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# Engineered nanoparticles. How brain friendly is this new guest?

Q1 Francesca A. Cupaioli [a,1], Fabio A. Zucca [a,1], Diana Boraschi [b], Luigi Zecca [a,*]

[a] Institute of Biomedical Technologies, National Research Council of Italy, Segrate, Milan, Italy
[b] Institute of Biomedical Technologies, National Research Council of Italy, Unit of Pisa, Pisa, Italy

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ABSTRACT

In the last 30 years, the use of engineered nanoparticles (NPs) has progressively increased in many industrial and medical applications. In therapy, NPs may allow more effective cellular and subcellular targeting of drugs. In diagnostic applications, quantum dots are exploited for their optical characteristics, while superparamagnetic iron oxides NPs are used in magnetic resonance imaging. NPs are used in semiconductors, packaging, textiles, solar cells, batteries and plastic materials. Despite the great progress in nanotechnologies, comparatively little is known to date on the effects that exposure to NPs may have on the human body, in general and specifically on the brain. NPs can enter the human body through skin, digestive tract, airways and blood and they may cross the blood-brain barrier to reach the central nervous system. In addition to the paucity of studies describing NP effects on brain function, some of them also suffer of insufficient NPs characterization, inadequate standardization of conditions and lack of contaminant evaluation, so that results from different studies can hardly be compared. It has been shown *in vitro* and *in vivo* in rodents that NPs can impair dopaminergic and serotoninergic systems. Changes of neuronal morphology and neuronal death were reported in mice treated with NPs. NPs can also affect the respiratory chain of mitochondria and Bax protein levels, thereby causing apoptosis. Changes in expression of genes involved in redox pathways in mouse brain regions were described. NPs can induce autophagy, and accumulate in lysosomes impairing their degradation capacity. Cytoskeleton and vesicle trafficking may also be affected. NPs treated animals showed neuroinflammation with microglia activation, which could induce neurodegeneration. Considering the available data, it is important to design adequate models and experimental systems to evaluate in a reliable and controlled fashion the effects of NPs on the brain, and generate data representative of effects on the human brain, thereby useful for developing robust and valid nanosafety standards.



**Contents**



*Abbreviations:* BBB, blood-brain barrier; CA, cornu ammonis; CNS, central nervous system; IL, interleukin; iPS, induced pluripotent stem; MRI, magnetic resonance imaging; NPs, nanoparticles; LPS, lipopolysaccharide; PEG, polyethylene glycol; PBCA, polybutyl-cyanoacrylate; PM, particulate matter; PS, polysorbate; ROS, reactive oxygen species.

* Corresponding author at: Institute of Biomedical Technologies, National Research Council of Italy, Via Cervi, 93, 20090 Segrate, Milano, Italy. Tel.: +39 02 26422616; fax: +39 02 26422770.
*E-mail address:* luigi.zecca@itb.cnr.it (L. Zecca).
[1] These authors contributed equally.

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## 1. Introduction

During the annual meeting of American Physical Society at the California Institute of Technology in 1959, the physicist Richard Feynman, in his lecture "There's plenty of room at the bottom", mentioned for the first time the possibility of manipulating and controlling things at the small scale (Feynman, 1960). More than fifty years later, today nanoparticles (NPs) are widely used in extensive range of applications in different fields. NPs are nano-objects with all three external dimensions in the nanoscale, where nanoscale is defined as a size range from approximately 1 to 100 nm (ISO/TS 27687:2008) (Fig. 1), and show size-dependent properties that strikingly differ from those of the bulk material.

The NPs can be natural or synthetic. NPs naturally present in the environment derive from natural events such as terrestrial dust storm, volcanic eruptions, erosion and forest fire. Moreover, human activities introduce NPs in the environment, as by-products of simple combustion or generated by combustion engines, power plants and other thermodegradation systems. On the other hand, the advent of the nanotechnological industry is now exposing man to a new category of NPs, the engineered NPs, which encompass multiple chemical compositions, shapes and sizes.

At variance with natural NPs, which are heterogeneous in material, size and properties, engineered NPs are synthesized as homogenous entities with controlled characteristics. These unique features make engineered NPs very versatile, thus they are nowadays used in a wealth of applications, either incorporated into products to enhance or improve their properties, or as new stand-alone products. For example, highly conductive NPs are used in the electronics and telecommunication fields to create sensors and small components in electronic devices, like smartphones (Shipway et al., 2000). Other NPs with special characteristics of hardness and friction are used as abrasives in the nanopolish of ultra-smooth surfaces (Guo et al., 2014); in addition, they can be used as additives in minimum quantity lubrication systems, allowing very low friction and wear, and leading to lower temperature in grinding zone with respect to lubricant devoid of NPs (Li et al., 2013; Guo et al., 2014). NPs may also be applied in materials engineering, since they can form chain aggregates with high plasticity and elasticity, thus improving mechanical properties of rubber and other polymeric materials (Rong et al., 2006). Moreover, NPs can be used as anti-reflection coatings, taking advantage of their specific optical properties (Du et al., 2010).

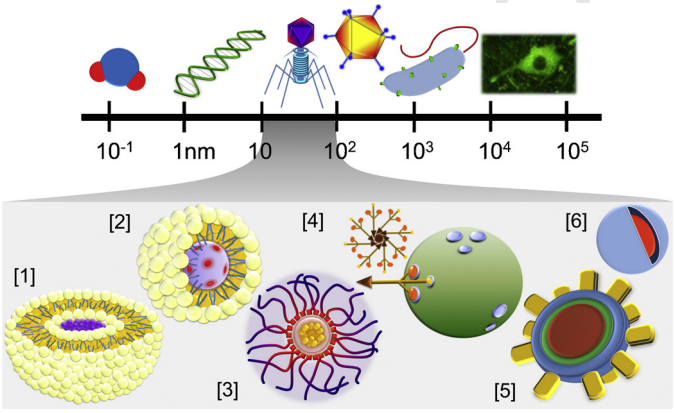


**Fig. 1.** Scale size of NPs. Length scale showing the size of NPs compared to biologic materials (*i.e.*, water molecule, DNA, bacteriophage, virus, bacterium and neuron) in the nano and micro size. In the bottom panel few types of NPs are represented: [1] liposome, [2] solid–lipid NP, [3] polymeric micelle, [4] dendrimer, [5] quantum dot and [6] iron oxide NP.

Because of their chemical chelation and antimicrobial capacity, some NPs are also used in environmental remediation technologies as removal agent of toxic metals and compounds, or as antimicrobial agents. For example, magnetite and zero-valent iron NPs are used for removal and retention of uranium from contaminated environmental water (Crane et al., 2011), and Ag NPs are effective in eliminating bacterial pathogen population from wastewater (Seo et al., 2012).

NPs, as nanoemulsions, have excellent sensorial and hydrating properties and for this reason they are widely used in the cosmetic industry, for example in lotions, moisture milks, crystal-clear gels, nail polish, hair products, toothpaste, and others (Hougeir and Kircik, 2012). Furthermore, metallic NPs are commonly used in sunscreen lotions ($TiO_2$ NPs) and as antimicrobial agents (Ag NPs) in detergents and other everyday cosmetic products (Bondarenko et al., 2013).

As in the case of cosmetics and textile products, NPs (*i.e.*, Ag NPs, $TiO_2$ NPs, etc.) are largely used in the food industry as preservatives that avoid microorganisms proliferation (Hajipour et al., 2012), and they are also employed to encapsulate food additives thereby enhancing flavours and brightening colours (Sekhon, 2010). NPs are also used in food packaging (Fuciños et al., 2012), embedded in the polymer matrix, where they act as gas sensors (UV-activated $TiO_2$ NPs oxygen sensors), or forming a protecting barrier from UV radiation, or inhibiting gas permeability (Duncan, 2011).

Finally, engineered NPs are largely used in the field of healthcare and life sciences, having numerous medical applications (*i.e.*, drug delivery, magnetic resonance imaging, hyperthermia treatment, etc.) and biotechnological uses (*i.e.*, biosensors, basic research technologies, biomedical engineering, etc.).

The development of nanotechnologies is evolving very rapidly and in parallel NP-containing products are becoming significantly

present in the market. As a consequence, human exposure to NPs, either in the workplace or upon release from NP-containing products, is progressively increasing. Therefore, understanding the environmental and health impact of engineered nanomaterials and by-products has become a priority both for ensuring health protection and for regulating the safe development of nanotechnologies.

The possible untoward effects of NPs on human health are still unclear, despite the fact that thousands of different kinds of engineered NPs are being developed and included in commercial products already on the market (Pautler and Brenner, 2010). In addition, the Food and Drug Administration has approved several nanopharmaceuticals, and many medical products and drugs are currently commercially available (Bawarski et al., 2008). From characterization studies it is evident that NPs are reactive entities, probably due to their large surface area compared to their overall mass. Furthermore, due to their small size, NPs are likely to interact with cells and subcellular structures in highly efficient but often poorly characterized ways.

It has been shown that NPs are able to enter the CNS (Kreuter, 1994). This capacity represents a huge advantage for diagnosis and treatment of CNS disorders, such as brain tumours, stroke and neurodegenerative diseases. Indeed, NPs-based delivery systems have been developed to facilitate the diagnosis/treatment of CNS diseases. On the other hand, the ability of NPs to enter the brain represents a realistic risk factor both in the case of chronic and accidental exposure, depending on the magnitude and intensity of exposure (Sharma, 2009a,b). Nanosized materials, such as nanoscale magnetic biominerals (*e.g.*, magnetite or $Fe_3O_4$ and maghemite or $Fe_2O_3$ associated with senile plaques and tau filaments), have been found in human brain and have been associated with neurodegenerative diseases (Dobson, 2001; Hautot et al., 2003). Moreover, several studies suggested the involvement of NPs in inflammatory processes in the CNS and in the pathogenesis of neurodegenerative disease (Sharma and Sharma, 2012).

