and free iron ions are released into the cell [91].

In a previous study, it was demonstrated that M-IONPs penetrate differentially into the neural cells (glial cells, primary neurons of the cerebellum, microglia, astrocytes, oligodendrocytes and Schwann cells), based on their dimensions: large size nanoparticles were absorbed by endocytosis, whereas small sized ones via pinocytosis [91, 93]. It was also proved that exposure to M-IONPs has an impact on iron homeostasis by upregulating the proteins responsible for iron storage or export from the cell and by downregulating the proteins expression involved in iron uptake [94].

Besides these positive features, application of an external magnetic field leads to accumulation of M-IONPs in target cells and potential toxicity. The accumulation of iron into the cells after exposure to M-IONPs seems to be dependent on several factors, such as (i) concentration and dose of M-IONPs (high concentrations require a longer period for elimination—several months, whereas M-IONPs in low concentrations can be eliminated within 3 weeks), size (small size nanoparticles cumulate in increased concentrations as compared to large size nanoparticles), shape (spherical nanoparticles present a longer degradation process due to a small contact surface), coating (some coating agents may prolong the degradation process or may increase it), the functional groups (the positively charged functional groups present in M-IONPs structure increase their uptake by the cells) and cell type (microglia have a higher affinity for M-IONPs, whereas into the brain endothelial cells penetrate less nanoparticles) [91].

A significant number of studies sustained that M-IONPs exerted *in vitro* and *in vivo* toxicity. The main players responsible for toxic effects are considered to be the iron ions released from M-IONPs at lysosomal level, which react with hydrogen peroxide and lead to ROS generation [91]. Exposure of neural cells to M-IONPs was associated with a low concentration of ROS, but a reduced level of glutathione and mitochondrial membrane hyperpolarization [95]. Other studies conducted on healthy cell lines (both human and animal origin) pointed out that bare M-IONPs may induce cytotoxic effects via ROS generation, leading to cell death [96–98].

The oxidation state of iron (Fe^{2+} or Fe^{3+}) plays a major role in determining the nanoparticles toxicity according to the studies that affirm that Fe^{3+} in Fe_2O_3 is more toxic than Fe^{2+} in Fe_3O_4 and causes more DNA oxidation [91, 99].

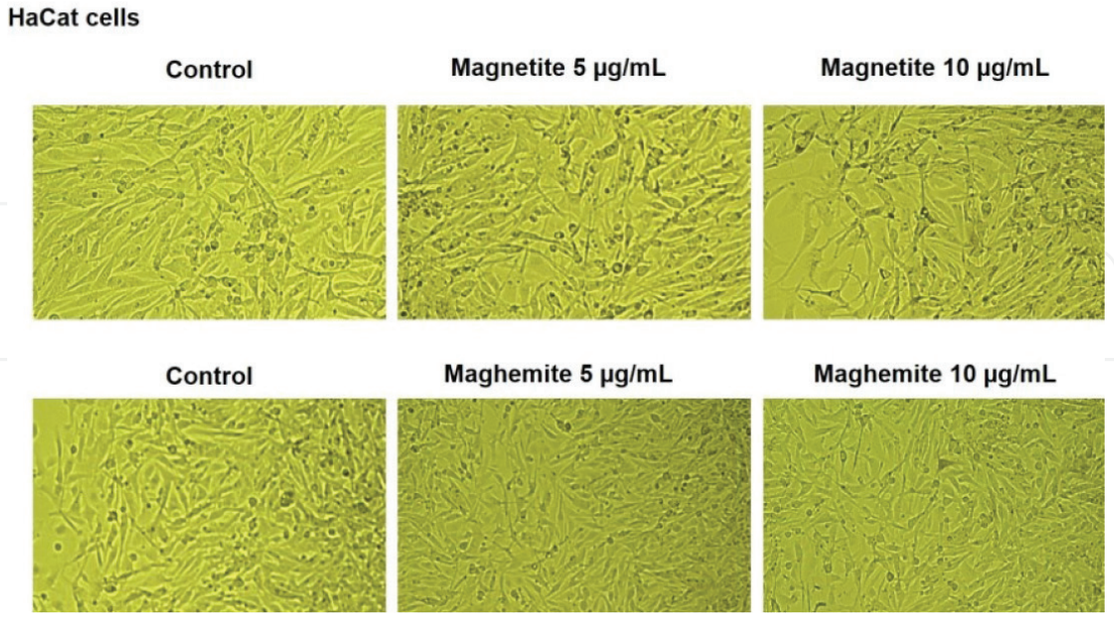

Figure 5. The impact of magnetite and maghemite obtained by combustion method on HaCat cell morphology after 24 h stimulation.

