Neuro-Cytotoxic profile of Poly Vinyl Pirrolidone (PVP) Iron Oxide nanoparticles, an in vitro assessment in SH-SY5Y cell line

Relatore:

Prof. Teresa Coccini

Tesi di Master

Dr. Lenin Javier Ramiréz Cando

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Abstract

Magnetic Iron Oxide nanoparticles as magnetite ones (Fe₃O₄ NP), due to their physic-chemical and superparamagnetic properties, are widely used in various industrial applications such as: audio speaker devices, position sensors, water purification (heavy metal elimination); and medical such as: drug and gene delivery, imaging, contrast agent. Although, several studies suggest that Fe₃O₄ NP may arrive to central nervous system (CNS) independently of the route of administration (i.e. inhalatory, intravenous or intra peritoneal), and there is a lack of data about molecular interaction of these NPs with cerebral cells. Actually, scientific evidence indicates that the presence of iron ions, iron oxides and iron oxide nanoparticles into nervous system tissues are linked to several neurodegenerative diseases (i.e. Alzheimer; Parkinson).

The aim of this work was to assess in vitro Poly Vinyl Pirrolidone (PVP) coated magnetic Iron Oxide nanoparticles (PVP-Fe₃O₄; Ø 20nm) toxicological profile and their mechanism of action, which was fixed over human derivate neuronal cell line (SH-SY5Y). PVP-Fe₃O₄ NPs inducted biological effects were evaluated after 4, 24, 48 hours at crescents doses (1-100 μg/mL) using the following endpoints: i) membrane integrity using calcein-AM/PI assay; ii) mitochondrial activity by MTT assay; iii) cellular morphology through phase contrast microscopy. Additionally, cellular uptake of PVP-Fe₃O₄ NPs was evaluated by Prussian blue assay.

**Membrane integrity**: PVP-Fe₃O₄ NPs have produced no effect over SH-SY5Y cellular membrane for every dose (1-100 μg/mL) and times (4-48 hours) evaluated.

**Mitochondrial activity**: SH-SY5Y cells have shown alteration over mitochondrial function only after 48 hours of exposure. Starting at 10 μg/mL with a decrease of cellular vitality of 25%, and a maximum decrease of 35% at highest dose (100 μg/mL).
Cellular morphology: SH-SY5Y cells have evidenced no alteration after 48 hours of exposure at doses equal and lower that 50 μg/mL, whereas at 100 μg/mL, the presence of precipitates has masked microscopy and cells were less visibly.

PVP-Fe₃O₄ NPs uptake: a doses- time depend accumulation has observed: blue spots have been founded at 10 μg/mL after 4 hours of exposure.

Mitochondria are apparently the target in SH-SY5Y cells: considering the toxic effect produced by PVP-Fe₃O₄ NPs after 48 hours exposure in a dose-time dependent manner and was evident only at MTT assay (which evaluates mitochondrial activity). Membrane integrity was not visually affected for PVP-Fe₃O₄ NPs exposure, low dead cell ratios have been observed that may be due to a normal apoptotic process.

Riassunto

Le Nanoparticelle magnetiche di ossido di ferro come la magnetite (Fe₃O₄ NP) grazie alle loro proprietà fisico-chimiche e superparamagnetiche, vengono ampiamente utilizzate in numerose applicazioni industriali quali: audio speaker, sensori di posizione, purificazione dell’acqua da metalli pesanti; e mediche come: drug o gene delivery, imaging, agenti di contrasto. Sebbene vi siano evidenze sperimentali che Fe₃O₄NP possa raggiungere il sistema nervoso centrale (SCN), indipendentemente dalla via di somministrazione (es inalatoria, intravenous o intraperitoneale), pochi sono ancora i dati relativi alle interazioni molecolari di queste NP nelle cellule cerebrali. Prove scientifiche indicano che la presenza di ioni ferro, ossidi di ferro e nanoparticelle di ossido di ferro nel SNC sono correlate con malattie neurodegenerative (es Alzheimer; Parkinson).

Lo scopo di questo studio e stato quello di valutare in vitro il profilo tossicologico e il meccanismo di azione di nanoparticelle di magnetite revestite con Poly Vinyl Pirrolidone (PVP-Fe3O4 NP; Ø 20nm) su una linea cellulare neuronale di derivazione umana (SH-SY5Y) mediante una bateria di test. Gli effecti biologici
indotti da PVP-Fe$_3$O$_4$ NP sono stati valutati dopo 4, 24, 48 ore a dose crescente (1-100 µg/mL) mediante la determinazione di: i) integrità di membrana con colorazione calceina-AM/PI; ii) attività mitocondriale con MTT assay; iii) morfologia cellulare in microscopia a contrasto di fase. È stato inoltre valutato l’accumulo intracellulare di PVP-Fe$_3$O$_4$ NP mediante colorazione con Blu di Prussia.

**Integrita di membrana:** Non è stato osservato alcun effetto di PVP-Fe$_3$O$_4$ NP sulle membrane delle cellule SH-SY5Y per ogni dose testata (1-100 µg/mL) e per tutti i tempi considerati (4-48 ore) testati.

**Attività mitocondriale:** la funzionalità mitocondriale delle cellule SH-SY5Y è alterata solo dopo 48 ore a partire dalla dose di 10 µg/mL con un decremento della vitalità cellulare pari a 25% con un maximo di decremento di 35% alla dose più alta testata (100 µg/mL).

**Morfologia cellulare:** le SH-SY5Y non mostrano nessuna alterazione morfologica fino alla dose di 50 µg/mL dopo 48 ore, mentre alla dose di 100 µg/mL, a causa dell’abbondante presenza di precipitati di PVP-F$_3$O$_4$NP le cellule erano poco visibili.

**Uptake di PVP-Fe$_3$O$_4$NP:** L’accumulo di PVP-Fe3O4NP all’interno delle SH-SY5Y è dose- e tempo-dipendente: spot blu iniziavano ad essere visibili dalla dose di 10 µg/mL già dopo 4 ore.

Le mitocondrie sembrano essere il bersaglio nelle cellule SH-SY5Y: visto che il effetto tossico prodotto da PVP-Fe$_3$O$_4$ NP dopo 48 ore solo e evidente solo nel MTT assay quello che valuta la attività mitocondriale. Gli altri endpoint valutati non hanno presentato un rapporto tra la dose e la presenza di uno effetto tossico. Si evidenza bassi livelli di cellule morte nella Calceina AM/PI assay, quelo debuto al normale all’apoptosi cellulare.
1. Introduction

Nanotechnology is the science that deals with matter at the scale of 1 billionth of a meter \((10^{-9} \text{ m} = 1 \text{ nm})\), and it is also the study of manipulating matter at the atomic and molecular levels. Image 1 illustrates the relationship of natural and anthropogenic substances, which are involved in this scale and are compared with the micro world that is as smaller as we cannot see it without technological support. A nanoparticle is the fundamental component in the fabrication of a nanostructure, and is far smaller than the world of everyday objects that are described by Newton’s laws of motion, but bigger than an atom or a simple molecule that are illustrated by quantum mechanics’ laws (Horikoshi and Serpone 2013).

Image 1. Scale comparison of materials, comparing natural and handmade objects taken from Buzea et al., 2007.

To make clear the concept: Nanoparticles are engineered particles with , at least, one of their three dimensions between 1-100 nm witches has unusual physic-chemical proprieties (i.e. High hydrodynamic diameter, lower melting
point, etc.) (Jiang et al., 2014; Arora et al. 2012). In any case that definition should not use to describe particle material produced in natural or incidental way. However, how a nanoparticle is viewed and defined depends on the specific application. In this regard, Table 1 summarized definitions of nanoparticles and nanomaterial from principal standards, health and safety institutions worldwide.

Table1. Definitions of nanoparticle and nanomaterial by various organizations: International Organization for Standardization (ISO), American Society of Testing and Materials (ASTM), National Institute of Occupational Safety and Health (NIOSH), Scientific Committee on Consumer Products (SCCP), British Standards Institution (BSI), and Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA). Modified from Horikoshi and Serpone (2013).