The present knowledge on NP neurotoxicology is still limited, and in depth studies are warranted, particularly when considering the recent emphasis in the use of nanocarriers for drug delivery in the brain (Costantino and Boraschi, 2012). Here, we review the current knowledge concerning the chemical–physical characteristics of engineered NPs and their effects on brain. The wealth of data and results obtained over the years regarding natural NPs has been exhaustively reviewed elsewhere (Block et al., 2012), and therefore will be only briefly discussed in this review.

## 2. NPs in air pollution

Air pollution is a complex mixture of gases and particulate matter (PM). The gases encompass by ozone, carbon monoxide, nitrogen oxide, sulfur oxide and volatile organic compounds. PM is composed of both organic and inorganic compounds, including aromatic hydrocarbons, biological materials, such as bacterial endotoxin and allergens, and toxic metals, such as vanadium, lead, nickel, copper and manganese. The components of PM that can be inhaled deep into the lung are monitored and sampled in many countries as $PM_{10}$, $PM_{2.5}$ and ultrafine particulate (a measurement of particles having an aerodynamic diameter less than 10 μm, 2.5 μm and 0.1 μm, respectively). In particular, the ultrafine NPs are more dangerous than the bigger ones, as they can translocate to the brain *via* nose-to-brain route, or can penetrate deep into lung alveoli upon inhalation, from where they can enter into the circulation and reach the brain (Phalen et al., 2010). Ultrafine particles mostly consist in a mixture of traffic- and incomplete combustion-derived carbon NPs. These airborne NPs can carry soluble organic compounds, polycyclic hydrocarbons and oxidized transition metals on their surface. The sustained and chronic exposure to air pollutants apparently has a key role in neuroinflammation, oxidative stress and cerebral vascular damage, and contributes to neurodegenerative disease development (Block and Calderón-Garcidueñas, 2009). Studies on feral dogs naturally exposed to high level of urban air pollution showed enhanced oxidative damage, premature presence of diffuse amyloid plaques and a significant increase in DNA damage in olfactory bulbs, frontal cortex and hippocampus (Calderón-Garcidueñas et al., 2003, 2002). Diesel exhaust, a major constituent of near-road and urban air pollution, caused neuroinflammation in animal models, with elevated early markers of neurodegenerative disease (Levesque et al., 2011a), perturbed dopamine neurochemistry and impaired motor behaviour. In rats exposed to diesel exhaust particles (0.5 and 2.0 $mg/m^3$; inhaled over 4 weeks), high levels of interleukin (IL)-6, ionized calcium-binding adaptor molecule 1, and significant elevation of protein nitration levels were observed in the whole brain. Moreover, increased levels of tumour necrosis factor-α, IL-1β, IL-6, macrophage inflammatory protein-1α, receptor for advanced glycation end-products, and fractalkine were detected in cortex, midbrain and olfactory bulb, with the most pronounced response in the midbrain. Single intra-tracheal administration of diesel exhaust particles (20 mg/kg; 6 h post treatment) increased microglial staining for ionized calcium-binding adaptor molecule 1 in the substantia nigra and elevated both serum and whole-brain tumour necrosis factor-α levels, indicating a key role of these particles in neuroinflammation and neurotoxicity (Levesque et al., 2011b).

## 3. Nano-strategies in drug delivery to the CNS for diagnostic and therapeutic purposes

The use of NPs is dramatically changing the future of clinical medicine, with expected applications in screening, diagnosis and treatment of diseases. Depending on the method of preparation, engineered NPs can be designed to display different properties of pharmacokinetics, targeting and cargo release useful for controlling encapsulation and delivery of therapeutic and diagnostic agents (Barratt, 2000; Couvreur et al., 1995; Moghimi et al., 2011). NPs can be designed to carry insoluble or poorly soluble drugs, thus avoiding the use of toxic organic solvents, and to control and/or trigger drug release, resulting in increased therapeutic efficacy. Moreover, NPs can have the capacity to stabilize labile molecules such as proteins, peptides or DNA, and can be tailored so as to carry out site-specific drug targeting. NPs can be used to maintain drug levels in a therapeutically desirable range by increasing half-life, solubility, stability and permeability of drugs. Compared to the classical approaches for drug delivery into the brain, the use of NPs would make diagnostic and therapeutic procedures better tolerated, since the more accurate targeting allows obtaining therapeutic effects with lower drug dosages and concomitantly reducing drug dependent side effects.

The NPs currently used for therapeutic drug delivery and for diagnostics systems (*e.g.*, brain imaging) can be lipidic (liposomes, nanoemulsions, solid lipid NPs), polymeric (polymeric NPs, polymeric micelles, nanogels and dendrimers) and organometallic compounds (*e.g.*, $Fe_3O_4$ NPs and quantum dots), and may be coated with inorganic or organic shells (Fig. 2). Depending on the method of preparation, the diagnostic/therapeutic agents may be adsorbed or covalently bound to the matrix or entrapped into the NPs (encapsulated or dissolved).

### 3.1. Lipid-based NPs for therapeutic use

Liposomes are concentric vesicles composed of biocompatible and biodegradable lipids similar to those of biologic membranes



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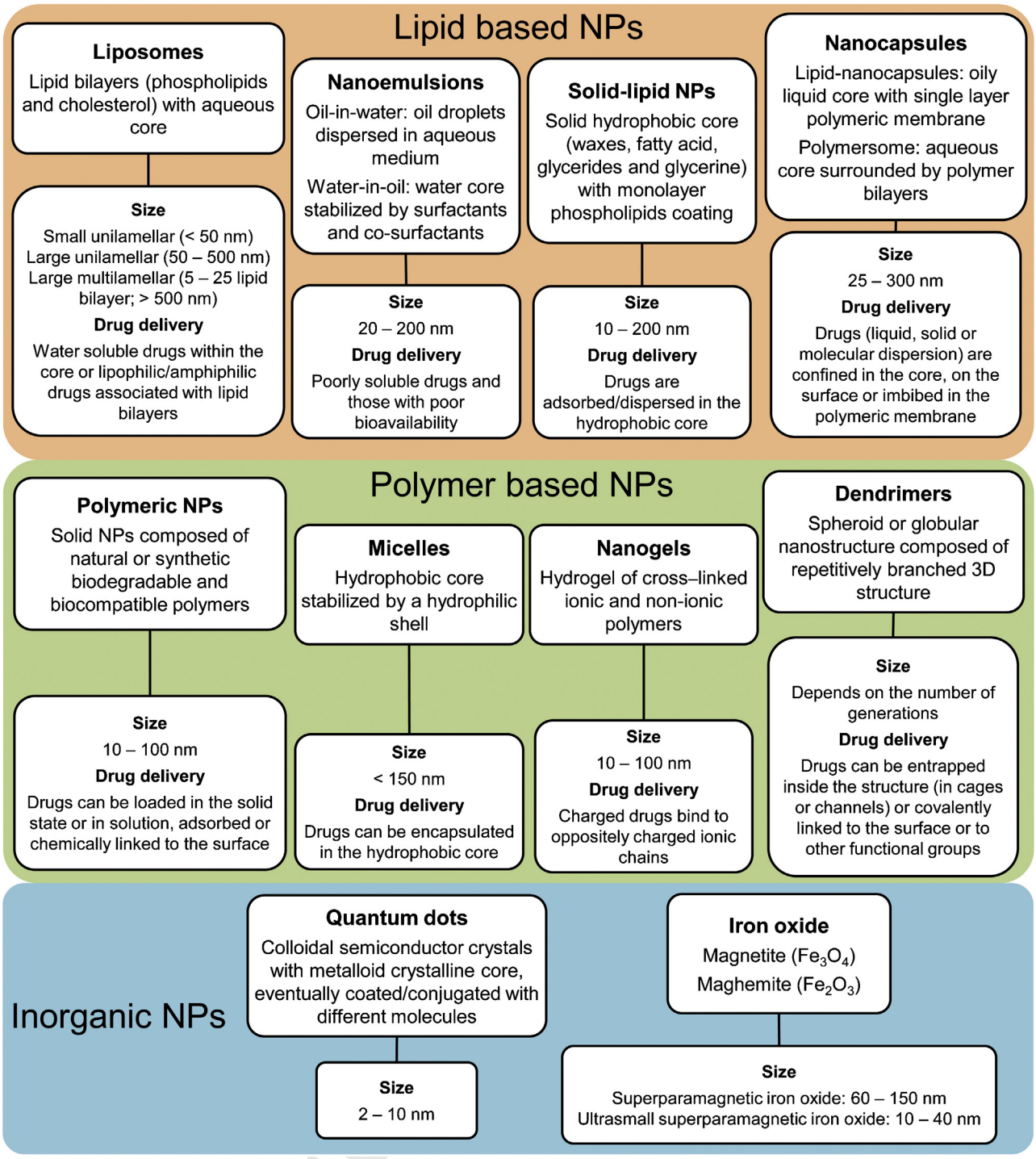


**Fig. 2.** Classification of NPs. This scheme summarizes the most commonly used NPs in drug delivery to the brain and their principal features.

