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REVIEW ARTICLE

Cell death induced by AC magnetic fields and magnetic nanoparticles: Current state and perspectives

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Abstract

This review analyses the advances in the field of magnetically induced cell death using intracellular magnetic nanoparticles (MNPs). Emphasis has been given to *in vitro* research results, discussing the action of radiofrequency (RF) waves on biological systems as well as those results of thermally induced cell death in terms of MNP cell interactions. Our main goal has been to provide a unified depiction of many recent experiments and theoretical models relevant to the effect of applied electromagnetic fields on MNPs after cellular uptake and the cytotoxicity assessment of MNPs. We have addressed the effects of RF waves used for *in vitro* magnetic hyperthermia on eukaryotic cells regarding physical modifications of the cellular local environment and cell viability.

Keywords

Cell death, inductive heating, magnetic hyperthermia, magnetic nanoparticles, superparamagnetism

History

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Introduction

The recent development of a multidisciplinary branch of nanoscience known as nanomedicine has resulted in a novel state of affairs related to the interaction of living organisms with synthetic entities of nanometer size. These man-made nanometric objects such as nanoparticles, nanowires and nanotubes can interact with cellular structures and substructures in unprecedented ways not only because their size, but also because of specific engineered properties that can be tailored by the current state of the art (e.g. morphology, electric/magnetic properties) [1–3]. When designed to penetrate the cell, they can disrupt or promote molecular exchange across cell membrane as well as to produce specific effects on the metabolic cell pathways, setting the basis for new biomedical therapies [4–7].

Magnetic fluid hyperthermia (MFH) is one of these novel clinical nanotherapies that rely on the use of magnetic nanoparticles (MNPs) as heat generators to induce localised cell death by the application of external radiofrequency (RF) radiation [8]. At the frequencies used for MFH (the low radiofrequency range, from approximately 100 kHz to 1 MHz, see Table I), the physical basis of heat generation involves the interaction between the magnetic moment of the MNPs and the magnetic component of the applied electromagnetic wave. At these frequencies the interaction of both the electrical and magnetic components of RF waves with living organisms is not relevant and can be neglected.

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The use of hyperthermia treatment as an adjuvant for cancer therapies has been known since 3000 BC when some cancer patients were treated by using hot sand baths and saunas [9]. During the 1980s more sophisticated hyperthermia protocols were developed for oncology applications after the verification that cancer cells were more heat-sensitive than normal cells [10–13]. From then on, a large number of studies have combined the use of different chemotherapeutical molecules or radiotherapy with hyperthermia to obtain a synergistic effect. The advent of nanoscience in later years made the use magnetic nanoparticles available as heating agents for magnetic hyperthermia. Since then, an increasing number of novel nanomaterials have been investigated as heating agents, aiming synergic effects with cytotoxic drugs on cancer cells.

In this review we discuss the current state of both theoretical models and experimental results about the *in vitro* utilisation of radio frequency and magnetic nanoparticles as intracellular power-releasing agents to trigger cell death. Given the amplitude of this field, we have limited our investigation to those works involving RF radiation within the MFH range, i.e. from approximately 100 kHz to 1 MHz, which comprises all the low frequency phenomena exploited by magnetic hyperthermia. We aimed at identifying what the key experimental results and theoretical models are that enjoy of general consensus, and to recognise those issues on this field that are currently either not completely understood or inconsistent and deserve further efforts to elucidate.

The interaction between electromagnetic fields and living organisms

The physical effects of electromagnetic (EM) fields on living organisms depend upon the frequency range of the

Table I. The different identifications of the RF sub-regions in terms of their frequency. Adapted from Radiation AGoN-i [14].