<table>
<thead>
<tr>
<th>Organization</th>
<th>Nanoparticle definition</th>
<th>Nanomaterial definition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO</td>
<td>A particle spanning 1-100 nm (diameter)</td>
<td>-</td>
<td>ISO/TS 27687:2008</td>
</tr>
<tr>
<td>ASTM</td>
<td>An ultrafine particle whose length in 2 or 3 places is 1-100 nm</td>
<td>-</td>
<td>ASTM E2456-06</td>
</tr>
<tr>
<td>NIOSH</td>
<td>A particle with diameter between 1 and 100 nm, or a fiber spanning the range 1-100 nm.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCCP</td>
<td>At least one side is in the nanoscale range.</td>
<td>Material for which at least one side or internal structure is in the nanoscale</td>
<td>SCCS/1484/12</td>
</tr>
<tr>
<td>BSI</td>
<td>All the fields or diameters are in the nanoscale range.</td>
<td>Material for which at least one side or internal structure is in the nanoscale</td>
<td>PAS 71:2011</td>
</tr>
<tr>
<td>BAuA</td>
<td>All the fields or diameters are in the nanoscale range.</td>
<td>Material consisting of a nanostructure or a nanosubstance</td>
<td>-</td>
</tr>
</tbody>
</table>

Nowadays, nanoparticles have become trendsetter materials, every day we could find a new discovery or application development around nanoparticles. Actually, there is a catalogue of commercial nanoparticles for example: NanoGard™ LL Zinc oxide, Zinc oxide NanoTek™, NanoArk™ cerium oxide,
NanoArk™ Copper oxide, and so on. They are used as biosensors or iron nanoparticles against cancer and more, these are examples of their multifunctional uses. In general, biotechnology and biomedicine are two of the most highlight fields to apply nanoparticles technology.

1.1. **History of nanoparticles**

Art and science are apparently no linked disciplines with different tasks, tools and schemes. Painting is a kind of the creative activity, where the most surrealistic human ideas and visions can be expressed fairly materialistically through color. Color can be taken either as a sensorial perception or as a physical phenomenon. However, it is directly related to the chemical and structural character of the material; therefore the same question, ‘Why it looks green or red?’ implies totally different sense for historical and natural scientists (Hadril et al., 2003). In particular, the optical property is one of the fundamental attractions and a characteristic of nanoparticles (e.g. a 20 nm diameter gold nanoparticle has a characteristic wine red color, a silver nanoparticle is yellowish gray, and platinum and palladium nanoparticles are black).

Not surprisingly, nanoparticles have been used from immemorial times in sculptures and paintings even before the 4th century AD, due to their optical characteristics. One of the most famous examples is the Lycurgus cup (4th century AD) illustrated in Image 2, Freestone and co-workers (2007) have described it that is made of a very special type of glass, known as dichroic glass, that changes color when held up to the light. The opaque green cup turns to a glowing translucent red when light is shone through it internally (i.e., light is incident on the cup at 90° to the viewing direction). Analysis of the glass revealed that it contains tiny (∼70 nm) metal crystals of Ag and Au in an approximate molar ratio of 14:1.
In the last century, the development of various techniques in microscopy, chemistry and physics, which contributed to know, design and apply of new technologies, was used to develop nanoparticles and nanomaterial technologies. Industrial production of nanomaterials saw its origins in the 20th century. For example, nanoparticles of black carbon (tire soot) have been used in the tires fabrication or pigments such as SiO₂ and TiO₂ have been prepared by high-temperature combustion method. The era of nanoscience began with the Nobel award winner, Richard Feynman, in 1959. Ever since, and thanks to the development of highly sophisticated characterization methods at the nanoscale, both the research and the technological aspects of nanoscience have grown explosively, particularly during the last two decades (Mahmoudi et al., 2011).

1.2. Characteristics and proprieties of nanoparticles

Nanoparticles exhibit unconventional or enhanced physico-chemical properties, which are not encountered in the corresponding bulk materials (e.g.,
lower melting points, higher specific surface areas, specific optical properties, mechanical strengths, and specific magnetizations). Their physical and chemical properties depend not only on their composition, but also on the particle size, shape and aggregation (Jiang et al., 2014).

All nanoparticles have different properties that might catch attention in various industrial fields. The intrinsic properties of nanocrystals, as an example, are mainly determined by their composition, crystallinity, and structure. It is of great importance to investigate the structure-sensitive catalytic activity for obtaining a profound understanding of catalytic processes. Much success has been achieved in the investigation of the structural effects of nanocrystals on catalysis and the results showed that the catalytic performance could be specifically regulated either by the crystal size or morphology with distinct crystallographic planes (Liu et al., 2011; Asati et al., 2009).

In general, the most relevant characteristics of nanoparticles are summarized below; **thermal function:** when the particle diameter is small (less than 10 nm), the melting point is also lower than a bulk metal. For example, electronic wiring can be made with nanoparticles that have a low boiling point; **electrical function:** since superconductivity transition temperature rises so that particle diameter is small (less than 1 nm), it can be used to make high temperature superconductivity material; **mechanical function:** since the mechanical characteristics improve, mechanical strength can be sharply raised by mixing the nanoparticles with metals or ceramics; **magnetic function:** the attractive force of a magnetic metal increases on reduction of the particle diameter, such that soft-magnetic materials can be made in the form of an alloy of nanoparticles (Asati et al., 2009).

Combinations or mixes of nanoparticles can also modify some properties, i.e. Kallen and co-workers (2014) have demonstrated that combined oxides of; iron, manganese and silica can give highly reactive oxygen carriers with the ability to release oxygen. This opens two interesting options. First, could be the use of
naturally minerals, as it is common to find manganese minerals with high concentrations of iron and silica. Second, the possibility is of course the manufacture of highly reactive oxygen carrier materials (nanoparticles) from low cost raw materials.

1.3. Magnetic nanoparticles

Most materials found in Earth are generally thought of as being nonmagnetic, for example, either diamagnetic (repelled weakly from a magnetic field, as is water and almost any fatty substance) or paramagnetic (weakly attracted to a magnetic field, as is deoxyhemoglobin in blood cells). For these types of materials, the direct physical influence of the earth’s magnetic field is extraordinarily weak (Kirschvink et al., 1992). However, nanomaterial’s magnetic property is based on its magnetic susceptibility, which is defined by the ratio of the induced magnetization to the applied magnetic field. The susceptibility of the material depends on their temperature, external magnetic field and atomic structure (Indira & Lakshmi, 2010).

Some of the most relevant magnetic nanoparticles are listed below: iron oxides (Fe₃O₄, Fe₂O₃, and γ-Fe₂O₃), iron-palladium and cobalt, ferrimagnetic spinels, cobalt ferrite, CoFe₂O₄, Mn-Zn and Mn-Zn-Gd ferrite particles, copper nickel, ferromagnetic perovskites La₁₋ₓSrₓMnO₃, Ni(1₋ₓ)Crₓ, gadolinium-, calcium-, and lanthanum complexes, and ferrimagnetic SrFe₁₂O₁₉/γ-Fe₂O₃ composites (Mahmoudi et al., 2011).

In small sizes, around 10 nm, ferric and ferromagnetic materials such as magnetic nanoparticles (MNPs), that maintain a large magnetic moment, show the phenomenon of superparamagnetism. It consists in not keeping magnetized after the action of a magnetic field, offering advantage of reducing risk of particle aggregation (Indira & Lakshmi, 2010). MNPs have served for applications with sizes from nanoscale up to bulk for hundreds years because of their unique
properties such as superparamagnetism, high field irreversibility, and high saturation field. MNPs have their own advantages that provide many exciting opportunities in biomedical applications (Chi et al., 2012).

These advantages are: first, they deliver controllable sizes ranging from a few nanometers up to tens of nanometers, so the optimization of sizes and properties easily matches with the study of interest; second, the movement of nanoparticles can be manipulated by an external magnetic force, which provides tremendous advantages for many applications involving biological detection (Gao et al., 2010), diagnosis (Chi et al., 2012; Yiu et al., 2011), and drug delivery (Chomoucka et al., 2010); third, magnetic nanoparticles have the ability of magnetic resonance contrast enhancement because the signal of magnetic moment of a proton around magnetic nanoparticles can be captured by resonant absorption (Karimi et al., 2013; Lu et al., 2007). Due to their unique and promising properties, the exploiting of magnetic nanomaterials as nanoprobes to detect bacteria, virus, proteins, and other active biomolecules has attracted high research interests (Xie et al., 2009; Perez et al., 2004).