(usually phospholipids and cholesterol) that self-assembly into bilayers to encapsulate an aqueous core. Drugs with widely varying lipophilicity/hydrophilicity can associate with liposomes. Water-soluble drugs can be encapsulated within the aqueous core, while lipophilic or amphiphilic compounds can associate with the lipid bilayers (entrapped in the phospholipid bilayer or at the bilayer interface) (Barratt, 2000). Liposomes can be classified depending on their size and the number of lipid bilayers (small unilamellar vesicles or large unilamellar vesicles and large multilamellar liposomes), or in terms of composition and delivery mechanism (conventional liposomes, pH sensitive liposomes, cationic liposomes, immunoliposomes, long-circulating liposomes). Cationic liposomes are used for gene transfer because of their efficiency in entrapping genetic material. The spontaneous electrostatic interaction between the positive charge of the liposome and the negative charge of DNA results in an efficient condensation of the nucleic acids, thus facilitating DNA encapsulation and subsequent transfection (Artzner et al., 2000; Caracciolo and Amenitsch, 2012). A recent study also showed the possibility of using cationic lipidic-NPs for the co-delivery of nucleic acid therapeutics along with gadolinium as contrast agent. In this way, it should be possible to combine gene transfer with non-invasive

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detection of vector localisation by magnetic resonance imaging (MRI) (Writer et al., 2012).

The mechanism by which liposomes pass the blood-brain barrier (BBB) is not fully understood. The transport likely occurs by diffusion or by endocytosis through the brain endothelial cells. Endocytosis is thought to represent an important route for the transport of smaller liposomes, with a diameter of 80–100 nm, because this size is comparable to that of vesicles normally transported by endothelial cells (Brasnjevic et al., 2009; Garcia-Garcia et al., 2005). The liposome technology is used since more than 40 years, but liposomes-based products have not significantly entered the market because of their instability, poor batch-to-batch reproducibility, difficulty in sterilization, low drug loading (Bawarski et al., 2008) and rapid clearance.

Lipid -based NPs delivery system also include nanoemulsions. Nanoemulsions are nanoscale emulsions, including oil -in-water emulsions (oil droplets dispersed in aqueous medium) and water -in-oil emulsions (in which water is the liquid core of the particle), stabilized by an interfacial film of surfactant and co -surfactant molecules. These formulations overcome the difficulty in delivering drugs with poor bioavailability and solubility by encapsulating them in the inner phase. Nanoemulsion droplets for drug delivery have diameters of 20–200 nm, are kinetically stable and are versatile: the selection of the oil phase, water phase and surfactants allows to control particle size and shape, surface charge and particle to particle interactions (Sarker, 2005). Moreover, the choice of the oil components can influence the nanoemulsion uptake in the brain. Edible plant–seed and fish oils, containing high percentage of polyunsaturated fatty acids such as omega -3 and omega-6-fatty acids, are able to enhance brain uptake of drug -loaded-nanoemulsions, probably due to their similarity to natural fatty acids up taken in the brain (Desai et al., 2008; Hamilton and Brunaldi, 2007; Vyas et al., 2008).

Another lipid -based nanoparticulate drug delivery system is represented by the solid -lipid NPs. These NPs are ideally suitable for optimized drug delivery with reduced toxicity. They have a lipidic core, which is in solid state at room and body temperatures, and is stabilized by surfactant and a phospholipid monolayer coating. The solid core encompasses, embedded in the lipid matrix, hydrophobic end of the phospholipid chains, and may contain dissolved or dispersed both hydrophilic and hydrophobic drugs. Solid lipid NPs are manufactured from synthetic or natural lipids, which are physiologically well tolerated and often already approved for pharmaceutical application in humans and "generally recognized as safe", such as fatty acids, mono -, di- or triglycerides, glycerine mixtures and waxes (Bondì and Craparo, 2010). Solid lipid NPs are less toxic than cationic liposomes, and have higher drug entrapment efficiency compared to other lipid based NPs. It was shown that drugs in the lipid core are stable and that the NPs can release the drug in a slow fashion for up to several weeks (Mishra et al., 2010). Recent evidence points at solid lipid NPs as a promising alternative drugs delivery system for the treatment of brain tumours (Martins et al., 2012).

Nanocapsules are another class of lipid-based NPs, characterized by a hybrid structure between polymeric NPs (see below) and liposomes, which display enhanced stability when compared to liposomes (Huynh et al., 2009). Lipid nanocapsules consist of a liquid oil core surrounded by a single layer polymeric membrane. Another type of nanocapsules, not containing lipids, are the polymersomes with an aqueous core surrounded by polymer bilayers (Letchford and Burt, 2007). Nanocapsules are being currently considered as useful drug carriers for brain glioma treatment (Bernardi et al., 2013; da Silveira et al., 2013; Fang et al., 2014).

### 3.2. Polymer-based NPs for therapeutic use

Polymeric NPs are solid particles on which drugs can be loaded in the solid state or in solution, adsorbed or chemically linked to the surface. The polymers used for polymeric NPs include natural and synthetic materials of proven biodegradability and biocompatibility, non-toxic, non-thrombogenic, non-immunogenic, and finally inexpensive. The synthetic polymers (polyesters and their copolymers polyacrylates and polycaprolactones) offer advantages over natural molecules (albumin, gelatin, alginate, collagen and chitosan) because they can be better modified to display a broad range of desirable properties. Other reasons why natural polymers have not been widely used to date are the batch-to-batch variability due to different purification processes, and the risk of denaturing the embedded drugs during the required cross-linking procedures (Hans and Lowman, 2002). At present, two polymers (polylactide-co-glycolide and polylactide) are approved by the Food and Drug Administration for the preparation of NPs, but clinical data evaluating polymer-based NPs as drug carriers are still lacking (Costantino, 2010; Costantino and Boraschi, 2012). The degradation of polylactide or polylactide-co-glycolide into oligomers and monomers of lactic and glycolic acids (substrates of the Krebs cycle) occurs by an autocatalytic cleavage of the ester bonds through spontaneous hydrolysis (Li, 1999). Depending on their molecular weight and their conjugation with other polymers [such as polyethylene glycol (PEG)], these biodegradable polymers show different clearance/elimination kinetics (Bazile et al., 1992; Li, 1999).

A commonly used polymer for the preparation of NPs for CNS drug delivery is polyalkylcyanoacrylate (Andrieux and Couvreur, 2009; Vauthier et al., 2007), not yet approved by the Food and Drug Administration for intravenous administration, but currently in phase II of clinical trial.

Polymeric micelles consist of amphiphilic polymers, having a block-structure with a hydrophilic head-group and hydrophobic tail which self-assemble in an aqueous environment into structures composed of a hydrophobic core stabilized by a hydrophilic shell (Torchilin, 2007). The hydrophilic shell provides steric stability and may be able to avoid rapid uptake by the reticuloendothelial system, resulting in prolonged circulation time in the body (Adams et al., 2003). The hydrophobic core allows the encapsulation of hydrophobic compounds with limited transport to the brain, protecting them from degradation and rapid blood clearance, thus achieving prolonged therapeutic activity. PEG, the most commonly used hydrophilic polymer, is a water soluble, highly hydrated, efficient steric protector, biocompatible and with limited toxicity.

Nanogels are nanosized hydrophilic polymer gels (hydrogels) of cross-linked ionic and non-ionic polymers (Vinogradov et al., 2002). These NPs are suitable for incorporating charged molecules such as siRNA, DNA, oligonucleotides and low molecular mass compounds, which bind to oppositely charged ionic chains.

Dendrimers are spheroid or globular nanostructures with very well defined chemical structures that are engineered to carry drugs encapsulated in their interior void spaces or attached to their surface. Dendrimers have a highly branched 3D structure constituted of three distinct domains (central core, internal branches, reactive surface groups) that provide a high degree of surface functionality and versatility. The core of dendrimers may be a single atom or a group of atoms having similar chemical characteristics or functions; the branches emanating from the core towards the periphery are composed of repeated units that have at least one junction of branching and connect the core to the surface groups; these groups play a key role in gene-complexing, drug-entrapping and targeting ability. The size can be precisely controlled during the polymerization process, as the number of



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branches and surface groups increase exponentially with each generation. Cavities in the core structure and folding of branches create cages and channels for drug delivery (Tomalia and Fréchet, 2002).

### 3.3. Polymeric NPs in brain imaging and diagnosis (dendritic NPs)

Dendritic NPs, in addition to therapeutic use, find application in brain imaging as contrast agents with improved performance. Bertin and collaborators recently developed a hydrophilic dendritic Mn(II) contrast agent for manganese-enhanced MRI of the brain (Bertin et al., 2009). Intense investigations is currently ongoing regarding the safety of the new dendrimer-based MRI contrast agents (such as polyamidoamine dendrimers loaded with gadolinium), as well as commercially available dendrimers with PEGylation, acetylation, glycosylation, and amino acid functionalization, in view of their possible use as dendrimer-based nanotherapeutics (Cheng et al., 2011).

### 3.4. Inorganic NPs in brain imaging diagnosis and therapy (quantum dots and superparamagnetic NPs)

Quantum dots are spherical nano-sized (range 2–10 nm) colloidal semiconductor crystals composed of a metalloid crystalline core and a shell that improves optical properties and enhances water solubility (Bailey et al., 2004; Bawarski et al., 2008). The most commonly used semiconductor material in quantum dots is CdSe, but other semiconducting materials are also used, derived from the II and VI elemental groups (CdTe, CdS, CdHg, ZnS) to III and V elemental groups (InAs, InP, GaAs) (Yang, 2010). Interaction with photons excites the semiconductors that emit energy in the UV, visible or near-infrared regions, which can be detected. For this distinctive feature, quantum dots are emerging as a new class of fluorescent probes for both *in vitro* and *in vivo* imaging. Perhaps one of the most attractive and promising applications of quantum dots is the *in vivo* imaging for diagnostic and therapeutic purposes. In bio-imaging, quantum dots are used as contrast agents due to their ability to adsorb white light and re-emit it within nanoseconds with different bulk band gap-energies, corresponding to different combinations of particles (Bawarski et al., 2008). In comparison with "classic" fluorophores, such as organic dyes and fluorescent proteins, quantum dots have a broad excitation spectrum, narrow emission spectrum, good photostability and long fluorescent lifetime (Chan et al., 2002). Moreover, the quantum dots surface can be modified by conjugation of surface molecules for targeted delivery, for example coating with cysteamine or PEG to enhance their penetrating ability in CNS or their stability in aqueous solution. Quantum dots are also excellent donors in Förster resonance energy transfer (Hildebrandt and Geissler, 2012).