Frequency	Wavelength	Designation	Abbreviation
3–30 kHz	100–10 km	Very low frequency	VLF
30–300 kHz	10–1 km	Low frequency	LF
300 kHz–3 MHz	1 km–100 m	Medium frequency	MF
3–30 MHz	100–10 m	High frequency	HF
30–300 MHz	10–1 m	Very high frequency	VHF
300 MHz–3 GHz	1 m–10 cm	Ultra high frequency	UHF
3–30 GHz	10–1 cm	Super high frequency	SHF
30–300 GHz	1 cm–1 mm	Extremely high frequency	EHF
300 GHz–3000 GHz	1 mm–0.1 mm	Tremendously high frequency	THF

electromagnetic spectrum considered. Living organisms are mostly composed of molecules with a dipolar electric moment, and therefore can interact with the electric component of the RF radiation [15]. This interaction at molecular level depends on the characteristic energy levels that can be excited at the frequency of the incident waves. Dielectric heating at higher frequencies is of course also possible and was developed, at microwave ranges, decades ago [15]. A large number of protocols for physical medicine based on direct emission and detection of EM radiation have been developed for nearly every region of the EM spectrum, including X-ray radiography, computed tomography scanning (CT scan) and gamma-ray radiotherapy from radioactive isotopes.

As for the magnetism, only a minor fraction of the molecules that compose living organisms have a permanent magnetic moment, and none of them display long-range magnetic ordering at room temperature, making their interaction with the magnetic field B component of RF radiation feeble or non-existent. To achieve the kind of strong interactions that would yield cellular heating (i.e. energy transfer from the magnetic field to living organisms) a heating agent is needed, i.e. magnetic nanoparticles carrying large magnetic moment must be introduced to the intracellular space. Regarding MFH, the above characteristics of the interaction between magnetic fields and living organisms constitute the source of both the potential high selectivity of this therapy and the complexity to achieve its clinical application.

The general public exposure to RF is of course a relevant matter of public health, and therefore much effort has been oriented to evaluate the impact of these emissions in public environments. There is currently a consensus that for urban areas the high frequency commercial bands such as the GSM900 (925–960 MHz) or UMTS (3G, at 2110–2170 MHz) used by mobile devices and station transmitters are the main sources of public potential health concern [16]. Although there is also a large diversity of RF-generating devices working below 10 MHz located at urban environments, those emission sources do not contribute significantly to the exposure of people and therefore few systematic exposure studies are available on these low frequency ranges [17].

The electromagnetic radiation

The electromagnetic (EM) radiation is composed of EM waves propagating at the speed of light and carrying electric and magnetic energy. Radiofrequency (RF) waves are a subset of the EM spectrum with wavelengths and frequencies within a specific range of values (see Table I). When a living organism is exposed to RF waves, a fraction of the energy is absorbed from the waves, yielding heating of the radiated part of the organism. The rate at which living organisms are heated is the most accepted parameter to assess and quantify the biological effects of RF radiation. In general, the potential effects of RF radiation on living organisms (both *in vitro* and *in vivo*) can be classified regarding their observable effects as:

(1) Thermal effects attributable to the heating of the living organism due to the absorption of RF energy. At lower frequencies this could include heating due to excessive current densities in the organism.
(2) Non-thermal effects, involving all those effects from the fields acting directly on biological tissue without any significant heating being involved.

Regarding thermal effects, they are governed and measured by a physical magnitude known as specific absorption rate (SAR), given in units of $W.kg^{-1}$. Some confusion is found in the literature since the expression $SAR = \frac{1\sigma}{2\rho}E^2? >$ is sometimes used, where σ and ρ are the electrical conductivity and density of the living tissue, respectively, and E is the electric field amplitude. This expression, however, applies only at those frequencies for which the electric interaction is dominant, i.e. the high frequency part of the RF radiation. In the case of magnetic hyperthermia, this expression does not give the actual power absorbed by the cells/tissues since the power absorption is mediated by the magnetic nanoparticles and thus it is this magnetic coupling that must be described.

An important consideration for the quantification of the absorbed energy is the penetration depth of the RF radiation. This frequency-dependent parameter is defined as the distance at which the electric/magnetic field intensity reduces to 37% of its surface value. The skin depth will depend on the electrical conductivity and permittivity of the matter, and will set a limit for the depth of the target heating region. However, at the low frequency region of the EM spectrum considered for magnetic hyperthermia (i.e. typically from 100 kHz to 800 kHz) this effect is negligible, since the wavelengths of the corresponding EM waves involved (i.e. from 10 km to 500 m, respectively) are much larger than cellular dimensions [18].