1.4. Iron oxides

The most common iron oxides phases are FeO (wüstite), Fe₃O₄ (magnetite; hematite) and γ-Fe₂O₃ (maghemite) (Santos et al., 2009). These oxides are often used as: magnetite thin films lend themselves to room temperature applications in the construction of different devices such as tunneling magnetoresistance, giant magnetoresistance and magnetic random-access memory devices; maghemite is used in magnetic resonance imaging, magnetic recording media, fabrication of biocompatible magnetic fluids, and electrochromic devices; hematite nanostructures have been explored in the development of electrochromic devices, as cathodes in lithium batteries, and in the construction of photoelectrochemical systems to produce hydrogen from water using solar
radiation; thin films of wustite/maghemite have been used in solar radiation filters (Martinez et al., 2009; Santos et al., 2009).

During welding, production, synthesis and other manufactory procedures that involve iron oxides or iron oxides precursors (i.e. iron, still and iron minerals), iron oxides fumes arise as a result of the process chain. The presence of those fumes represents a potential risk for the human health. According to New Jersey Department of Health and Senior Services, iron oxides fumes can cause metal fume fever. This is a flu-like illness with symptoms of metallic taste, fever and chills, aches, chest tightness and cough. The principal way of exposure is by inhalation, the primary acute effect of exposure is irritation of nasal passages, throat and lungs. Long-term exposure may cause iron pigmentation of the lungs, a condition known as siderosis.

According to National Institute for Occupational Safety and Health (NIOSH), there is no information about skin and eyes irritation, mutagenicity and reproductive toxic effects. NIOSH presents acute toxicity data in the Registry of Toxic Effects of Chemical Substances (RTECS). In this report; the lowest published toxic dose, subcutaneously injected in rat, was 135 mg/kg, and tumors at site of application were detected; the lowest published toxic concentration given by inhalation to rat was 50 mg/m$^3$/12 hour, and the most relevant effects were excitement, fluid intake, hypermotility and diarrhea, after 60 hours at the same dose level the effects were equals; the lowest published toxic dose given intratracheally in rat was 12 mg/kg, and changes in lungs, thorax, respiration rate were observed, that associated with enzymatic inhibition, and changes in serum composition. All doses were evaluated using Iron Oxide Fume (as Fe$_2$O$_3$).

In human volunteers, the acute intrapulmonary instillation of small ferric oxide particles induced a transient subclinical inflammation that resolved within four days (Lay et al., 1998) and the inhalation of breathable iron oxide particles at an average concentration of 12.7 mg/m$^3$ for 30 minutes by human volunteers was reported to not significantly affect alveolar epithelial permeability, lung
diffusing capacity, or pulmonary function (Lewinski et al., 2013; Lay et al., 2001).

Occupational Security and Health administration (OSHA), NIOSH and other safety organizations, which are involved in human health and security, have established limit values for occupational exposure to iron oxide fumes that are summarized in Table 2. NIOSH also have released an Immediately Dangerous to Life or Health (IDLH) concentration: 2,500 mg/m³ (established as iron).

Table 2. Exposure limits and health effect of iron oxide (established as Fe₂O₃) in different exposure sceneries, summarized by hours of exposure (HE) and limit values, information taken directly from OSHA official web site.

<table>
<thead>
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<th>Limit Values</th>
<th>HE Codes</th>
<th>Health Factors and Target Organs</th>
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<tr>
<td>OSHA Permissible Exposure Limit (PEL)-General Industry</td>
<td>10mg/m³ TWA</td>
<td>HE10</td>
<td>Pulmonary siderosis (benign pneumoconiosis)</td>
</tr>
<tr>
<td>OSHA PEL - Construction Industry</td>
<td>10 mg/m³ TWA</td>
<td>HE10</td>
<td>Pulmonary siderosis (benign pneumoconiosis)</td>
</tr>
<tr>
<td>OSHA PEL - Shipyard Employment</td>
<td>10 mg/m³ TWA</td>
<td>HE10</td>
<td>Pulmonary siderosis (benign pneumoconiosis)</td>
</tr>
<tr>
<td>National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Limit (REL)</td>
<td>5 mg/m³ TWA</td>
<td>HE10</td>
<td>Pulmonary siderosis (benign pneumoconiosis)</td>
</tr>
<tr>
<td>American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV)(2006)</td>
<td>5 mg/m³ TWA (respirable fraction) ; A4</td>
<td>HE10</td>
<td>Pulmonary siderosis (benign pneumoconiosis)</td>
</tr>
<tr>
<td>CAL/OSHA PEL</td>
<td>5 mg/m³ TWA</td>
<td>HE10</td>
<td>Pulmonary siderosis (benign pneumoconiosis)</td>
</tr>
</tbody>
</table>
1.5. Iron oxide nanoparticles

The rich history of scientific interest in Iron Oxides Nanoparticles (IONPs) has been fuelled by valuable applications taking advantage of catalytic, electronic, and magnetic properties of these materials. At present, IONPs are part of nanomaterial’s science and engineering and they, in particular, are developed based on their unique properties: colloidal stability, hydrodynamic diameter (HDD) and capacity to response to magnetic fields (superparamagnetism) (Zhang et al., 2014). However, IONPs are actually in process of development in order to obtain new applications reducing their potential toxic effect.

1.5.1. Translocation of iron oxide nanoparticles

In order to achieve an effective level of nanoparticles in the target tissue or tumor site, targeted nanoparticles should translocate from circulating blood to the tissue of interest and bind to its molecular target as a first step in nanoparticle retention or cellular internalization (Sibov et al., 2012; Shubayev et al., 2009). Unfortunately, many types of systemically injected nanoparticles are rapidly cleared from the blood stream by the reticuloendothelial system (RES) and the mononuclear phagocytic system (MPS) mainly through liver, spleen, and bone marrow resulting in a low therapeutic index (Lee et al., 2010).

Weissleder and co-workers (1989) have illustrated the full body distribution of IONPs in an in vivo study. In this work; one hour after oral administration of IONPs (superparamagnetic iron oxide AMI-25; diameter 80 nm), radio-labeled with $^{59}$Fe, to rats (1 mg as Fe/kg), the majority of the administered dose was sequestered in the liver and spleen, the highest concentrations of Fe were found in liver after 2 hours and in the spleen after 4 hours. Fe was slowly cleared from liver (half-life, 3 days) and spleen (half-life, 4 days) and was incorporated into hemoglobin of erythrocytes in a time-dependent fashion. In another in vivo study, Wu and co-workers (2012) have evaluated the Central Nervous System (CNS)
regional distribution of IONPs (bare Fe₃O₄-NPs; diameter 30 nm) observing their presence in rat brains following IONPs intranasally instillation for seven days. The particles were found to be deposited at particularly high concentrations in the rat striatum and hippocampus. Over half of the IONPs were retained in the striata for a minimum of fourteen days, and induced local oxidative damage.

IONPs are cleared by human alveolar macrophages, which measured between 14 to 21 μm and thus can engulf particles of a size comparable to their own dimensions, but are significantly less effective with particles that are much larger or smaller (Shen et al., 2011; Buzea et al., 2007). Peters and co-workers (2006) explained that compared with larger particles, nanoparticles smaller than 200 nm are more capable of evading alveolar macrophages phagocytosis entering pulmonary interstitial sites, and interacting with epithelial cells to get access to the circulatory and lymphatic systems.

In the gastro-intestinal tract, that is the most important route for macromolecules to enter the body, the epithelium of the small and large intestines is in close contact with ingested material. Cellular uptake at gastro-intestinal level of nano and micro particles have been the focus of many investigations, the earliest dating from mid-17th century, while more recently entire issues of scientific journals have been devoted to the subject (Buzea et al., 2006). The extent of particles absorption in the gastro-intestinal tract is affected by size, surface chemistry and charge, length of administration, and dose (Yah et al., 2012).