Other inorganic NPs widely used in many applications are the iron oxide NPs. Depending on their size, these NPs are classified in superparamagnetic iron oxide (60–150 nm in diameter) and ultrasmall superparamagnetic iron oxide (10–40 nm) NPs. These NPs have an iron core and can include either inorganic materials (silica, gold) or synthetic/natural organic materials (phospholipids, fatty acids, polysaccharides, natural polymers such as dextran or chitosan, biodegradable polymers such as PEG, non degradable polymers such as polyvinyl alcohol, peptides or surfactants).

The superparamagnetic iron oxide NPs are already used as MRI contrast agents and are under investigation as drug delivery systems, while the ultrasmall superparamagnetic iron oxide NPs are of interest for gene-delivery (Hofmann-Amtenbrink et al., 2010). Superparamagnetic iron oxide NPs are also used for hyperthermia treatment of gliomas. In fact, these NPs have an optimal intratumoural distribution and are able to generate, under appropriate magnetic field, controlled heating of the pathological brain areas with minor side effects (Silva et al., 2011). Interestingly, superparamagnetic iron oxide NPs are being used for the non-invasive localization of inflammatory and neurodegenerative processes, and brain tumours by loading monocytes/macrophages with these NPs that then accumulate in the site of ongoing reactions (Petry et al., 2007). In addition, these NPs can be used for the selective detection of brain gliomas upon their conjugation with lactoferrin (Xie et al., 2011).

### 3.5. Important chemical–physical characteristics of NPs

NPs exhibit properties that are a bridge between those of atoms and those of corresponding bulk materials. The small size of NPs influences their chemical–physical properties and their interaction with the environment. In fact, in case of bulk material (larger than 1 μm) there is a very low ratio between the number of atoms on the surface and those in the bulk, while at nanoscale range the ratio between the surface area and volume is higher. Thus, the characteristics of atoms on the surface of NPs strongly determine their chemical–physical properties and reactivity. For example, the melting behaviour is one of the chemical–physical parameters that differs in nanoscale and in the corresponding bulk materials. Small particles have lower melting points than bulk material: due to an increased ratio of surface to internal atoms as the size of particles decreases, in many NPs the melting temperature decreases with their particle size and the heat of fusion as well (Qi and Wang, 2004; Sun and Simon, 2007). Furthermore, the geometry of NPs strongly influences their interaction with the environment. For instance, the charge distribution on the surface of spherical inorganic NPs is quite different from that on rod or urchin shaped particles (Hutter et al., 2010).

The chemical–physical characteristics of NPs determine their biodistribution, biological effects, and consequently their toxicity. In the case of engineered NPs for drug delivery, the chemical–physical characteristics influence drug loading, drug release, NPs stability and cellular uptake. The parameters that influence the biological behaviour of NPs are size, shape and composition. As cited before, the small size of NPs increases NP reactivity and, as a consequence, confers them new biological properties (*e.g.*, catalysts for chemical reactions, etc.). Furthermore, the surface atoms or molecules play a dominant role in determining material properties (Amato, 1989). The large area and the chemistry of NPs surfaces promote their aggregation (Limbach et al., 2005) and the interaction with biomolecules such as proteins and DNA in biological environments. The presence of mono- or divalent cations favours NPs aggregation in phosphate buffered saline and cell culture medium (Long et al., 2006), with aggreagates of different size depending on the type of aqueous medium (*e.g.*, different average diameters of clustered $CoFe_2O_4$ NPs in different media such as diethylene glycol, phosphate buffered saline and cell culture medium) (Marmorato et al., 2011).

The features of nanomaterial determine the type and amount of blood components that bind to NPs surface. NPs can interact with and adsorb media components, such as ions and proteins present in the serum, thereby changing their size (due to aggregation), biodistribution and consequent interaction with cells. NPs initially adsorb a significant amount of serum proteins as well as lipids and sugars. However, the composition of the protein "shell" changes with time, with the type and amount of protein stably adsorbed on the NPs surface being material-, time- and temperature-dependent. Also the hydrophobicity of NPs influences their capacity to bind proteins. It has been hypothesized that interaction of NPs with proteins might alter protein conformation leading to exposure of new epitopes and/or abnormal functions (Mu et al., 2009). In addition, interaction of NPs with cells is dictated by the



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type and amount of proteins adsorbed on the NP surface, which could influence NPs uptake by cells, cellular signalling pathways and viability (Monopoli et al., 2011).

Due to their size, NPs should be easily taken up into living organisms; smallest NPs penetrate the cellular barrier (such as BBB) more rapidly than larger ones (Chen et al., 2010). Together with size, shape also interferes with the NP biodistribution and organ-specific accumulation. Recent evidence shows that sub-micrometric discoidal particles accumulate in lungs, liver, heart and spleen more than spherical, quasi-hemispherical and cylindrical particles (Decuzzi et al., 2010). In the CNS, relatively thin rods (12 nm in diameter) have a more favorable morphology for the internalization in neurons, while urchin NPs of about 77 nm are easily phagocytosed by microglia (Hutter et al., 2010).

The influence of the crystalline phase on NPs toxicity is still discussed. It has been shown that $TiO_2$ NPs with the same dimension are able to generate different amount of ROS depending on the crystal phases. ROS production was highest with amorphous particles, followed by anatase, and then anatase/rutile mixtures, and lowest for rutile samples (Jiang et al., 2008). However, the body of *in vivo* literature suggests that all spherical $TiO_2$ NPs are of low toxicological concern, except when used at excessively high doses and independent of crystal phase (see for example Bonner et al., 2013; Sayes et al., 2007).

## 4. NPs entry into the CNS

With the extension of human life expectancy, the incidence of neurological diseases is bound to increase significantly in the 21st century. Therefore, the modern ageing societies require a broad spectrum of successful diagnostics tools and treatments for neurological diseases. Drug delivery to the CNS is difficult due to the presence of BBB and today only a small class of "classical drugs" or small molecules with high lipophilicity and low molecular mass (<400–500 Da) are able to enter the CNS (Pardridge, 2003). NPs have long been known to enter the CNS (Kreuter, 1994), thus they can represent a promising vehicle for delivery of drugs for diagnosis and therapeutics scopes. On the other hand, the massive use of NPs in daily-use products, and the continuous exposure to environmental NPs may pose a risk, as these NPs could interfere with the CNS functions or display toxic effects on neural cells.

Human exposure to NPs may occur by different routes: dermal uptake, ingestion, inhalation and intravenous injection (Borm et al., 2006), but all end up in the blood circulation, and through this way NPs may reach the CNS crossing and/or damaging the BBB. Another important mechanism by which the inhaled NPs may enter the CNS is the nose-to-brain transport through the olfactory epithelium. Also the blood cerebrospinal fluid barrier could represent a way to enter the brain for NPs, however its surface is 1000-folds smaller than the surface area of BBB and it does not seem to be significantly involved in the penetration of drugs into the brain (Pardridge, 1997).

The ability of NPs to penetrate the CNS and their kinetics depend on the NPs surface chemistry, coating with surfactants or specific peptides/lipids, and on the possible *in vivo* surface modifications (Kreuter, 2004).

### 4.1. Uptake mechanism of NPs across the BBB

NPs can enter the CNS through the BBB *via* systemic distribution. The BBB is protective network of vessels and cells that strictly regulates the transport from blood to the brain and *vice versa*. Endothelial cells, clamped together by tight junctions, form the main physical barrier of the BBB. The BBB also include astrocyte end-feet and pericytes. Astrocytes can modulate the BBB functionality in several ways: (i) physical barrier, by tightening junctions; (ii) transport barrier, by regulating the expression and polarization of transporters; (iii) metabolic barrier, by modulating specialized enzyme systems (Abbott et al., 2006). Pericytes seem to regulate the BBB-specific gene expression patterns in endothelial cells and to induce the polarization of astrocyte end-feet (Armulik et al., 2010).