Behaviour of MNPs under EM fields

Many theoretical models about the behaviour of an assembly of single-domain magnetic nanoparticles in an external

alternating magnetic field have been developed along the last years [19–22]. The two main physical situations that are of importance for biomedical applications are:

(1) Single-domain MNPs that are 'physically fixed' (i.e. cannot physically rotate) within some solid medium. In this case, the magnetic moment of each single MNPs that rotates as a consequence of the alternating magnetic field, against the effective magnetic anisotropy of the nanoparticle (mainly due to the particle shape or its crystal magnetic anisotropy).
(2) MNPs that are dispersed in a magnetic colloid of a given viscosity, implying that the particles can physically rotate as a whole. In this situation both the MNPs and the magnetic moment can rotate under the influence of both the torque from the AC magnetic field and the thermal fluctuations (Brownian movement) on the small MNPs by the surrounding liquid.

In either case, the description of the power absorption process is made through the relaxation of the MNPs magnetic moment governed by the thermal fluctuations at finite temperatures, known as the Neel-Brown relaxation process. Currently, general consensus can be found in the literature on what are those physical and magnetic parameters of the nanoparticles that are important for maximising the Specific Power Absorption (SPA) of a magnetic colloid: the SPA dependence on the average MNP volume, magnetic anisotropy, size distribution and surface composition has been extensively studied both theoretically and experimentally [19–23].

A simple model for interpreting the absorption mechanisms, first proposed by Rosensweig [19], was based on the relaxation process occurring when a single-domain MNP was under AC magnetic fields. In this model the Néel and Brown relaxation processes were considered as two independent processes occurring in parallel so that the effective relaxation time was given by Equation 1.

$$\tau_{eff}^{-1} = \tau_B^{-1} + \tau_N^{-1}. \quad (1)$$

The discussion about the details of this model is beyond the scope of this review, and the reader is referred to the excellent publications where the model is improved and the outcomes explored [21,23]. When considering *in vitro* applications of this model the main results are that, for the heat generation from MNPs, at least one of the following conditions must be fulfilled: (1) superparamagnetic behaviour at the experimental frequency used and at room temperature, and/or (2) a small hydrodynamic diameter (e.g. diameters <100 nm for Fe_3O_4) and no aggregation [22]. Therefore it is clear that, because agglomeration in biological media is unavoidable, the heating properties of a given magnetic colloid are likely to change when studied inside cells.

When comparing SPA values from different authors, the field and frequency dependence of this parameter constitute a drawback for obtaining a physical magnitude that could be directly compared. An interesting attempt to overcome this obstacle has been to define an intrinsic loss power (ILP) parameter ($SPA/[H^2.f]$), which offers a system-independent physical magnitude that could allow comparison between different experimental situations [24]. This normalisation, however, has a limited applicability range: it remains valid as long as the RF frequencies of the experiments are within the LF range (i.e. approximately <800 kHz) and the amplitude satisfy $H \ll H_C$, where H_C is the coercivity of the magnetic material.

RF-induced cell death

As discussed in the previous sections, the interaction of radio-frequency (RF) with living organisms critically depends of the frequency range considered. Indeed, electromagnetic waves are continuously being emitted by a number of ubiquitous industrial and communication devices into public areas so that the impact of environmental RF on human health is currently a matter of public health safety.

Therapeutic applications of low frequency RF using magnetic nanoparticles require continuous application of high amplitude magnetic fields during time intervals from minutes to hours, and therefore exposure assessments for these specific fields have to be considered. In particular, dosimetry guidelines have to be yet developed to determine the relevant physical quantities to be measured and also the measurement procedures. The amount of energy absorbed by cells, tissues and organs under low frequency RF is certainly one parameter that must be considered. When a structured tissue or organ is involved, the tissue–tissue or tissue–air interfaces are important since they can both absorb and reflect the RF radiation. In this review only the case of cell cultures will be considered (*in vitro* studies), and because at the low frequency range of RF (i.e. around 100 kHz) implies wavelengths of the order 1–10 km the penetration depth problem for micrometre-range cells is clearly not relevant.