The passage of nanoparticle to the nervous system is possible via the blood-brain-barrier (BBB). BBB is a physical barrier with negative electrostatic charge between the blood vessels and brain, selectively restricting the access of certain substances (Wang et al., 2011). This anionic barrier is believed to stop most anionic molecules, while the cationic molecules increase the permeability of the BBB by charge neutralization. This route has been extensively studied for the
purpose of drug delivery to the brain. Actually, a highlight IONPs property is the capability to enter the brain, under some conditions, either by direct transport (through BBB) or using an indirect route via the olfactory bulb (Kim et al., 2012; Cengelli et al., 2006).

At moment, in CNS structures, cellular uptake is not well known. However Petters and co-workers (2014) have performed an in vitro study and their data have demonstrated that oligodendroglial cells accumulate efficiently IONPs through an endocytotic process, which is strongly affected by the temperature and the presence of serum, and that IONPs (labeled by fluorescent dye BODIPY) are a reliable tool to monitor by fluorescence microscopy the uptake and cellular fate of IONPs. Microglia might behave like phagocytic cells in the brain that respond rapidly to alterations in order to remove harmful material and dying cells. Since IONPs are used for diagnostic and therapeutic applications in the brain and CNS, the consequences of an exposure of microglial cells to IONPs are of particular interest and need to be assessed (Luther et al., 2013).

1.5.2. Biomedical application of iron oxide nanoparticles

Nanomaterials are multifunctional, specifically for the development of medical, biotechnological and pharmaceutical applications; Table 3 shows examples of nanomaterial, diameter and their potential biomedical applications.

The materials in nanostructured form are the excellent candidates as probes because they can achieve high response to very small targets in practical conditions. Nanomaterials (e.g., nanoparticles, nanowires, nanotubes, and even nanodevices) have been explored in many biomedical applications (e.g., biosensing, biological separation, molecular imaging, and anticancer therapy) because their novel properties and functions differ drastically from the bulk counterparts (Chi et al., 2012).
Table 3. **Principal types of nanomaterial used in biomedicine**, based on their size and core type, modified from Duguet et al., (2006).

<table>
<thead>
<tr>
<th>Core type</th>
<th>Examples</th>
<th>Size (nm)</th>
<th>Current or future biomedical application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>Based on lipids</td>
<td>50-500</td>
<td>Drug carriers, potential thermo and photo sensitive</td>
</tr>
<tr>
<td>Micelles</td>
<td>Based on surfactants or block copolymers</td>
<td>&lt;50</td>
<td>Drug carriers, potential thermo and photo sensitive</td>
</tr>
<tr>
<td>Biodegradable polymeric capsules</td>
<td>Polylactide and polyalkylcyanocrylate</td>
<td>10-100</td>
<td>Drug carriers, potential thermo and photo sensitive</td>
</tr>
<tr>
<td><strong>Inorganic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semiconductor quantum dots</td>
<td>CdS</td>
<td>2-8</td>
<td>Fluorescent probes for optical diagnosis</td>
</tr>
<tr>
<td>Noble metal colloids</td>
<td>Gold and silver</td>
<td>2-20</td>
<td>Fluorescent probes for optical diagnosis</td>
</tr>
<tr>
<td>Magnetic nanoparticles</td>
<td>Maghemite, magnetite, iron cobalt and nickel</td>
<td>5-50</td>
<td>Fluorescent probes for optical diagnosis, MRI contrast agent, drug carriers and so on</td>
</tr>
</tbody>
</table>

The regularly employed iron nanomaterials in drug delivery consist of nanoparticles, nanospheres, liposomes and microspheres. In these systems, the drugs are bound to the iron nanomaterials' surface or encapsulated in magnetic liposomes and microspheres. IONPs designed for drug delivery should be above all biodegradable and biocompatible (Chomoucka et al., 2010). IONPs-assisted drug delivery systems have been designed to deliver peptides, DNA molecules, chemotherapeutics, radioactive and hyperthermic drugs. IONPs have been actively investigated as the next generation of targeted drug delivery for more than thirty years. The importance of targeted drug delivery and targeted drug therapy is to transport a drug directly to the center of the disease under various conditions and thereby treat it deliberately, with no side effects on the body (Chi et al., 2012).
At present, Fe$_3$O$_4$ iron oxide nanoparticles (Fe$_3$O$_4$-NPs) are involved in biomedical and biotechnological application, this is due to their good tolerance evidenced by in vivo studies and lower toxic effect compared with Fe$_2$O$_3$ iron oxide nanoparticles (Wang et al., 2011; Pisanic et al., 2007). In particular, the excellent properties of Fe$_3$O$_4$-NPs give rise to numerous multitask applications including magnetic resonance imaging (MRI) contrast agents, multimodal imaging, ferrofluid technology for thermotherapy, targeted drug delivery, cancer tumor detection via magnetometry, gene therapy, biomolecular separation, in vivo biomolecular detection, and tissue repair (Deng et al., 2014; Wu et al., 2013; Singh et al., 2010; Eun & Myung, 2007).

The surface engineered Fe$_3$O$_4$-NPs (e.g. with targeting ligand/molecules attached to their surfaces) used together with the aid of an external magnetic field is recognized as a modern technology to introduce particles to the desired site, where the drug is released locally, and it represents a great advantage to Fe$_3$O$_4$-NPs per se. Engineered Fe$_3$O$_4$-NPs, such a system, has the potential to minimize the side effects and to release the required dosage of the drugs at the target site/tissue (Kim et al., 2011). However, once the engineered Fe$_3$O$_4$-NPs are inside the cells, the coating is likely digested leaving bare particles exposed to cellular components and organelles thereby potentially influencing the overall integrity of the cells. Fe$_3$O$_4$-NPs with appropriate surface chemistry exhibit many interesting properties that can be exploited in a variety of biomedical applications such as MRI contrast enhancement, tissue repair, hyperthermia, drug delivery and in cell separation (Mahdavi et al., 2013; Singh et al., 2010).

1.6. Nanoparticles toxicity

Several diseases with unknown cause, including; autoimmune, Crohn’s, Alzheimer’s, and Parkinson’s diseases, appear to be correlated with nanoparticles exposure. Conversely, several nanoparticles’ properties considered as toxic ones actually may be beneficial, as they are thereby able to fight disease at a cellular
level, and could be used as a medical treatment, for example targeting and destroying cancerous cells (Buzea et al., 2007).

Nanoparticles size allows them to translocate from these entry portals into the circulatory and lymphatic systems, and finally to body tissues and organs. Some nanoparticles, depending on their composition and size, can produce irreversible damage to cells by oxidative stress or/and organelle injury (Arora et al., 2012; Buzea et al., 2007; Oberdoster et al., 2005). Therefore, the processes of generating nanoscale materials in the gas phase, or using or producing nanoscale materials as powders or solutions pose the risk for releasing nanoparticles (Oberdorster et al., 2005). Potential exposure may arise during their production, development, use, or discarding. Also there is likely exposure to NPs if it involves disturbing deposited nanoscale material (Yah et al., 2012).

The principal working activities that cause an exposure to nanoparticles are: working with ultrafine particles; working with nanoscale materials in solution; working in welding place; generating NPs in the gas phase; industry that uses ultrafine powders; maintenance on equipment and processes used to produce or fabricate nanosize materials; cleaning of dust collection systems; machining, sanding, drilling, or other mechanical disruptions of materials containing nanoscale materials (Peters et al., 2006). There is a serious lack of information concerning the toxicity of these nanoparticles at the cellular and molecular level (Ahamed et al., 2011).