Considering that the dimension of most NPs is larger than that of low molecular weight molecules or nutrients (*i.e.*, aminoacids, peptides, proteins, sugars, etc.), they cannot pass across the BBB through any of the identified pathways (Abbott et al., 2006). Since the tight junctions in the BBB have 4–6 nm gaps, only NPs smaller than these size can pass *via* inter-endothelial tight junctions, whereas the majority of NPs pass through the endothelial cell plasma membranes by transcytosis (Sharma and Sharma, 2007; Zensi et al., 2009). In a model based on the hCMEC/D3 human endothelial cells line, which form tight junctions between adjacent cells, it has been demonstrated that 50 nm $SiO_2$ NPs were transported *via* transcellular processes (Fig. 3) (Ragnaill et al., 2011). In a co-culture of rat brain microvessel

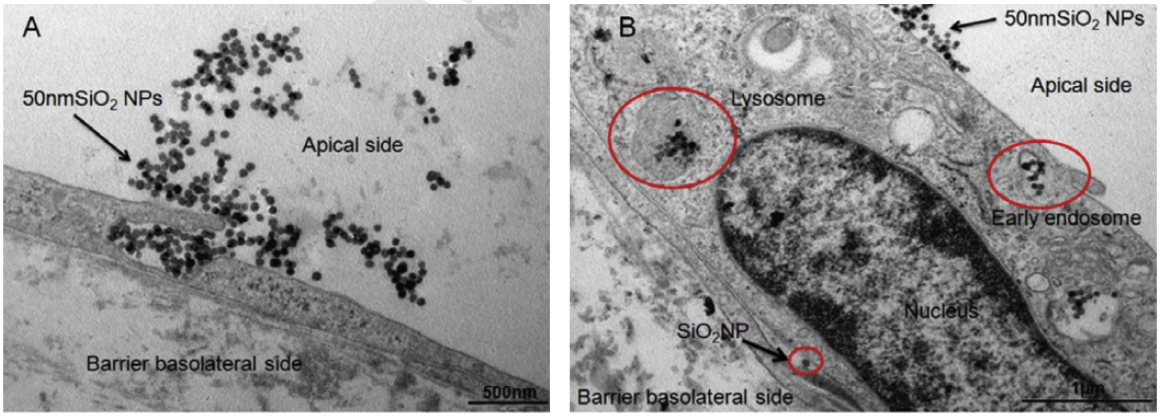


**Fig. 3.** NPs in a BBB model. Electron microscopy image of 50 nm $SiO_2$ NPs transport through the hCMEC/D3 molayer that mimics the BBB, 4 h post exposure (100 μg/ml; 1 h exposure). (A) In this panel the plasma membrane envelops the 50 nm $SiO_2$ NPs demonstrating the uptake process at the apical side of the monolayer. (B) The above mentioned NPs are visualized in endosomes and lysosomes organelles. A process of exocytosis with release of NPs at the basolateral side is also shown. Scale bar 500 nm (A) and 1 μm (B).
Reprinted from Eur. J. Pharm. Biopharm. 2011 77 (3), Ragnaill, M.N., Brown, M., Ye, D., Bramini, M., Callanan, S., Lynch, I., Dawson, K.A., "Internal benchmarking of a human blood-brain barrier cell model for screening of nanoparticle uptake and transcytosis", 360–367, Copyright (2013), with permission from Elsevier Limited.

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endothelial cells and astrocytes, Ag NPs (100 μg/ml, for 4 h) crossed this barrier model, probably by transcytosis, and accumulated inside brain vascular endothelial cells (Tang et al., 2010). Experimental evidence showed that NPs are able to accumulate not only in endothelial cells but also in pericytes. In a mouse model of cerebral ischemia, superparamagnetic iron oxide NPs were observed in vascular pericytes in the region of BBB injury (Liu et al., 2012a).

Although the exact mechanisms of NP influx across the BBB is not fully understood, receptor- and adsorptive-mediated transcytosis are supposed to be the main routes (Fernandes et al., 2010; Hervé et al., 2008; Kreuter, 2001). The first mechanism is specific, while the second one is non-specific (Fig. 4). The precise mechanism of NPs transcytosis from luminal to abluminal membrane of the endothelial cells is not completely identified, given the fact that the majority of transported material is degraded in the endosomal/lysosomal compartments that should be avoided to preserve transported NPs from degradation (Hervé et al., 2008). Transcytosis can occur *via* clathrin coated pits/vesicles (numerically most abundant and found associated with NPs also in an *in vivo* model, see Fig. 5C) and caveolae (rare in brain capillaries), which are opened at the luminal surface of the BBB endothelial cells (Fernandes et al., 2010; Hervé et al., 2008). Also transport proteins in the endothelial cell membrane may contribute to NPs passage through the BBB. Recently, in an *in vitro* model of BBB (brain endothelial cells and astrocytes co-cultures) carrier-mediated endocytosis of glutathione-coated NPs has been suggested as potential tool to deliver drugs, *e.g.*, for brain cancer treatment (Geldenhuys et al., 2014).

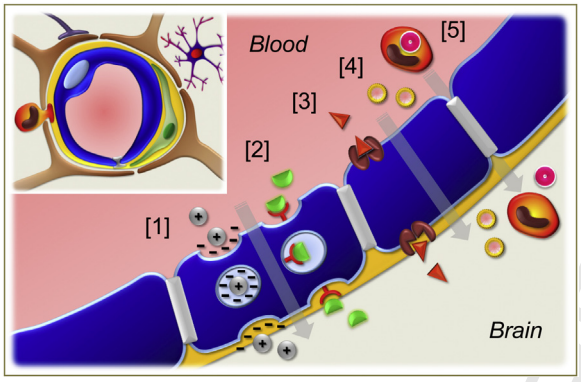


**Fig. 4.** Possible mechanisms by which NPs can pass through the BBB. The schematic section of the neurovascular unit forming the BBB is represented in the upper panel. The BBB is a selective barrier formed by capillary endothelial cells (blue) joined each other by tight junctions (grey) and surrounded by a basal lamina (yellow). Brain capillaries are surrounded by or closely associated with several cell types, including the perivascular endfeet of astrocytes (brown) opposed to the outer surface of the basal lamina, pericytes (green) which are enclosed in the basal lamina and neuronal processes (purple) that can reach the BBB releasing vasoactive neurotransmitters and peptides. The cartoon also shows microglia (blue-red; the resident immunocompetent cells of the brain) and perivascular macrophages (orange-red; located on the parenchimal side of the endothelial cells near astrocytic endfeet), which derive from systemic circulating monocytes. The figure shows possible mechanisms by which NPs can cross the BBB. [1] Adsorptive-mediated transcytosis. This pathway does not require specific interactions such as ligands to membrane receptors, but starts with a charge–charge interaction of positively charged NPs with negative charges of the luminal membrane. [2] Receptor-mediated transcytosis. NPs can be conjugated/coated with specific ligands (*e.g.*, insulin, transferrin, lactoferrin, ceruloplasmin, apolipoproteins, diphteria toxin, etc.,) in order to be captured by receptors present on the endothelial cell surface. This way requires the following steps: receptor mediated endocytosis of the NPs at the luminal side (blood), movement across the polarized endothelial cell, exocytosis of the NPs at the abluminal side (brain). [3] Carrier-mediated transport. NPs coniugated/coated with specific molecules (*i.e.*, gluthatione, amino acids, etc.) can be uptaken through specific transport proteins. [4] Transcellular lipophilic diffusion. Lipid based NPs could potentially use this pathway to cross the BBB endothelium. This pathway is only available to molecules with strictly defined criteria (*e.g.*, low molecular weight, unionized, *etc.*). [5] Cell-mediated transcytosis. Monocytes/macrophages can endocytose high amounts of NPs, cross the BBB and slowly release them by exocytosis into the brain parenchyma acting as Trojan horses.

Considering that perivascular macrophages come from circulating mononuclear phagocytes (monocytes), which cross the intact BBB with high turnover, a cell-mediated transport has been recently proposed as a possible route of NPs passage across the BBB (Fig. 4). Circulating monocytes could take up NPs in the blood and translocate them to the brain like Trojan horses (Chen and Liu, 2012). It should be considered that all these mechanisms of NP transport across the BBB might be enhanced in some circumstances: (i) during brain pathological conditions, such as neurodegenerative/neurovascular disorders, brain tumours, metabolic disease or inflammatory states, in which the BBB structure and integrity is impaired (Chen and Liu, 2012; Lafuente et al., 2012; Lossinsky et al., 1995); (ii) in the case of increased BBB permeability by means of biological, chemical and physical stimuli that temporarily open the tight junctions to enhance the transport of NPs (Chen and Liu, 2012).

Different factors may influence the NP uptake: size, type and surface properties. Transport of lipid based NPs, such as liposomes, across the BBB endothelial cells seems to be achieved by passive diffusion (only possible with molecules having specific properties), by endocytosis (especially for liposomes <80–100 nm) or by fusion with plasmalemma membrane (Brasnjevic et al., 2009). Polymer based NPs, such as dendrimers, could be taken up by both clathrin coated pits/vesicles and caveolae, as shown by decrease NP captation with the use of inhibitors of these pathways in *in vitro* model of BBB (Dhanikula et al., 2009).

### 4.2. Strategies to enhance the NPs targeting to the CNS

To enhance NPs bioavailability and to facilitate drug delivery to the target (tissue or cell type), NPs can be coated and/or modified by the addition of specific ligands on the outer surface, such as antigens, antibodies or receptor ligands (Biswas et al., 2011; Koshkaryev et al., 2011; Torchilin, 2010). Furthermore, it is necessary to prolong NP permanence in the circulation by limiting their uptake by the reticuloendothelial system and by liver cells. One of the most used methods is surface coating with PEG (Blasi et al., 2007; Mishra et al., 2010; Yang, 2010). PEGylated NPs display a minimal surface charge that reduces NP opsonization leading to low level of uptake by the reticuloendothelial system (Li and Huang, 2009). This is the case of Doxil®, one of the two nanoparticulate cancer drugs (together with Abraxane®) approved by the Food and Drug Administration for the treatment of recurrent ovarian cancer, AIDS-related Kaposi's sarcoma and multiple myeloma. Doxil® is the anti-cancer drug doxorubicin repackaged into PEG coated liposomes. The use of PEGylated liposomes improves the pharmacological profile of doxorubicin prolonging its half-life, slowing drug release, increasing tissue and tumour concentrations. Thus, drug uptake by the tumour is improved, possibly through the enhanced permeability and retention effect, and the anti-cancer therapeutic effect is enhanced (Gabizon, 2001; Gabizon et al., 2003).