Several names can be found in the literature for describing the biomedical application of RF-based inductive heating using MNPs: magnetic fluid hyperthermia, magneto-thermo-cytolysis, magnetically induced hyperthermia, magneto-thermoablation or intracellular hyperthermia. This lack of consensus reflects both the variety of experimental situations (cell cultures, organs, animals) and the immature state of this novel application. The term magnetic fluid hyperthermia comprises three basic characteristics of this technique: (1) the magnetically driven power absorption mechanisms, (2) the need of a magnetic fluid for achieving heating, and (3) the requirement of increasing the local temperature to achieve the desired therapeutic effects. The latter point will be revised later in this review (see 'General consensus and open issues' section), since several groups have recently reported cell death that can be related to non-thermal effects of the RF radiation.

MFH is currently being used as an approved clinical protocol for cancer treatment, and therefore a fast-growing number of scientific reports on *in vivo* MFH experiments, trials and efficacy analysis are being produced. Reviewing this large body of *in vivo* applications would be beyond the scope of this work, and therefore the reader is referred to the many excellent works on this topic [25–28].

In vitro MFH experiments: current status

Experimental *in vitro* research offers a wide playground where different physical and biochemical scenarios could be tested as a first step in the design of new clinical therapies.

These scenarios include different types of nanoparticles [29], lower/higher values of field amplitude or frequency [30], for example. As mentioned in the introduction, tumour cells have been demonstrated to be more heat sensitive compared to normal cells [10–13]. However, thermotherapy treatment is usually combined with other oncology treatments, such as radiotherapy and chemotherapy due to the fact that the synergistic response observed is higher compared to thermotherapy treatment alone. So firstly, we are going to discuss the use of the MFH combined with other treatments, and later we will focus on the MFH alone which is also the field our group works in and where the per se MFH implication in cell death will be discussed, showing that it is efficient enough to provoke it.

Regarding the synergistic effect of chemotherapy and MFH, thermosensitive magnetoliposomes (ML) charged with paclitaxel and magnetite NPs have been successfully tested as heating agents to kill HeLa cells, showing the synergistic effect of the chemotherapy and MFH [31]. Similar experiments have been reported using ferrogels as thermoresponsive elements. Ferrogels are MNP-loaded, cross-linked networks of some polymers (also known as hydrogels) that can respond to temperature changes [32]. These ferrogels, previously loaded with paclitaxel, have been applied to NIH-3T3 murine fibroblasts with a detectable decrease in cell viability, thus constituting good candidates for cancer treatment [33].

A possible explanation of why combining MFH and chemotherapy results in a synergistic effect has been recently given by Alvarez-Berrios et al. [34] in a study where they have reported that MFH significantly increases cell membrane fluidity relative to hot water hyperthermia and untreated cells, and this could contribute to an enhance potentiation of chemotherapy in MFH-treated cells [34]. These experiments were made using cisplatin and Caco-2 cells, but it would be very interesting to extend this work to different cell lines to assess whether this effect depends on cell type or MFH parameters, or is a general mechanism contributing to the synergistic effect explanation.

Not only has tumour cell death been observed when treating cells with both MFH and chemotherapy, but also stimulation of the immune response has been detected which could be an efficient therapy for treating not only primary cells but also distant metastasis ones. After combined treatment, melanoma cells have been shown *in vitro* to enhance the production of heat shock proteins, specifically Hsp72 which is responsible for triggering the immune response against tumour cells [35]. Related to heat shock protein production, some *in vivo* researches have demonstrated that combining the MFH with a previous increased production of Hsp70 results in a more efficient therapeutic strategy inducing systemic anti-tumour immunity [36]. With these results it can be observed that thermotherapy, apart from causing cell death, is a good strategy for stimulating the immune system against tumour cells, which is a great advance of this treatment compared to classic ones such as chemotherapy and radiotherapy alone.