1.6.1. Fe$_3$O$_4$-NPs toxicity

Nowadays, IONPs remain the only magnetic nanoparticles that have been approved by Food and Drugs Administration (FDA) (Busquets et al., 2014; Kim et al., 2012) for clinical use and are commercially available (Mahmoudi et al., 2009). However, accumulating evidence indicates that exposure to IONPs causes apoptosis and alters the functionality of macrophages. In fact, exposure of the
murine macrophage cell line J774 to IONPs resulted in an increased production of intracellular ROS, with subsequent cell injury and apoptosis (Shen et al., 2011). Experimental studies have shown that metal and metal oxide nanoparticles induced DNA damage and apoptosis through ROS generation and oxidative stress (Ahamed et al., 2011). Fe₃O₄-NPs, in particular, could produce; oxidative stress following a direct route through Fe⁺² and Fe⁺³ ions liberation from nanoparticles themself (Sun et al., 2013), by a surface catalytic action over H₂O₂ (Lu et al., 2007) producing ‘OH and OH’ wich are highly reactive species (Lou et al., 2014); and producing inflammation reactions (Ahamed et al., 2011; Choi et al., 2008).

The direct route to produce ROS by Fenton, Fenton-like and Heber-Weiss reactions is described below. The following equation illustrated reaction models and subsequent products (Luo et al., 2014; Yan et al., 2013). Equations (1), (2) and (3) show the reactions mediated by Fe⁺².

\[
\begin{align*}
(1) & \quad \text{Fe(s)} + \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow \text{Fe}^{+2} + 2\text{H}_2\text{O} \\
(2) & \quad \text{Fe}^{+2} + \text{O}_2 \rightarrow \text{Fe}^{+3} + \cdot\text{O}_2 \\
(3) & \quad \text{Fe}^{+2} + \cdot\text{O}_2 + 2\text{H}^+ \rightarrow \text{Fe}^{+3} + \text{H}_2\text{O}_2
\end{align*}
\]

The presence of iron Fe⁺³ ions at the end of the reactions chain produce new reactions involved in ROS production described by Yah and co-workers (2012). In this case, this reaction could reintroduce iron Fe⁺² ions to the reaction chain and increase the ROS amounts. In the reaction (5) we could see, at the end of the reaction, the presences of Fe⁺² ions that might enter in (2), (3) reaction, bringing an endless reactions chain.

\[
\begin{align*}
(4) & \quad \text{Fe}^{+3} + \text{H}_2\text{O}_2 \rightarrow \text{FeOOH}^{+2} + \text{H}^+ \\
(5) & \quad \text{FeOOH}^{+2} \rightarrow \text{Fe}^{+2} + \cdot\text{O}_2\text{H}
\end{align*}
\]
Both, inflammation route and the surface catalytic capacity are not described well at cellular level. On the other hand, Table 3 summarized several studies about Fe₃O₄-NPs induced toxic effect in in vitro systems (i.e., different cell lines).

Table 3. IONPs Toxicity information, selected from several studies (last 5 years) evidencing a potential IONPs toxic effect.

<table>
<thead>
<tr>
<th>Coating material</th>
<th>Cell lines</th>
<th>Doses Range</th>
<th>End points</th>
<th>Relevant results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂/ amine modified SiO₂</td>
<td>A549/</td>
<td>0.5-5 nM</td>
<td>DNA damage, Oxidative stress.</td>
<td>Bare NPs showed a strong viability reduction at high NPs concentration (2.5, 5 nM) in both cell lines. Passivized NPs did not show any sign of toxicity.</td>
<td>Malvindi et al., 2014.</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>THP-1</td>
<td>1–25 μg/cm²</td>
<td>Inflammation, migration of macrophages, Cell viability, mitochondrial stress and DNA damage</td>
<td>Induced the production of a pro-inflammatory secretome and also induced an increased migration of macrophages. Caused cell membrane damage, only at a high concentration (100 μg/mL); a lower concentration (10 μg/mL) increased the production of reactive oxygen species, and increased oxidative damage to DNA</td>
<td>Andujar et al., 2014</td>
</tr>
<tr>
<td>-</td>
<td>A549</td>
<td>0-100 μg/mL</td>
<td>Cell viability, mitochondrial stress and DNA damage</td>
<td></td>
<td>Watanabe et al., 2013</td>
</tr>
<tr>
<td>Tetraethylorthosilicate (TEOS)</td>
<td>HDFs/</td>
<td>0-1000 μg/mL</td>
<td>Cell viability,</td>
<td>Reduction of cell viability at &gt;600 μg/mL over HT-1080 cells and 1000 μg/mL to HDFs (due to APTMS Fe₃O₄ NPs)</td>
<td>Yang et al., 2013</td>
</tr>
<tr>
<td>TEOS/3-aminopropyltrimethoxysilane (APTMS)</td>
<td>HT-1080</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>mES</td>
<td>0-1000 μg/mL</td>
<td>Cell viability,</td>
<td>The amount of uptake at the dose of 1000 μg/mL was lower than</td>
<td>Shundo et al., 2012</td>
</tr>
</tbody>
</table>

\[(6) \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot \text{OH} \]
that at 750 μg/mL. Two reasons can be considered for this finding: (i) mES cells excreted or did not take up excess nanoparticles, and (ii) mES cells died due to the addition of a high dose. However, the viability at 1000 μg/mL was not lower than that for the control experiment, which suggests that the low uptake was caused by the reason (i).

| - | HAECs/ U937 | 2, 20, 100 μg/mL | Inflammation, cell viability and mitochondrial stress. | cytoplasmic vacuolation, mitochondrial swelling and cell death were induced in HAEC, significant increase in nitric oxide (NO) production was induced which coincided with the elevation of nitric oxide synthase (NOS) activity in HAECs | Zhu et al., 2011 |
| Polivynil alcohol (PVA) | L929 | 0.2-20 nM | Oxidative stress | PVA causes growth of the particle hydrodynamic size and causes lower cell toxicity effects. | Mahmoudi et al., 2009 |
| BSA | CDBgeo | 0-8 μg/mL | Cell viability | At >4 μg/mL reduction of viability were observed | Bajaj et al., 2009 |

There are contrasting in vivo data regarding the toxic effects induced by Fe$_3$O$_4$-NPs (Wu et al., 2013; Wang et al., 2008; Weissleder et al., 1989). Shen and co-workers (2011) have reported Fe$_3$O$_4$-NPs induced a differential effect on antigen-specific cytokine expression by T cells. In addition, the suppressive effect of Fe$_3$O$_4$-NPs on interferon-γ was closely associated with the diminishment of glutathione. However, further information about toxicity of Fe$_3$O$_4$-NPs is missing.
1.6.2. Neurotoxicity of Fe₃O₄-NPs

A better understanding of how properties of nanoparticles define their interactions with cells, tissues, organs and systems in animals and humans is a considerable scientific challenge, but one that must be addressed to ascertain the feasibility of using nano-biotechnologies in biomedical applications (Cegnelli et al., 2006). In general, IONPs represent a risk because they may release iron ions, this might produce a disruption of normal iron metabolism in the brain that is a characteristic of several neurodegenerative disorders such as: Alzheimer's disease (AD), Parkinson's disease and progressive supranuclear palsy (Bajaj et al., 2009). For example, excess biogenic iron oxide accumulation is known to occur in AD patients, particularly in AD plaques and total iron levels are elevated in the hippocampus, amygdala and cerebral cortex (Hautot et al., 2003; Dobson 2001). However, functionalized Fe₃O₄-NPs are active investigated as a T1 and T2 MRI agent, and this characteristic is one of the principal strategies for develop an early AD diagnosis tool (Busquets et al., 2014), based on detection or identification of amyloid plaques, using IONPs as negative contrast agents.

On the other hand, Schafer and co-workers (2000) affirm that various experiments have also demonstrated that iron-oxygen complexes may be even more effective catalysts for free-radical damage in brain tissue than the Fenton reaction, so the potential deleterious effect of magnetite is possibly even more significant. As described above, the normal brain contains approximately 60 mg of non-heme iron distributed in the parenchyma and an increase in this value could represent a risk. Because of the relative accessibility of blood, liver and bone marrow, there have been many more direct studies of iron metabolism in these organs than of that in the brain and, the great deal is known of the role of iron in the physiology and pathology of tissues outside the CNS and their diseases related with an increase of iron oxides amounts (Schenck and Zimmerman, 2004).
The striatum and hippocampus are important structures in the brain and are associated with the development of Parkinson’s and Alzheimer’s diseases (Wu et al., 2013; Bonson 2001). *In vitro* studies have demonstrated that, bare Fe$_3$O$_4$-NPs may decrease neuron viability, trigger oxidative stress, and activate JNK- and p53-mediated pathways to regulate the cell cycle and apoptosis. These results suggest that environmental exposure to Fe$_3$O$_4$-NPs may play a role in development of neurodegenerative diseases. Xue and co-workers (2012) have conducted a study testing in PC12 cells four different NPs (SiO$_2$-NPs, TiO$_2$-NPs, HAP-NPs and Fe$_3$O$_4$-NPs) demonstrating enhanced secretion of cytokines by microglia. Several of these soluble factors produced by NP-treated microglia affected dopamine synthesis through the suppression of Th expression and also caused cytotoxicity to PC12 cells. This study provides important evidence into the potentially adverse effects on neurons via microglia exposed *in vitro*.