In the case of CNS, several approaches have been attempted for enhancing the NPs passage through the BBB. It has been shown that the NP transport across the BBB is enhanced by surfactants, as in the case of polybutyl-cyanoacrylate (PBCA) NPs coated with polysorbate (PS)-80, which can cross the BBB, while uncoated NPs remain in the blood vessels (Kreuter et al., 1995; Ramge et al., 2000). PBCA NPs are among the most studied nano-enabled drug delivery systems for the brain. By testing twelve different surfactants it was found that only PBCA NPs coated with PS-20, -40, -60 and -80 can cross the BBB, the maximum effect being detected with PS-80 (Kreuter et al., 1997, 1995; Schroeder et al., 1998). The PBCA/PS-80 NPs are transported through the BBB *via*



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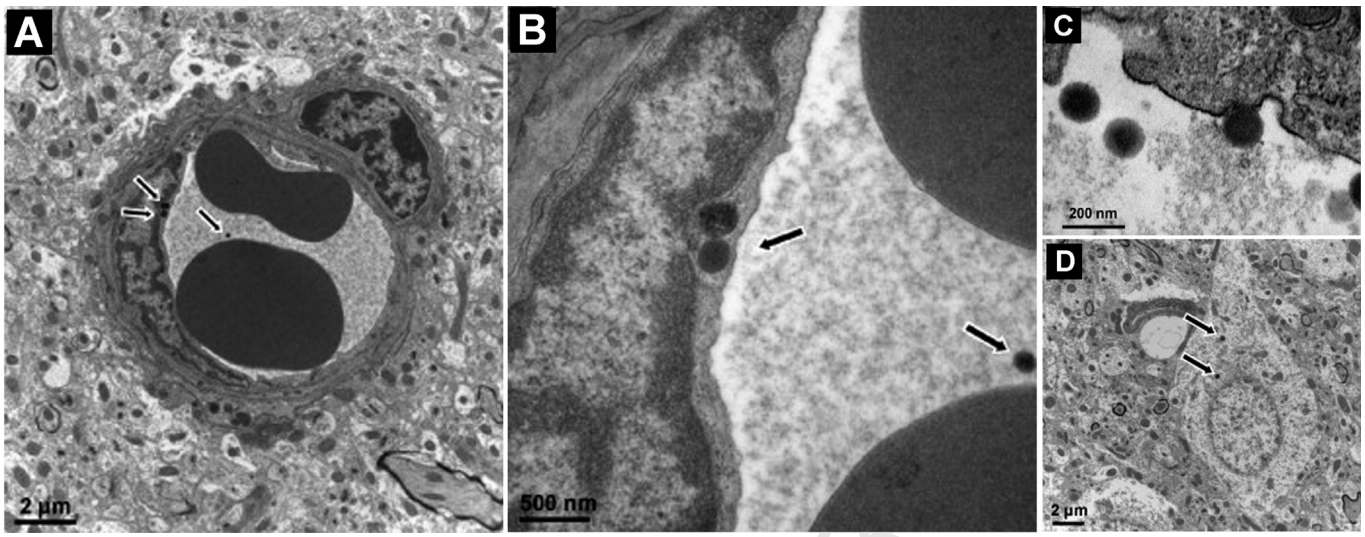


**Fig. 5.** NPs cross the BBB in *in vivo* model and reach neurons. Electron microscopy image of SV129 mice cortex after injection of apolipoprotein E coated human serum albumin NPs (see details in the text). The injected NPs (arrow in A) are present in the lumen of blood vessel and enter in the endothelial cells 15 min post injection (A and B). The higher magnification panel (C) shows the formation of coated pit in the endothelial cell membrane in close contact with NPs. These particles enter the neurons 30 min after injection (arrows in D), indicating the transcytosis of NPs across the BBB endothelium and their infiltration in brain parenchima. Scale bar 2 μm (A, D), 500 nm (B), 200 nm (C). Modified from J. Control Release, 2009, 137 (1), Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Wagner, S., Buchel, C., von Beriesen, H., Kreuter, J., "Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and delivered to neurons", 78–86, Copyright (2014), with permission from Elsevier Limited.

low density lipoprotein receptor-mediated endocytosis. After intravenous administration, the circulating apolipoproteins are adsorbed on the surface of the PBCA/PS80 NPs, and the apolipoprotein E makes the NPs being mistaken for low density lipoproteins particles, causing their uptake and internalization *via* low density lipoprotein receptor (Kreuter et al., 2003, 2002). This Trojan horse mechanism was demonstrated to occur in mice injected with human serum albumin NPs covalently bound with apolipoprotein E (199 nm particles; 10 μl suspension, containing 200 μg NPs, per g of body weight in jugular vein). Only this type of apolipoprotein E coated NPs is rapidly taken up by endothelial cells of BBB by endocytosis followed by transcytosis, and can reach neurons of different brain areas (Fig. 5) (Zensi et al., 2009).

It should be noted that several reports emphasize the danger of complement activation by PEG and surfactants, a fact that may cause serious detrimental effects both systemically and locally in the brain (Moghimi et al., 2010; Weiszhár et al., 2012; Andersen et al., 2013). Thus, surface modifications of NPs for facilitating their entry into the brain should take into account the safety aspects as much as the delivery/transport issues.

Other targeting strategies include the use of peptides that are used as ligands for receptors present on the brain endothelium, for example the peptide T7 (sequenced HAIYPRH), which is recognized by the transferrin receptor with an affinity comparable to that of transferrin. This peptide sequence can be conjugated to dendrimers and used to deliver MRI contrast agents (Han et al., 2011). In an *in vitro* model of BBB, endocytosis of transferrin-coated polylactide-co-glycolide NPs was about 20-fold greater than that of uncoated NPs. The specificity of receptor-mediated endocytosis was evident and the transferrin-coated polylactide-co-glycolide NPs entered the cell *via* the caveolae mediated endocytic pathway (Chang et al., 2009). Also lactoferrin, covalently conjugated to NPs, can facilitate the transport across the BBB. It has been recently demonstrated *in vitro* and *in vivo* that lactoferrin-coated $Fe_3O_4$ NPs exhibited an enhanced ability to cross the BBB compared to PEG-coated $Fe_3O_4$ NPs and the mechanism involved in the delivery was the lactoferrin-receptor mediated transcytosis (Qiao et al., 2012).

Due to the negative electrostatic charge of brain microvasculature endothelial cells at physiological pH, the electrostatic charge of NPs influences the penetration of the BBB. Positively charged molecules increase endothelial cell permeability, in fact, cationic NPs translocate more readily to the brain compared to anionic or neutral particles (Fenart et al., 1999). The same effects have been observed for NPs coated with modified proteins, such as cationic bovine serum albumin (Lu et al., 2007). On the other hand, cationic NPs have an immediate toxic effect on the BBB, as explained below (Lockman et al., 2004).

It is important to say that strategies aiming at enhancing NP passage through the BBB are increasingly being considered undesirable. Breaking the BBB or increasing the non-physiological passage of molecules and particles is a very dangerous strategy, which may allow the passage of many potentially dangerous cells, agents and molecules together with the NPs. This could end up causing about more damages than advantages. The more recent strategies under development focus on the functional modulation/activation of endothelial cells by NPs at the luminal level, with the aim of inducing the release of endothelial signals and mediators at the abluminal site, which can modulate brain functions, without affecting the BBB integrity (Moghimi SM, personal communication).

### 4.3. NPs uptake by nerve endings embedded in the airway epithelia

The nose-to-brain transport of NPs is a critical issue, especially in humans under high environmental or occupational NPs exposures, but also in chronic drug treatments. This route mainly involves the olfactory and/or the trigeminal nerve. The axons of olfactory receptor neurons, localized in the olfactory epithelium that lines a portion of nasal cavity, enter in the olfactory bulb forming the olfactory nerve while the trigeminal nerve passes in the respiratory epithelium and mouth. The inhaled NPs are taken up by sensory nerve endings and may reach the CNS by axonal translocation (Kreuter, 2001; Kreuter et al., 1995) bypassing the BBB. NPs (ultrafine solid particle) can translocate from the axon of the nerves to olfactory bulbs within 24 h, as demonstrated in the rat. However, this way appears to be relatively inefficient in delivering inhaled NPs to more distant brain structures (Oberdörster et al., 2004). In addition, in rhesus monkeys it has been

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demonstrated that there is no direct translocation of Mn NPs from the olfactory bulb to the globus pallidus, the target area for Mn neurotoxicity (Dorman et al., 2006). The exact mechanism of nose-to-brain NPs transport is not fully understood and requires further investigations, as it could become a non-invasive drug delivery system to the brain.

To date the nose-to-brain transport has been mainly studied using metals and carbon NPs in rodent models after inhalation or intranasal instillation (Oberdörster et al., 2005), but it should be considered that airway epithelia and turbinate anatomy is quite different between rodents and humans.

The time required for the passage from nerve endings to the CNS depends on the NPs nature and size. It is also important to note that certain metals, such as inhaled tungsten and iron (in the form of sulfate salt), are poorly transported by the nose-to-brain pathway (Radcliffe et al., 2009; Rao et al., 2003).

## 5. Neurotoxicity of NPs

In addition to the desired effects of engineered NPs, it is important to consider carefully the toxic effects of NPs. An effort is being taken worldwide to identify risk, design predictive assays and establish a solid regulatory framework, so that technological advancement of nanotechnology can develop in a knowledge-based environmentally friendly fashion, by adopting a safe-by-design NP production strategy. This is still far, but it has fostered a wealth of studies in the nano-safety area, including modelling and structure-function relationship. The consequences of exposure to NPs may be relevant for all biological systems, from simple environmental organisms up to man. *In vitro* and *in vivo* studies revealed that NPs can interact with main systems, like respiratory, cardiovascular, immune and CNS, as well as several organs. However, the conclusions of many studies are partially reliable, mainly due to the incomplete characterization of NPs, largely missing information about putative mechanisms of uptake and action, and to the unrealistically high doses used in the experiments. In addition, the life-cycle of NPs within biological systems must be considered including for instance their dissolution and ion release, as this obviously influences the toxicity output.