MFH on its own has also been demonstrated as a convenient approach to killing cancer cells. For this application to work it is necessary to assess some previous *in vitro* studies concerning the internalisation of MNPs in the cells, determining the final fate of the particles inside the cell, as important data which will determine the cell death pathway, and also for analysing how the MNP uptake affects the biology of the cells in terms of viability. A successful *in vitro* application using Poly(lactic-co-glycolic acid) PLGA-functionalised magnetic nanocapsules has been proven against neoplastic 4T1 and MCF-7 breast cell lines [37], for which apoptotic cell death was reported. Also, experiments using Herceptin-conjugated MNPs delivered to SK-BR-3 cells have shown that, after exposure to an alternating magnetic field (AMF), these cells died mainly by apoptosis [38]. Regarding *in vivo* applications, recent reports have demonstrated that mice with pancreatic cancer locally injected with 20-nm MNPs after receiving MFH for 30 min suffered from a localised temperature increase up to 47–51 °C, which caused inhibition of further tumour growth and survival prolongation [39]. Similarly, iron oxide-based nanocubes of about 100 nm have been used to successfully eradicate cancer cells through caspase-mediated apoptosis and to reduce tumour growth [40]. Folic acid, a well-known targeting ligand for breast cancer cells, has been used to functionalise MNPs for simultaneous drug delivery and MFH [41]. However, although SPA values reported for these bifunctional MNPs in the pure colloid were quite large, no actual temperature measurements were made of the cell cultures during AMF application. Recently, a novel approach has been proposed to use the rise in temperature from MFH experiments to induce controlled gene expression [42]. Although this strategy could be used to trigger the apoptotic self-destruction cell mechanism, it is clear that the concept has more appeal for gene-based therapies. Either way, the use of this approach in therapies will require the co-encapsulation of cells and MNPs, and the modification of the cells to express therapeutic genes under heat-inducible promoters.

Summarising the above findings, it can be said that considerable evidence has been collected that supports the *in vitro* synergistic effect of MFH and different cytostatic drugs, so that more effective and selective clinical applications are envisaged.

General consensus and open issues

As discussed in the section ‘Behavior of MNPs under EM fields’ above, notable advances have been produced during the last few years on theoretical models explaining the heating mechanisms of MNPs when submitted to AC magnetic fields [20–22,43]. However, the translation of these models into *in vitro* hyperthermia is far from trivial. One of the reasons for this difficulty is the lack of precise knowledge of the final state of the MNPs once they enter the cell, such as for example the degree of agglomeration; the hydrodynamic size, and/or the final intracellular distribution [44]. It is nowadays accepted that the idea of individual MNPs entering the cells during uptake is an over-simplification, since the composition of the cell culture medium yields to opsonisation that in turn results in agglomeration and larger hydrodynamic sizes [45]. Fortunately, uptake mechanisms in most cell types are capable of dealing with large cluster sizes, even micrometre-sized ones as reported by Baba et al. [46]. Because of the complex nature of the interaction between MNPs and biological media, there are still many aspects of MFH that

remain yet to be explored relating to how the local power release of the MNPs could affect cell structures and biological pathways in *in vitro* MFH. It is accepted that nanoparticle–cell interactions are influenced by the presence of proteins absorbed from biological fluids on the nanoparticle, but the effects of nanoparticle surface on protein adsorption is not yet fully understood, and these question must be answered before a complete understanding of the MNP–cell dynamics is established.

The knowledge of the final intracellular distribution of MNPs (before and after MFH) is another key piece to get precise understanding of the biological mechanisms involved. Detailed studies in this regard are scarce, so the question of whether the uptake MNPs are incorporated into or attached to subcellular structures/organelles is still open. From the growing literature on diverse biological systems, it is becoming clear that the fate of intracellular MNPs will strongly depend both on the type of MNP formulation and the cellular line involved. Changes in these parameters are likely to modify the MNPs' heating properties, and although some models for MNP clusters have been proposed [47], there are actually no complete theoretical formulations for the heating properties of intracellular clusters. Because this '*in vitro* effect' of MNP clustering will be present for virtually any cell-based experiment, considerable caution has to be taken when discussing the heating properties of intracellular MNPs in terms of their properties as free particles in the magnetic colloid.