Neuro-cytotoxicity and coating relationships with toxic effect of IONPs need to be considered. According to Deng and co-workers (2014) after exposure to different concentration of Silk Fibroin (SF)- Fe$_3$O$_4$-NPs, the reactive oxygen species generation in PC12 cells were reduced compared with uncoated Fe$_3$O$_4$-NPs. One to 5 days of treatment with SF- Fe$_3$O$_4$-NPs did not destroy cell membrane and cyto-skeleton, and could improve the neurons extension in a dose-dependent manner at lower concentration (6.25 – 50 µg/mL), because SF peptide coating could delay the release of iron ions and the increase of surface crystal defects of Fe$_3$O$_4$-NPs. Intact mitochondria in a neurite indicate extension activity of neurites of cells treated with SF-NPs.

At present, there is a considerable lack of information about neurotoxicity of Fe$_3$O$_4$-NPs, this lack makes imperative to assess its risk. The potential multifunctional application and new developments in biomedical application based on Fe$_3$O$_4$-NPs increase the risk of exposure. Emerging industries bring multiple work-place risk of exposure and, disposition after use of Fe$_3$O$_4$-NPs also brings an environmental risk of exposure or release. At the moment, there is no
information about limits of exposure or referential values that can use as reference in any exposure scenery. In this regard, Fe3O4-NPs induced-neurotoxicity must be estimated also based on the Fe3O4-NPs translocation capability, potential applications, approved used and incidental exposure. The first logical approach is use alternative models such as in vitro cellular models to assess their potential neurotoxic effect in human origin cell.

2. Thesis aim

To assess in vitro neurotoxicity outline of Polyvinylpyrrolidone-coated Iron Oxide nanoparticles in human origin astrocytes (SH-SY5Y) measured at different time points (short exposure time 4-48 hours), focusing the attention to mitochondrial function, membrane integrity, cellular morphology and intracellular nanoparticles accumulation.

3. Materials and methods

3.1. Chemicals

All cell culture reagents, culture mediums, and chemicals were purchased from Sigma-Aldrich, (Milan- Italy), excluding calcein AM (calcein acetoxymethyl), which was obtained from Life Technologies (Milan-Italia).

3.2. Nanoparticles

Polyvinylpirolidone coated iron oxide (Fe3O4) nanoparticles (PVP-Fe3O4-NPs) were obtained from nanoComposix (San Diego, CA, USA), PVP-Fe3O4-NPs have the following characteristics: Diameter of 20 nm, Zeta potential -39.9 mV, 20.6 mgNPs/mL of concentration and a spherical morphology (information
provided from the seller). Stock solution were prepared by dissolving PVP-Fe3O4-NPs in culture medium, formerly cells were exposed to concentrations from 1 to 100 µg/mL. Nanoparticles solutions were prepared shortly before every experiment.

3.3. Cell line and culture medium

Human neuroblastoma (SH-SY5Y) cell line was purchased from ECACC, Sigma-Aldrich, (Milan- Italy), and cell culture reagents were obtained from Sigma-Aldrich (Milan-Italia). SH-SY5Y cells were cultured in Eagle’s minimum essential medium and Ham’s F12 (1:1) with 15% FBS, 2nM l-glutamine, 50 IU/mL penicillin and 50 µg/mL of streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

3.4. Cellular uptake

Perl’s Prussian blue assay is employed for localization of iron into a cellular cytoplasm. This is an extremely reliable and sensitive technique that can detect even small quantities of iron microscopically (Sivaprakash et al., 2003). A general idea about the reaction was presented in the equation (7):

$$\text{Fe}^{2+} + [\text{Fe(CN)}_6]^{3-} \leftrightarrow \text{Fe}^{3+} + [\text{Fe(CN)}_6]^{4-}$$

In order to evaluate cellular uptake we performed the following protocol: 1x10⁵ SH-SY5Y cells/mL were seeded in 60 mm diameter petri plate in duplicate and were incubated for 24 h at 37°C and 5% CO₂ humidify atmosphere. After that, the cells were treated with PVP-Fe3O4-NPs at different concentrations (1, 10, 25, 50 and 100 µg/mL of PVP-Fe3O4-NPs). After treatment with PVP-Fe3O4-NPs, cells were placed in an incubator maintained at 37°C and 5% CO₂ humidify atmosphere, then at various time points (4 and 24 hours), culture
medium was extracted and cells were washed twice with phosphate-buffered saline (PBS) to remove unbound nanoparticles. Cells were lysed by the addition of 5 mL of 4% of p-formaldehyde solution for 20 min, and PVP-Fe3O4-NPs content was determined based on the Prussian’s blue reaction described below. Cell lysing solution were removed and cells were washed with PBS twice, PVP-Fe3O4-NPs remains were colored with 5 mL of iron staining solution prepared using 1:1 solution 2% of K₄[Fe(CN)₆] and solution 6% of HCl for 30 min, at the end of coloring process cell were cleaned up with deionized water three times, the contrast coloration agent (solution 0.5% of neutral red) were added and settled for a minute, before observation, cell were washed with water until eliminated contrast agent and dry at environmental conditions. Results were obtained observing under a Zeiss Axiovert 25 attached with a digital camera (Canon Powershot G8).

3.5. Cell morphology

Phase contrast microscopy is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in images. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

This assay was conducted following this protocol: SH-SY5S cells were seeded in 96 wells plate at a concentration of 1x10⁴ cell/mL in complete medium, after 24 hours of incubation at 37°C and 5% CO₂, the cells were exposed to various concentrations of PVP-Fe3O4-NPs (1, 10, 25, 50 and 100 µg/mL), in three periods of time (4, 24, 48 hours) under a Zeiss Axiovert 25 and attached with a digital camera (Canon Powershot G8).

3.6. Cytotoxicity assays
In this work, mitochondrial function and membrane integrity were assessed using MTT and Calcein AM/ Propidium Iodine staining assays respectively. Cell-based assays are often used for screening collections of compounds to determine if the test molecules, compounds or nanoparticles have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death.

MTT assay uses tetrazolium salts to assess mitochondrial activity. The reaction begins when mitochondrial dehydrogenases adheres the tetrazolium ring and reduce MTT to insoluble dark blue formazan crystal. Only functional mitochondria contain these enzymes, in that case, mitochondrial function is affected, consequently, there is no enzyme production and the enzymatic reduction does not occur. Coloured complex is formed according to the following diagram:

The Live/Dead viability test is use in toxicology to detect cell membrane integrity and measure the number of damage cells, live cell (cell with intact membrane), are distinguished by an intense green fluorescence generated by enzymatic hydrolysis of calcein AM (calcein acetoxyethyl). Dead cells or cell, whose membrane integrity has been damage, are distinguished by a brilliant red fluorescence resulting from propidium iodine binding to nucleic acids. The following chemical equation illustrates the general idea about the reaction.
Both assays were done using the following order of steps: SH-SY5Y cells were seeded in 96 wells plate at a concentration of \(1 \times 10^4\) cell/mL in complete medium, after 24 hours of incubation at 37\(^\circ\)C and 5\% CO\(_2\), the cells were exposed to various concentrations of PVP-Fe3O4-NPs (1, 10, 25, 50 and 100 \(\mu\)g/mL), in three periods of time (4, 24, 48 hours). For each assay, respectively protocols were done and they are described below.