The concentration of M-IONPs is also important in the assessment of M-IONPs toxicity. In one of our previous studies developed on HaCat cells (human keratinocytes), it was shown that concentrations lower than 25 µg/mL did not induce toxicity in terms of viability and cytoskeleton changes (**Figure 5**) [100].

Shelat and coworkers indicated a dose-dependent cytotoxic effect of M-IONPs on mouse embryonic fibroblast (NIH 3 T3) [101]. It was also assessed the effect of negatively charged superparamagnetic iron oxide nanoparticles on heart cells and no changes in actin cytoskeleton were observed, whereas in the case of brain and kidney cells, a disruption of the actin cytoskeleton was detected, but some increased vascular permeability was seen after exposure [102].

Another sign of toxicity that was described after neural cells exposure to M-IONPs was represented by protein aggregation. In addition, it was shown that M-IONPs induce apoptosis of hepatocytes in a mitochondrial-dependent way consisting of upregulation of pro-apoptotic markers (Bax and Bad) and downregulation of bcl-2 (anti-apoptotic); decrease of mitochondrial membrane potential followed by the release of cytochrome c into the cytosol what leads to activation of caspases cascade and apoptosis induction (**Figure 6**) [91].

Based on the data that were presented in this section, it could be said that the mechanisms involved in M-IONPs toxicity are accumulation of iron ions, oxidative damage by generating reactive oxygen species, protein aggregation and apoptosis.

Mutagenic effects of M-IONPs on different murine and mammalian normal cell lines were clearly synthesized in an extensive review [103].

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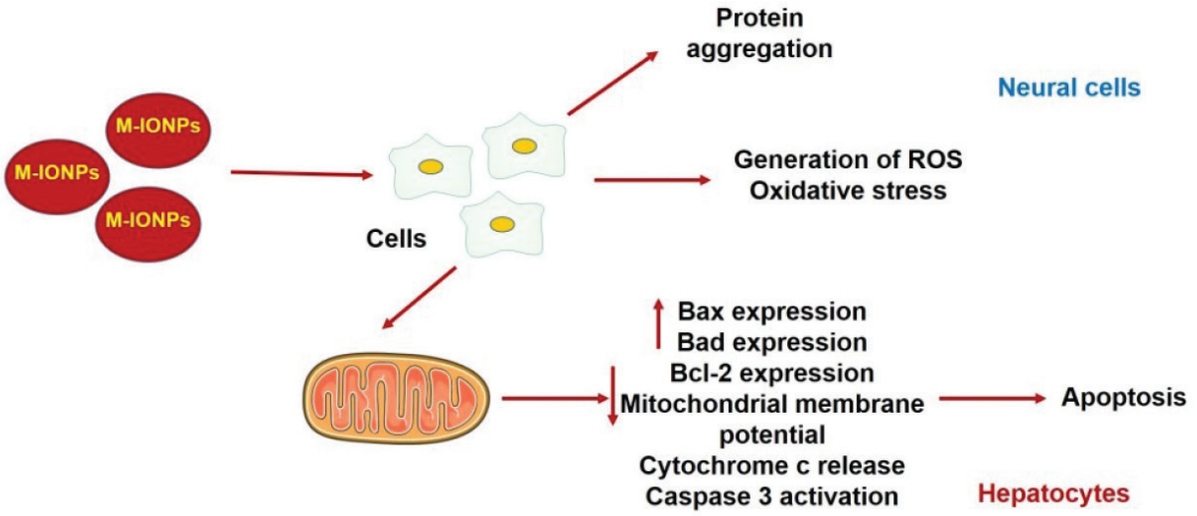

Figure 6. Mechanisms of toxicity induced by M-IONPs to normal cells (neural cells and hepatocytes): oxidative stress and apoptosis.

4.2. Magnetic iron oxide nanoparticles effect on cancer cells

SPIONs (superparamagnetic iron oxide nanoparticles) are the most frequently used iron oxide nanoparticles in the biomedical applications due to their proper size (range between 50 and 200 nm) and the magnetic properties responsible for the lack of particle aggregates *in vivo.* Another type of iron oxide nanoparticles is represented by USPIONs (ultra-small superparamagnetic iron oxide nanoparticles), which have a diameter lower than 50 nm. The mandatory features of M-IONPs that must be analyzed in order to establish the bioavailability and the possible interactions with endogenous compounds (proteins, immune system cells, etc.) are: (i) size (the recommendable size for biomedical applications is between 10 and 200 nm; the ones that are too big will be assimilated by liver and spleen cells, the ones that are too small will be filtrated by the kidneys and their life in the bloodstream is reduced); (ii) superparamagnetism and (iii) presence of a coating agent [104].

The affinity of liver, spleen, bone marrow and lymph nodes for SPIONs after their removal from the blood by the mononuclear phagocytic system (MPS) after intravenous administration represents the reason for the study of this type of nanoparticles as contrast agents but also for their use as delivery tools for chemotherapeutic agents. USPIONs due to their small size possess the capacity to escape macrophages of MPS surveillance and their circulation time is higher, but they also encounter macrophages in deeper compartments.