Irrespective of the detailed characteristics of the heating mechanisms by MNPs under AC fields, the effects of hyperthermia are known to complement those of radio- or chemotherapy in the sense that tumour cells are radio-sensitised by heat. Since the first studies on the effect of heat on the radioresistant S-phase cells associated with the induction of chromosomal disturbances, a large number of *in vitro* hyperthermia experiments have allowed the identification of the thermodynamic basis of this process [48]. For example, from the thermodynamics of cell death progression as a function of temperature, it is known that increasing the temperature by 1 °C requires a decrease of treatment time by a factor 2 for an isoeffect. From this relationship, the obtained value of 140 kcal/mol for the activation energy can be related to protein denaturation as one possible mechanism during hyperthermic damage. Since the thermal effects play a role in hyperthermia, a precise quantification has been proposed by the concept of 'thermal dose', as a key factor to determine its efficacy [49]. The standard expression for the thermal dose involved in hyperthermia protocols is based on a time–temperature equivalency relation to a reference temperature, namely the cumulative equivalent minutes at 43 °C (CEM_{43}). Several theoretical models have been proposed for analysing mild-temperature thermotherapy experiments, including advanced numerical models based on parallel processes to analyse the thermal damage [50]. As for intracellular heating using MNPs, no theoretical model is yet available to describe the key parameters that determine the heating efficiency for a given type of MNPs when incorporated by a cell line. Moreover, it is not clear whether a single model could apply for different cell types, or to what extent the cell function can determine what are the key parameters to take into account [51]. Using an ingenious experimental design for *in vitro* hyperthermia, Fortin et al. [52] first demonstrated that the magnetic properties of MNPs determine the efficacy for intracellular heating, and also that the heating efficiency of MNPs can be modified by the change of physicochemical parameters of the intracellular medium [53].

Table II. Experimental *in vitro* results of MNP-based magnetic hyperthermia on different cell types.

Cell line	T-increase	f (kHz)	H (kA/m)	Reference
A549 and MDA-MB-231	Yes	386	6	[54]
ECA-109	Yes	110	50–110	[55]
Caco-2 and MCF-7	Yes	238	20	[56]
MCF-7	Yes	250	N/A	[46]
Caco-2	Yes	237	20	[34]
BT20	Yes	520	13.2	[57]
PC3	Yes	700	24.7	[58]
WEHI-164	No	265	26.7	[59]
MDA-MB-468 and MCF-7	No	233	37.5	[60]
He-La	No	100	11.9	[61]
MDA-MB453	No	148	87.5	[62]
Dendritic cells	No	260	12.7	[63,64]

A most significant difference among the available data on *in vitro* hyperthermia is related to the observation of non-thermal effects during the experiments: the absence of temperature increase up to the threshold temperature required for apoptosis could imply that direct effects from the RF radiation on the cellular medium are taking place. Some of these works on diverse cell lines are listed in Table II together with the corresponding AMF parameters in each case. In all cases, the fall of cell viability from moderate to nearly complete was reported for cells previously loaded with MNPs and then exposed to AMF. Two aspects must be taken into account; the presence or absence of non-thermal effects and the necrotic or apoptotic cell death. Some authors have described necrotic cell death when cells were incubated with 12-nm MNPs and exposed to AMF (6 kA/m, 386 kHz) for 30 min, achieving higher cell death rates compared to 30 min of hot water hyperthermia (46 °C) [54]. Again, differences in cell death when comparing MFH and hot water hyperthermia are shown in Caco-2 and MCF-7 cells, observing an apoptotic cell death mechanism after MFH and a cell-type-dependent significant reduction in cell viability by MFH when compared to hot water hyperthermia [56].

Based on the known differences in metabolic pathways of MNPs for different cell types, it seems reasonable to assume that the cell death mechanisms involved during MFH protocols should depend on the cell line investigated. It is also likely that some experimental parameters such as the amount of MNPs incorporated per cell, and the amplitude and frequency of the AMF, could trigger different mechanisms for cell death. Therefore attempts to merge different experimental data onto a 'universal cell death mechanism map' are neither possible nor useful.