At the end of each period of exposure, treated cells were compared with no treated cells as a negative control, at same culture conditions, the mitochondrial function was assessed by 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), incubated for 3 hours at 37\(^\circ\)C and 5\% CO\(_2\), before, formazan crystals were quantified at 550nm in Biorad microplate spectrophotometric reader.

Membrane integrity was tested by co-incubation with 2\(\mu\)M calcein AM and 2.5 \(\mu\)g/mL of propidium iodine (PI) for 5 min at 37\(^\circ\)C, cells were observed under a Zeiss Axiovert 25 fluorescence microscope equipped with a triple filter set (Excitation: 400, 495, 570 nm; Beamsplitter 410, 505 585 nm; Emission 460, 530, 610 nm) and attached with a digital camera (Canon Powershot G8). Integrity was expressed as percent of cells retaining calcein compared to the total cells counted.
3.7. Statistics

Results are expressed as mean ± SD. Data from short-term exposure were obtained from two independent experiments each carried out in six replicates. Statistical analysis was performed by one-way ANOVA followed by Tukey’s test (for each time-point). A value of P < 0.05 was considered statistically significant.

4. Results

4.1. Cell morphology

Evaluation of cell morphology by phase contrast microscopy showed no alterations in SH-SY5Y cells after exposure to PVP-Fe₃O₄ NPs concentrations 1-100 µg/mL. Something important to emphasize was the fact that PVP-Fe₃O₄ NPs precipitates, those were observed in the culture medium at doses equal and over 25 µg/mL, this phenomenon could mask the light microscopy cell visualization, and was remarkably evident at 50 and 100 µg/mL due to greater quantity of aggregates visualized. However visual cell modifications were not observed during the experiment, which was conducted for 48 hours and measured at three time points (i.e. 4, 24 and 48 hours).

One of remarkable observations was the fact that PVP-Fe₃O₄ NPs form aggregates in a dose-time dependent manner. These aggregates were observed clearly at 25, 50 and 100 µg/mL. All results are illustrated in the Image 3 below. The lowest concentration (1 µg/mL) showed no difference with the control, for this reason these data were not showed.
Image 3. **Phase contrast microscopy random images**, SH-SY5Y cell exposed to 10-100 µg/mL of PVP-Fe₃O₄ NPs in three periods of time (4, 24 and 48 hours). Arrows indicate the zone that are amplified 2X.

**SH-SY5Y exposed to PVP-Fe₃O₄ NPs (Phase contrast microscopy)**
4.2. Cellular uptake

Assessment of cellular uptake by Prussian blue assay showed internalization of nanoparticles in SH-SY5Y cells after 24 hours of exposure to PVP-Fe₃O₄ NPs (1-100 µg/mL) in a dose-dependent fashion. Blue cores inside cells (Image 4) have indicated a dose-time dependent cellular uptake. Something important to emphasize is the fact that, PVP-Fe₃O₄ NPs precipitates were observed in the culture medium at doses equal and over 25 µg/mL, this phenomenon could mask the light microscopy cell visualization, this were more evident at 50 and 100 µg/mL (Image 4), due to greater quantity of aggregates. In the lowest concentration (1 µg/mL) uptake phenomenon was not evident at microscope level. However visual cell modifications were not observed during the experiment and nanoparticles cores were clearly observed into the cellular cytoplasm.

The experiment was conducted for two time points (i.e. 4 and 24 hours). Notably PVP-Fe₃O₄ NPs forms aggregates in a dose-time dependent way that was concordant with the result of cellular morphology. These aggregates and PVP-Fe₃O₄ NPs cores were observed clearly at 25, 50 and 100 µg/mL, these results were illustrated in the Image 4 below. The lowest concentration shows no difference with the control (data not showed).
Image 4. **Cellular uptake (Prussian blue) random images**, SH-SY5Y cell exposed to 1-100 µg/mL of PVP-Fe$_3$O$_4$ NPs in two periods of time (4 and 24 hours). Arrows indicate the zone that are amplified 2X.

**SH-SY5Y exposed to PVP-Fe3O4 NPs (Cellular Uptake)**

4 Hours

Control 10 µg/ml 25 µg/ml 50 µg/ml 100 µg/ml

24 Hours

Control 10 µg/ml 25 µg/ml 50 µg/ml 100 µg/ml
4.3. Membrane integrity

Cell membrane integrity and morphology of SH-SY5Y cell line were not affected by PVP-Fe\textsubscript{3}O\textsubscript{4} NPs treatments for doses 1-100 µg/mL considering exposure times within 4-48 hours. All treatments showed a green fluorescence uniformly diffused as presented in Image 5, in this case, aggregates did not mask results and were not visible. After 4 hours exposure, no dead cells were observed or any else alteration, these results are presented in Graphic 1.

After 24 hours of exposure few dead cells were founded, although they it did not represent over 2% compared with control, these dead cells could be due to a normal apoptotic process, which arose at concentrations higher than: 25 µg/mL and are exposed in Graphic 2.

After 48 hours of exposure results were congruent with 24 hours showing dead cells ratios around 2%, the difference was the beginning of the effect that was at 10 µg/mL level and those results are represented in Graphic 3.

Graphic 1. Quantitive analysis after 4 hours of exposure, there is no signal of dead cells in any concentration.

![Graph 1](image1)

*PVP-Fe\textsubscript{3}O\textsubscript{4} NP (µg/ml)*

![Graph 2](image2)

*Live cells (%) Dead cells (%) [0, 100]*

33
Graphic 2. Quantitative analysis after 24 hours of exposure, there is low dead cells percent not over 2% in concentrations over 25 µg/mL.

Graphic 3. Quantitative analysis after 48 hours of exposure, there is low dead cells percent not over 2% in concentrations over 10 µg/mL.
Image 5. Fluorescence microscopy random image. SH-SY5Y cell exposed to 1-100 µg/mL of PVP-Fe₃O₄ NPs in three periods of time (4, 24 and 48 hours).

SH-SY5Y exposed to PVP-Fe₃O₄ NPs (Fluorescence microscopy)

<table>
<thead>
<tr>
<th>4 Hours</th>
<th>1 µg/ml</th>
<th>10 µg/ml</th>
<th>25 µg/ml</th>
<th>50 µg/ml</th>
<th>100 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="" /></td>
<td><img src="image2" alt="" /></td>
<td><img src="image3" alt="" /></td>
<td><img src="image4" alt="" /></td>
<td><img src="image5" alt="" /></td>
</tr>
<tr>
<td>24 Hours</td>
<td>1 µg/ml</td>
<td>10 µg/ml</td>
<td>25 µg/ml</td>
<td>50 µg/ml</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Control</td>
<td><img src="image6" alt="" /></td>
<td><img src="image7" alt="" /></td>
<td><img src="image8" alt="" /></td>
<td><img src="image9" alt="" /></td>
<td><img src="image10" alt="" /></td>
</tr>
<tr>
<td>48 Hours</td>
<td>1 µg/ml</td>
<td>10 µg/ml</td>
<td>25 µg/ml</td>
<td>50 µg/ml</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Control</td>
<td><img src="image11" alt="" /></td>
<td><img src="image12" alt="" /></td>
<td><img src="image13" alt="" /></td>
<td><img src="image14" alt="" /></td>
<td><img src="image15" alt="" /></td>
</tr>
</tbody>
</table>
4.4. Mitochondrial function

In order to evaluate mitochondrial function as a toxicity endpoint, we used MTT assay, which is a colorimetric method, to assess this parameter in SH-SY5Y cell line. Graphic 1 showed dose-time results expressed as viability percent of control obtained after 4, 24 and 48 hours exposure to PVP-Fe$_3$O$_4$ NPs.

After 4 and 24 hours exposure, any of PVP-Fe$_3$O$_4$ NPs concentrations induced toxic effect. However, after 48 hours exposure mitochondrial function was affected: a decreasing viability within 25-35% compared with control was observed at PVP-Fe$_3$O$_4$ NP concentrations of 10 to 100 µg/mL. In this case a dose-dependent effect was evidenced (Graphic 1). It is important to emphasize, the fact that PVP-Fe$_3$O$_4$ NP precipitates in the culture medium were observed at doses higher 10 µg/mL and could interfere to colorimetric method.