Thus, in many instances toxicological studies report conflicting results, even in cases when the same biological model is used and allegedly the same nanoparticulate material is employed. With the increase of nanomedicine applications, the need of an accurate assessment of toxic effects on human health has become imperative. The controlled administration of NPs to human beings, as it occurs with nanomedicine, provides an important source of relevant data (*in vivo* in human beings) to the nanotoxicologist, who can relate possible detrimental effects to NPs that are characterized in terms of chemical–physical properties, composition, purity, dose, route and schedule of administration. This is an ideal situation that never occurs in the case of accidental exposure to engineered NPs or exposure to environmentally-borne NPs. Even in the case of nanomedicine, a number of variables should be carefully considered, as they can significantly influence the outcome of the interaction of NPs with the human body systems. The first of these variables is the interaction of the NPs with the proteins and other molecules present in the blood and tissues. There is solid experimental evidence that NPs injected intravenously are immediately coated with plasma proteins and other soluble molecules, forming on NPs surface the so called "protein or biomolecular corona" (Casals et al., 2010; Monopoli et al., 2012). This macromolecular bioshell varies in composition and thickness depending on the NPs chemical and surface properties, but also depending on the physical conditions of the patient (plasma composition can be very different, for example in hypercolesterolemic, autoimmune, diabetic, aged or infected individuals). The composition of the bioshell, which is dynamic and may change with time, then determines the features of the interaction with cells, starting from blood cells and endothelial cells up to the tissue target cells. After interaction with the cell surface and the consequent internalization, the biomolecular corona of NPs may change, as NPs in the endocytic vesicles find a different environment (different pH, different proteins and other macromolecules), thereby either allowing NP degradation or determining a different range of possible interactions (Wang et al., 2013). While most administered NPs are rapidly excreted, a significant fraction can be trapped by the reticuloendothelial system, mainly monocytes and tissue macrophages, and only a small fraction of the injected dose can reach the target tissue (*e.g.*, this can be between 0.01% and 0.5% in the case of targeted nanocarriers for drug delivery across the BBB). The impact of NPs on the CNS is poorly studied, and the few data available are mostly from NPs employed in nanomedicines and everyday products. This is the case, for example, of chronic exposure to metal NPs that may induce neuroinflammation and neurotoxicity (Sharma and Sharma, 2012). For this reason nanoneurotoxicity evidences should be carefully evaluated and future experimental plan should be designed *ad hoc* before environmental and human application of NPs. In the light of above issue, it is still not clear how much the "pure" NPs really contributed to the observed effects, in the absence of an accurate characterization of the NPs that could exclude the presence of inflammatory/toxic contaminants, aggregation, etc.

One important point is the possible contribution of NPs to the pathogenesis of brain diseases. Here we summarize the effects on BBB, neurons and glial cells that have been reported for the engineered NPs commonly used and present in the environment.

The reported data on toxic effects of NPs have to be evaluated with criticism considering that most of the studies regarding NP toxicity do not consider the possible contamination of the NPs batches with toxic or biologically active agents [*i.e.*, the most common is bacterial endotoxin, lipopolysaccharide (LPS)]. The latter, when present, may be fully responsible for the observed toxic or activating effects (Oostingh et al., 2011; Vallhov et al., 2006). Thus, all the studies that show significant detrimental effects of NPs (*e.g.*, see below for BBB alterations, toxic and inflammatory effects) should be considered as preliminary until full characterization of NP-associated toxic/bioactive contaminants has conclusively ruled out the possibility of unspecific non NP-dependent effects.

In this review, we mainly focus on the effects of metallic NPs, which are largely used in several fields, thus leading to a continuous exposure for humans. For example, silver finds applications in medical and consumer products mainly in the form of Ag NPs for its antimicrobial effects. The NPs large surface-to-volume ratio increases the oxidation activity due to the release of monovalent Ag ions, which are responsible for the antimicrobial effects (Damm et al., 2008). Ag ions can diffuse in the organs primarily through the vascular system, but also by retrograde axonal transport (Danscher and Locht, 2010), reaching the CNS. In adults, neurotoxicity by Ag ions occurs only upon very high levels of exposure (Lansdown, 2007), and the toxic effect can be higher in the developing brain. Moreover, the same cytotoxic mechanisms that elicit the antimicrobial activity of Ag may damage neuronal cells. Ag NPs may produce neurotoxicity by generating free radical-induced oxidative stress and by altering gene expression thereby inducing apoptosis (Rahman et al., 2009).

### 5.1. Effects on BBB

As cited before, the BBB protects the CNS, therefore an alteration of the endothelial cell functionality may change the



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permeability of the BBB, allowing the brain influx of toxins, vasoactive material, neuroinflammatory molecules, and other immunologically active substances that consequently affect neurons and glial cells (Sharma and Sharma, 2013). Furthermore entry of proteins promotes the passage of water in the brain parenchyma causing edema formation, cell injury and eventually cell death (Sharma et al., 2009; Sharma, 2009a).

It has been shown that metallic NPs (Al, Ag and Cu, 50–60 nm) can significantly alter BBB function and permeability in animal models and induce pronounced cerebral edema in brain areas associated with BBB leakage. Moreover, in these brain areas neuronal cell injuries, glial cell activation, heat shock protein up-regulation and loss of myelinated fibers are quite frequent (Sharma et al., 2009), indicating that the alterations of BBB may also lead to neuroinflammatory and neurodegenerative processes. These effects of NPs were most pronounced in the case of intravenous injection of Ag and Cu compared to Al NPs (Sharma et al., 2010), since Ag and Cu are redox-active metals. Another study shows that in *in vitro* model of the rat BBB the Ag NPs accumulate and interact with the cerebral microvasculature and induce the release of various inflammatory mediators like tumour necrosis factor-α, IL-1β and prostaglandin E2. Moreover, in primary cultured endothelial cells from rat brain microvessel, Ag NPs induce, in a size dependent way, alterations in cellular morphology and damage with the appearance of perforations in the cell monolayers with increased permeability (Trickler et al., 2010).

The BBB electrostatic barrier presents an overall luminal negative charge and the interaction with cationic particles results in an opening of inter-endothelial routes (Nagy et al., 1981). For this reason, neutral, cationic and anionic NPs have been compared and it has been demonstrated that neutral NPs (different size NPs; 10 and 20 μg/ml; 60 s perfusion at 10 ml/min) and low concentrations of anionic NPs (10 μg/ml) have no effect on rat BBB integrity, whereas higher concentrations of anionic and both 10 and 20 μg/ml of cationic NPs disrupted the BBB; in particular the cationic NPs have immediate toxic effects on brain microvasculature endothelium altering BBB integrity and permeability (Lockman et al., 2004). *In vitro* studies also reported that the application of PBCA/PS80 NPs leads to a reversible disruption of BBB integrity probably due to changes in endothelial cell morphology (Rempe et al., 2011).

The interaction of NPs with cytoskeleton in endothelial cells may also alter the tight junctions and, as a consequence, can affect the BBB permeability. In fact, in endothelial cell lines of human brain microvasculature the exposure to Al NPs (8–12 nm particle size; 10 μM; up to 24 h) resulted in rearrangements of F-actin with increased deposition of this protein at the cell–cell borders, while the exposure to 1 mM Al NPs induced marked cytoskeleton rearrangements and alterations in protein expression of tight junctions. The loss of F-actin and the formation of gaps between the cells was observed; moreover the junctional adhesion molecules-A, zonula occludens proteins 1 and 2 were markedly decreased in tight junctions (Chen et al., 2008). In rats the same Al NPs, intravenously infused at the dose of 29 mg/kg, after 20 h caused a marked fragmentation and disruption of integrity of claudin-5 and occludin in the cerebral vessels leading to an alteration of tight junctions (Chen et al., 2008).

Nowadays many studies have focused mainly on the effects of NPs on endothelial cells both in *in vitro* and *in vivo* models. The BBB is not composed only of endothelial cells, and the correct interaction between all cellular components of the BBB is crucial for the maintenance of BBB functionality; thus future studies should focus on the effects of NPs on the complete neurovascular unit. A recent study showed that in a human BBB model using hCMEC/D3 endothelial cells the carboxylated polystyrene NPs (100 nm, 100 μg/ml, 24 h) induced secretion of low levels of pro-inflammatory RANTES protein if compared to control. These NPs in the same BBB model but in presence of astrocytes induced a significant release of pro-survival signalling (up-regulation of epidermal grow factor signalling). This suggests that *in vivo* the paracrine signalling may be able to modulate the levels of pro-inflammatory/pro-surviving proteins (Raghnaill et al., 2013). This result demonstrates the need to investigate the interaction between NPs and BBB using complex models that include all cell types of the BBB.

### 5.2. Effects on neurons

Neurons are particularly vulnerable due to their biology, their axons and dendrites being thin and fragile. Ag NPs (20 nm, 1, 5, 10 and 50 μg/ml) in primary cell cultures from rat cortical neurons reduced cell viability of both neurons and glia, inhibited neurite outgrowth of premature neurons, and induced degeneration of neuronal processes in mature neurons (Xu et al., 2013).