The previously discussed differences in cell death mechanisms observed after MFH or hot water bath, for the same cell type and experimental conditions, strongly suggest that cell killing by AMF is not just a matter of temperature increase, but an AMF-triggered mechanism could be also present, yielding a different cell death pathway. This is

consistent with the observation, reported by several groups, of measurable cell death after AMF treatments without observing any macroscopic temperature increase. For example, Villanueva et al. [61] have shown that MFH experiments on HeLa cells using perovskite-based MNPs resulted in apoptotic cell death after 30 min of AMF ($f = 100$ kHz, $H = 15$ mT) while they observed a temperature increase in the culture medium of less than 0.5 °C. Also, longer exposures to AMF (i.e. up to 2 h at 37.5 kA/m, 233 kHz) resulted in a significant reduction in cell viability (99%) for MDA-MB-468 and MCF-7 cells loaded with magnetic material without a perceptible temperature rise [60,61].

Experiments on WEHI-164 cells loaded with magnetite-based MNPs exposed to AMF (265 kHz, 26 kA/m) for 10 min, have demonstrated a decrease of cell viability with a concurrent temperature increase of only 5.5 °C from the initial value (28 °C) of the culture medium [59]. Similar results have been reported in magnetically loaded dendritic cells (DCs) when submitted to AMF (260 kHz and 12.7 kA/m) for 15 min; in this case almost 100% of cell death was observed with only a 1 or 2 °C temperature increase of the cell culture [65]. Moreover, the amount of cell death could be controlled by changing either the amplitude or the application time of the AMF. Interestingly, the toxicity of the supernatant of AMF-exposed cells was demonstrated to have the potential to kill control cells (see Figure 1) [64]. To explain these results, it was proposed that the MNPs confined to endocytic vesicles/lysosomes can provoke the disruption of the vesiclés membranes during AMF exposure, releasing the toxic vesiclés content to the cytoplasmatic region and triggering cell death.

Non-thermal effects have also been reported to occur when tissues involved in fractures or wounds were subjected to low frequency pulsed electromagnetic fields [66]. The mechanisms for these RF waves to improve cell response seem to be related to calmodulin (CaM)-dependent nitric oxide signalling pathways. In a series of meticulous experiments on human articular chondrocytes, Pilla et al. [67] showed that a low frequency RF pulse can be designed and configured as a non-thermal trigger to act as first messenger in CaM-dependent signalling pathways (including NO and cyclic nucleotides) relevant to tissue growth, repair and maintenance. Within a different experimental framework, non-thermal effects of low/medium frequency AMF have also been reported to affect cell membrane integrity in MDA-MB-453 breast cancer cells [62]. Using large AMF amplitudes ($f = 148$ kHz, $H = 87.5$ kA/m), Thomas et al. demonstrated that cell-bounded MNPs can produce a modest increase in cell membrane permeability by non-thermal mechanisms. However, subsequent results from the same group [68], using DU145 prostate cancer cells, revealed that when these cells having up to 199 pg(Fe)/cell were submitted to AMF, the observed heating of the pellet (up to approximately 43 °C) scaled consistently with that expected from a heat diffusion model of continuous media. However, a continuous media model seems too naïve to account for the complexity of the cell response. For example, changes in cell membrane fluidity due to hyperthermia have been suggested to trigger a stress response which could influence the heat shock response and thus thermoresistance of tumoral cells, affecting the efficacy of the synergistic cancer therapies [65,69].

A possible explanation for the results discussed above is based on a non-thermal mechanism recently proposed by Carrey et al. [70]. This group has shown theoretically that MNPs can generate ultrasound waves when subjected to AMF, provided some general conditions are fulfilled. If experimentally corroborated, the production of ultrasound