Graphic 4. MTT assay results show cell vitality variation 65-120% compared with control. (* $0.05<p$)
5. Conclusions and discussion

5.1. Discussion

Iron oxide nanoparticles are widely used in the biomedical fields such as: MRI agent, drug and gene carriers, hyperthermia treatment agent, and as magnetic separation tool (Liu et al., 2013; Colombo et al., 2012). It is of significant meaning to assess the potential risks of IONPs considering their extensive applications. IONPs have become increasingly evident a factor, that might contribute to the development of neurodegenerative diseases, such as Parkinson’s and Alzheimer’s disease (Calderon-Garciduenas et al., 2004). Regardless of the route used to deliver drugs into the brain, the diffusion of the drug delivery devices into the brain parenchyma must be controllable and must avoid activation of microglial cells, since the brain possesses its own macrophage population, the microglia, which is involved in the development of neurodegenerative disorders (Cegnelli et al., 2006). At present, interaction and toxicity of IONPs are not well known and need to be assessed.

Peters and co-workers (2006) stress the importance of characterizing nanoparticle-related oxidative stress in the brain. It is also important to evaluate the connection between neurodegenerative diseases and nanoparticle exposure. Fe3O4-NPs applications continue to be found in the life science and industrial fields, the risk of occupational nanoparticle exposure rises correspondingly. It was reported that the inhalation of ultrafine particulate material in polluted air contributes to protein fibrillation; the enhancement of Ab42 and a-synuclein fibrillation, these may play a role in the development of Alzheimer’s and Parkinson’s diseases (Wu et al., 2013).

Once nanoparticles enter CNS, they immediately encounter a complex environment of resident microglial immune cell and neurons, in this regard, assess iron oxide nanoparticles interaction with these cells is fundamental. For
this reason, many researchers have reported the toxic effects of IONPs *in vitro* and *in vivo* (Deng et al., 2014; Wu et al., 2013; Mahmoudi et al. 2012; Singh et al. 2010; Weissleder et al 1989) and them revels a high grade of variation between bare IONPs and coated IONPs. Nevertheless, the results concerning to the toxicity of IONPs are not consistent due to different experimental conditions and characteristics of the nanoparticles (i.e. Different diameter, coating material, surface charge, etc.) (Luo et al., 2014).

Rat PC12 cell line (embryological origin with neuroblastic cells) is a closer *in vitro* model to SH-SY5Y cell line and we have certain toxicological information of this. For this reason, the following toxicity studies present PC12 results after exposure to different coated IONPs. Xue and co-workers (2012) have demonstrated that Fe$_3$O$_4$ NPs did not induce greater cytotoxicity after 48 h exposure at 500 µg/mL; while Wu and co-workers (2013) have reported that Fe$_3$O$_4$ NPs induced cytotoxic effect, which was observed at 100 and 200 µg/mL after 24 h exposure. Comparing these two works we can note different results reported in literature so far.

In another study that contrast pure Fe$_3$O$_4$ NPs with Silk-Fibroin coated Fe$_3$O$_4$ NPs, Deng and co-workers (2014) have presented this; after 24h, SF-Fe$_3$O$_4$ has showed reduced iron ions release and ROS production at 100 µg/mL. MTT results of PC12 cells treated with SF-NPs or pure NPs at different concentrations indicate that after cells were exposed to SF-NPs or pure NPs for 3 days, the cell viability of each group treated with SF-NPs is higher than that with pure NPs.

All these findings have evaluated cytotoxicity using MTT assay similarly to the method currently applied in our study aimed at evaluating the cytotoxicity caused by PVP-Fe$_3$O$_4$ NPs. Comparing our results with those reported by Xue and co-workers (2012), Wu and co-workers (2013) and Deng and co-workers (2014) some discrepancy can be observed. In particular, our study has demonstrated toxic effect after 48 hours exposure and at concentration equal and over 10 µg/mL, this might be due to Fe$_3$O$_4$ coating material
(Polyvinylpirrolidone) of our nanoparticles, and also the type of cells we used (such as SH-SY5Y) since no current studies on SH-SY5Y, so far, exposed to PVP-IONPs are existing to make any specific comparison with our MTT results in a certain way.

We also evaluated cellular uptake, in this regard, few information is reported. Deng and co-workers (2014) say that anionic NPs had a high affinity for the cell membrane due to electrostatic interactions; PVP-Fe₃O₄ NPs have a negative value of zeta potential, which means that the NPs can be easily adsorbed by cells and subsequently be internalized into the cells. However, according to Valdiglesias and co-workers (2014) cellular uptake efficiency of IONPs is dependent on surface coating of the nanoparticles, irrespective of the cell line used. Hence, a strategy to adjust the cellular uptake efficiency and precision of IONPs is to modify their surface coating, and several studies have demonstrated that surface modification modify IONPs, influence their toxic effect (reduction) and reduce iron ions release compared with bare IONPs. These modifications could explain literature inconsistencies and our results which apparently differ from some literature data.

In contrast, Hoskins and co-workers (2012) have reported that, increased cellular uptake was observed in the PEI-IONPs compared with PEI-PEG-IONPs which was attributed to the higher positive surface charge (+55.6 mV) attracting to the negative cell membrane and enhancing endocytosis. The cell viability data clearly showed that the cytotoxicity, after pegylation of the PEI-IONPs, was significantly reduced. The primary amines on the surface of the PEI-IONPs give rise to the large positive surface charge (+55.6 mV); this is completely different from the idea presented by Deng and co-workers (2014). Another report suggests the idea that positive charged NPs pass through cellular membrane more easily that negative or neutral ones. This study has evaluated the mechanism in neuronal cell, and has shown a macro-pinocytosis as a primal uptake mechanism (Kenzaoui et al., 2012). Deng´s findings are consistent with our results, in that we
found a negative charged PVP-Fe$_3$O$_4$ NPs (-39.9 mV) that were apparently internalized into cell (not quantitative established).

In this work, we used *in vitro* approaches aimed at demonstrating potential cellular morphology changes and damage to membrane integrity after PVP-Fe$_3$O$_4$ NPs exposure. We clearly showed no effects related to the PVP-Fe$_3$O$_4$ NPs exposure, excepting for mitochondrial function, which was noticeably the target in SH-SY5Y cells. *In vivo* studies (Deng et al., 2014; Wu et al., 2013; Kong et al., 2012; Weissleder et al., 1989); affirm that IONPs are generally good tolerated, and coated IONPs are less toxic than bare ones. These affirmations and the fact that some IONPs (with different coating materials) are approved by FDA, implies IONPs are safe for human uses, biomedical applications and nanobiotechnological developments (Kim et al., 2012).

5.2. Conclusions

The present study assessed neurotoxicity of PVP-Fe$_3$O$_4$ NPs over human neuroblastoma cell line (SH-SY5Y) and results indicate that:

- Mitochondria is apparently the target in SH-SY5Y cells: considerable toxic effect is produced by PVP-Fe$_3$O$_4$ NPs after 48 hours exposure in a dose-time dependent manner and it was evident only at MTT assay;

- Any microscopic change in cellular morphology was observed;

- Membrane integrity was not visually affected for PVP-Fe$_3$O$_4$ NPs exposure, low dead cell ratios may due to a normal apoptotic process;

- In general, PVP-Fe$_3$O$_4$ NPs at 1 µg/mL or higher concentrations after 48 h exposure was able to trigger toxic effect (i.e. mitochondrial function, but no effect on Membrane integrity and Cellular);

- Prussian blue assay revels that, PVP-Fe$_3$O$_4$ NPs enter to cytoplasm, but at concentrations equals and over 25 µg/mL in a dose time dependent manner;
- Interaction between culture medium and PVP-Fe₃O₄ NPs unleash aggregates formation, this cause interference in several approaches such as: spectrophotometric and optical instruments, this formation is apparently dose time dependent;

- Due to variability on IONPs diameter, coating material used and surface charge, toxicity results on this type of NPs show a greater variability in literature. Thus for this reason, it is necessary to assess one by one, every single IONPs nanoparticle used or in development.
6. References


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