The changes concerning the surface of the nanoparticles by using a coating agent proved to exert multiple roles: to improve colloids stability, to enhance the bioavailability and the bloodstream half-life and to reduce precipitation and formation of conglomerates [104, 105]. M-IONPs were used as drug delivery agents and as contrast agents based on their potential to activate at cellular and molecular levels [105].

Due to the multiple applications of M-IONPs in biomedical fields (drug delivery, as contrast agents, hyperthermia treatment), it was also verified the effects of the hollow nanoparticles (without payload) on different tumor cell lines.

As mentioned in the previous section, M-IONPs mediate DNA lesions in normal cells, and this property is also exerted in the case of tumor cells. The effect observed was dose-dependent and time-dependent and consisted of damage of tail length and DNA strand breaks. The results were similar in all the tumor cell lines tested: human breast cancer cell line (MCF-7), human fibrosarcoma cells, lung cancer and cervix carcinoma cells [103].

Another mechanism of M-IONPs by which are able to harm cancer cells is represented by the ability to induce magnetic hyperthermia in the form of heat generated by the release of energy after applying a high-frequency alternating magnetic field. The principle of action of this technique consists in raising the cell temperature abnormally to 41–45°C, which leads to significant detrimental effects that can be reversible in the case of normal cells whereas irreversible for cancer cells [105].

A novel proposed mechanism for M-IONPs-induced cell death is enucleation described by Paunescu and coworkers, process observed after exposure of breast cancer cells (MCF-7) and human melanoma (SK-BR-3) to magnetic iron oxide nanoparticles obtained by combustion synthesis [106]. The enucleation phenomena is well described for erythroid terminal differentiation process and there is also used a term in the literature "enucleation sign" that is specific for enhanced computed tomographic images of the ruptured hepatocellular carcinoma. The definition for this term is "the separation of tumor content with intraperitoneal rupture into the perihepatic space, which is seen as low attenuating lesion from peripheral enhancing rim on arterial phase imaging" [107]. The process observed by Paunescu et al. was described as a non-physiological process and it was unrelated with the process described for erythroblast enucleation [106].

The M-IONPs proved a cytotoxic effect against murine melanoma cells B16, cytotoxicity evaluated by the means of MTT viability assay [108].

Other mechanisms of action as anticancer agents may be attributed to M-IONPs, mechanisms that are related with the effects induced by the chemotherapeutical agents loaded in the engineered nanoparticles. The large surface-to-volume ratio characteristic for M-IONPs make them suitable to adsorb proteins or load drugs and attractive for *in vivo* applications, such as MRI, drug and gene delivery, cancer treatment, hard tissue repair and tissue engineering and biosensors [105].

Recent studies mention the use of M-IONPs as improved contrast agents in the diagnosis of cardiovascular pathologies, mainly in atherosclerosis for detection of unstable plaques by the means of MRI (magnetic resonance imaging) [104]. The commercial products based on M-IONPs applied as contrast agents in MRI are: Ferumoxytol (Feraheme—detection of primary tumors and cancer lymph node metastasis), Ferumoxides (Feridex—detection of liver lesions), Ferucarbotran (Resovist—detection of small focal liver lesions), Ferumoxtran—10 (Combidex or Sinerem—detection of metastatic disease in lymph nodes), etc. [104]. Some of these products are included in clinical trials for additional effects, such as Endorem—for tracking monocytes and inflammation cells, Feridex—to keep track of adult bone

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marrow-derived stromal cells for severe cases of Multiple Sclerosis therapy and Supravist (ferucarbotran—small size nanoparticles)—as enhancing blood pool agent [104, 109].

5. Concluding remarks

The intrinsic magnetic properties, the biocompatibility and biodegradability and the capacity to respond to an external magnetic field are unique features that recommend magnetic iron oxide nanoparticles as promising nanomaterials in biomedical applications. The recent advances in this field led to the synthesis of engineered and targeted M-IONPs that might be successfully applied for smart therapies, including controlled drug release, hyperthermia treatment, magnetofection and gene delivery, mapping of lymph nodes and tissue engineering. M-IONPs could be considered theranostics tools based on their capacity to combine their use in diagnostic, treatment and follow-up of a pathology. Despite all these beneficial effects, an important matter should be taken into consideration when M-IONPs are administered *in vivo*, this matter consisting in the thorough analysis of the factors that might induce toxic reactions like size, charge, coating agent, functional groups and shape. There are still some challenges to achieve M-IONPs optimum efficacy and safety, but the existent drawbacks can be corrected by the improvement of their properties by the means of appropriate methods, further studies and inclusion in clinical trials.

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