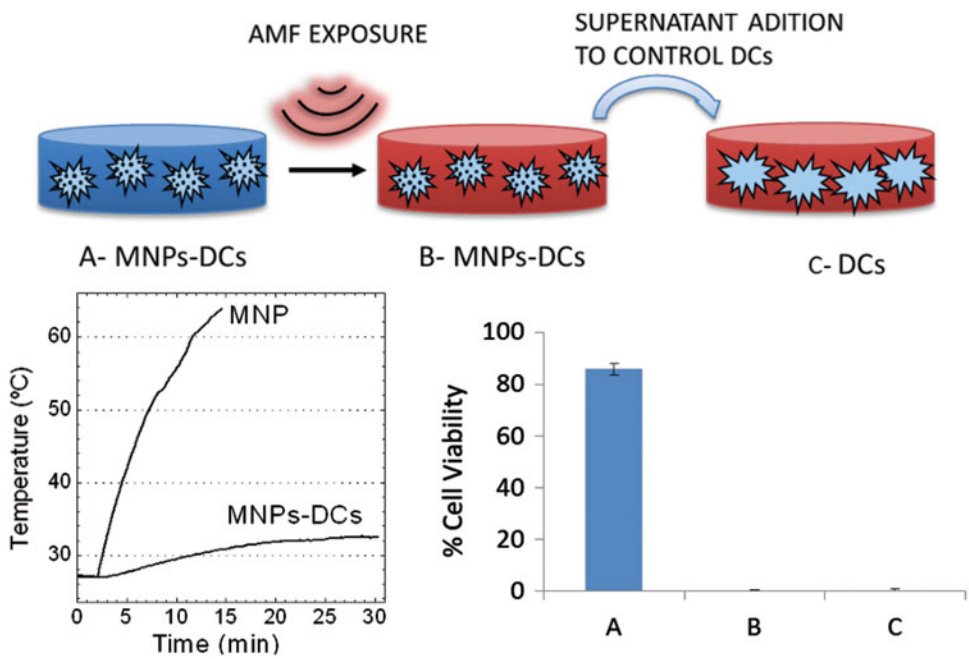

Figure 1. (Upper panel) Drawing of the proposed experiment for testing the toxicity of the cell culture supernatants loaded with NPs after AMF exposure. The sample A was submitted to AMF and the supernatant from the treated cells (sample B) was collected. The sample C consisted of viable DCs without any contact from MNPs or AMF that received the supernatants from the B samples and were incubated 30 min. Lower left panel: Temperature increase observed for pure magnetic colloid (MNP) and magnetically loaded cell culture (MNPs-DCs) under the same AMF. Lower right panel: Cell viability as measured by TB staining 15 min after AMF application. Adapted from Asin et al. [64].

waves by MNPs under an AMF could open new possibilities to the therapeutic applications based on this new paradigm.

The body of experimental results discussed above suggests that for a given cell type and experimental conditions, either thermal or non-thermal mechanisms could be triggered by the application of AMF. Although not yet identified, it seems plausible that the experimental conditions yielding one or another process would be cell-type dependent. For those cells having the capacity to incorporate large amounts of MNPs, it is likely thermal effects are to be observed due to classical heat diffusion mechanisms. On the other hand, as the MNPs are capable of affecting those cell membranes by which they are bounded, this could result in non-thermal cell death mechanisms through disruption of vital pathways of the cell metabolic network.

As a final remark, it is interesting to mention that the ultimate *in vivo* application of these mechanisms could have a profound impact on nanomedicine. Such a conceptual breakthrough on clinical protocols will probably be based on the design of new nanomaterials for specifically designed properties on target cells and tissues [71–77].

Conclusions and perspectives

From the compilation of experimental results presented above and the background of models of specific power absorption in MNPs, it can be inferred that there is still much room to improve the heating efficiency of MNPs, maybe orders of magnitude, by further theoretical investigation of the SPA model as well as future improvements in chemical synthesis. From the point of view of biomedical applications, it is also clear that our understanding of MFH at the cell level is far from complete. So far, most research about MFH on living organisms has been conducted *in vitro*, but the number of *in vivo* studies is growing. There are several promising areas for future MFH research, including multifunctional devices composed of magnetic cores and a releasable cytotoxic drug on the surface aimed at specific tumour therapies. At the cellular level, experimental evidence supports that acting locally on cell membranes by the power released from previously attached MNPs is possible. In more general terms, both thermal and non-thermal effects of the MNPs under AMF application have been observed, and it is yet to be determined whether these effects have the same physical origin and mechanisms. If these mechanisms could be understood and controlled in the future, the potential of magnetic hyperthermia will allow us to expect great breakthroughs in biomedical science.

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Declaration of interest

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