NPs can enter neurons at two different sites: in axons NPs are internalized only at axon terminals and in the dendrites by passing through the entire plasma membrane. NPs are transported inside endocytic vesicles by a combined effect of dynein-driven transport and diffusion (Kuznetsov, 2011). The brain areas in which NPs mainly accumulate depend on the route of administration and NPs characteristics. In rats, intravenous administration of Au NPs (20 nm, single tail-vein injection of 0.01 mg/kg, 15.1 μg/ml) did not show brain NPs accumulation (after 1 day up to 2 months) (Balasubramanian et al., 2010), while inhaled Au NPs (7 and 20 nm, 15-day whole-body inhalation, $10^6$ particles/cm$^3$, 6 h per day, 5 days per week, over 3 weeks) resulted in their accumulation in the brain with the major concentration in olfactory bulb (Balasubramanian et al., 2013). In this area the concentration of 7 nm Au NPs was more than 2-folds than that of 20 nm NPs. Lower but significant accumulation of both 7 and 20 nm Au NPs was also found in hippocampus, striatum, frontal cortex, entorhinal cortex, septum and cerebellum (Balasubramanian et al., 2013). Also intranasal instillation of 15 nm $SiO_2$ NPs (20 μg/rat; for 1 day and 7 days, once a day) resulted in a significant increase in $SiO_2$ NPs content in the brain, and the ranking of interested areas was olfactory bulb > striatum > hippocampus > brain stem > cerebellum > frontal cortex (Wu et al., 2011). Instillation of another NPs type at high concentration ($TiO_2$; 80 and 155 nm; 500 μg/mouse; every other day for 30 days), led to a different region-specific accumulation: hippocampus > olfactory bulb > cerebellum > cerebral cortex (Wang et al., 2008a). The high accumulation of inhaled NPs in hippocampus and frontal cortex suggests that NPs exposure may damage the behavioural and memory networks.

*In vitro* and *in vivo* studies demonstrated that NPs, depending on their chemical–physical characteristics, have different effects on neurons. For example, exposure to Cu and Ag NPs administrated into systemic circulation or in brain ventricular space have a more marked effect on brain pathology than Al NPs, probably because Cu and Ag are redox-active metals (Sharma et al., 2009, 2010). Moreover Ag NPs (15 nm; 50 μg/ml; 24 h) in PC12 cell line produced cell shrinkage and irregular membrane borders while Mn NPs (40 nm) at the same concentration did not greatly change cellular morphology (Hussain et al., 2006).

The exposure to metal NPs leads to dramatic neuronal loss in the brain areas with higher accumulation rate. In mice, after $TiO_2$ NPs exposure *via* nasal instillation (80 and 155 nm; 500 μg/mouse; every other day up to 30 days), 25–30% of cellular loss has been observed in the cornu ammonis (CA)-1 region and dentate gyrus (Wang et al., 2008b). Morphological changes of hippocampal neurons were also observed in the CA4 region after NPs instillation (same conditions above mentioned) together with increased glial fibrillary acidic protein-positive astrocytes, which indicates an activation of astrocytes; irregular and dispersed arrangements of

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neurons in olfactory nerve layers were also demonstrated (Wang et al., 2008b). Au NPs (17 and 37 nm; 0.5–14.6 mg/kg; single injection) injected intraperitoneally in mice passed through the BBB, affected dopaminergic and serotoninergic neurons and significantly altered dopamine and serotonin levels in the brain of mice after 21 days. The 17 nm Au NPs accumulated in cytoplasm of pyramidal cells in the CA region of the hippocampus while 37 nm Au NPs were excluded. Thus, in these mice treated with Au NPs a size-dependent impairment of cognition was observed (Chen et al., 2010), probably due to the hippocampal neuronal network damage.

NPs may alter gene expression. Ag NPs (15 nm; 50 μg/ml; 24 h) were able to moderately decrease dopamine content with an increase in reactive oxygen species (ROS) production in the PC12 cells line (Hussain et al., 2006). In a later study Wang and colleagues, using RT-PCR, explained the reduction of dopamine by showing that in PC12 cells treated for 24 h with 10 μg/ml Mn-40 nm, Ag-15 nm, or Cu-90 nm NPs the expression of 11 genes associated with the dopaminergic system and ROS production was altered. The alterations in gene expression induced by the three types of metal NPs are quite different, indicating that each metal-based NP has a unique mechanism of action (Wang et al., 2009). Ag NPs intraperitoneally injected in mice (25 nm; 100 mg/kg, 500 mg/kg or 1000 mg/kg; sacrificed after 24 h) caused in the brain an alteration in oxidative stress and antioxidant defence gene expression, such as oxidative metabolism, glutathione metabolism and antioxidant enzyme genes. These genes were up-or down-regulated, depending on the brain region: in the caudate nucleus most of the significantly altered genes were up-regulated, in the hippocampus were down-regulated, while in the frontal cortex genes were both up- and down-regulated (Rahman et al., 2009). It seems that exposure to these Ag NPs is able to generate ROS by throwing off balance the oxidant and antioxidant neuronal pathways, and to induce ROS formation and oxidative stress conditions leading to DNA and protein damage (Rahman et al., 2009). Also, primary murine frontal cortical networks on microelectrode array neurochips treated for 24 h with $TiO_2$ NPs (<100 nm; 10 μg/cm$^2$) showed a concentration-dependent increase in ROS formation in both neuronal and glial cells, although carbon black NPs and $Fe_2O_3$ NPs at the same concentration had no effect on ROS production (Gramowski et al., 2010).

It should be considered that NPs, once inside the brain parenchyma, could interact and enter into other cells of the CNS, such as astrocytes. Human astrocytoma cell lines U87 showed an intracellular localization of NPs after treatment with $CoFe_2O_4$ NPs (Fig. 6).

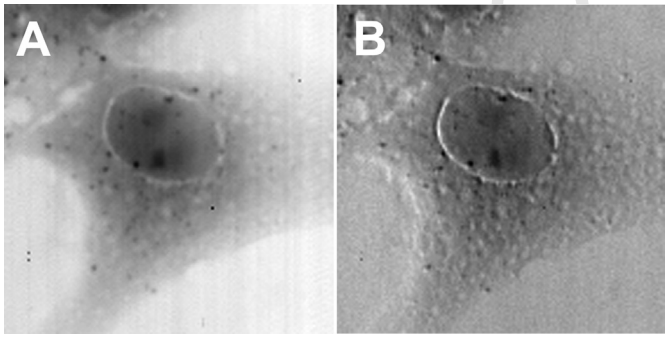


**Fig. 6.** Specific intracellular localization of NPs in human astrocytoma cell line. X-ray absorption (A) and contrast (B) images of U87 human astrocytoma cells treated with $CoFe_2O_4$ NPs (for conditions see Marmorato et al., 2011). An even intracellular localization is visible. Images were obtained with synchrotron radiation X-ray microscope TwinMic (Elettra Synchrotron) operating at 1.15 keV, on 40 μm × 40 μm area with 285 nm space resolution. Images were kindly provided by Giacomo Ceccone (Joint Research Centre, Ispra, EU) and Alessandra Gianoncelli (TwinMic team leader, ELETTRA, Sincrotrone Trieste, Italy).

### 5.3. Effects on cellular components

Once endocytosed the NPs interact with cellular components. It has been observed that NPs of various size and chemical–physical characteristics preferentially accumulate in mitochondria (Oberdörster et al., 2005), and this uptake may be correlated with the increased ROS production linked to NPs effect. The exposure to NPs causes impairment in the mitochondrial functions both in *in vivo* models and in cell lines with heavy consequences on cell viability. *In vitro* Ag NPs (diameter from 5 to 45 nm; 10, 25, and 50 mg/l; 1 h) inhibit the activity of mitochondrial respiratory chain complexes I and III from rat brain tissue (Costa et al., 2010). In isolated rat liver mitochondria, 40 and 80 nm Ag NPs (2 and 5 μg/mg protein) impaired the oxidative phosphorylation capacity and increased the permeability of the inner membrane to protons (Teodoro et al., 2011). Iron oxide superparamagnetic NPs (10 μg/ml; 6 h) in astrocyte cell cultures significantly increased mitochondrial activity, likely because of mitochondrial uncoupling, as measured by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay (Au et al., 2007). Decrease in mitochondrial membrane potential was also observed in a microvascular endothelial cell line from human brain treated with Al NPs (8–12 nm; 10 μM; up to 24 h) (Chen et al., 2008). In human lung fibroblasts (IMR-90) and human glioblastoma cells (U251) the disruption of the mitochondrial respiratory chain by Ag NPs (6–20 nm; 25–100 μg/ml; 48 h) increased ROS production and interrupted ATP synthesis, thus leading to DNA damage; the interaction of Ag NPs with DNA led to cell cycle arrest or apoptosis induction (AshaRani et al., 2009). Moreover, in NIH 3T3 mouse fibroblasts the treatment with Ag NPs (1–100 nm; 50 μg/ml; 24 h) induced the release of cytochrome c into the cytosol and the translocation of Bax to mitochondria, thus promoting apoptosis (Hsin et al., 2008). Similar effects of Ag NPs (5–35 nm; 0–9 μg/ml, in a dose dependent manner; 24 h) have been also observed in human Chang liver cells (Piao et al., 2011). Also ZnO NPs (30 nm; 14–20 μg/ml; 9–12 h) in human liver carcinoma cells HepG2 induced a decrease in mitochondria membrane potential and an increase in the ratio of Bax/Bcl-2 leading to mitochondria mediated apoptosis (Sharma et al., 2012). The NPs effects on mitochondria have been widely studied in *in vitro* models with transformed cell lines (Schrand et al., 2010) and not in animal models. *In vivo* studies are essential to supply useful information on the realistic impact of the NPs on respiratory chain and mitochondrial condition.

Mitochondria are not the only organelles affected by NPs, in fact also the autophagic-lysosomal system is a NPs target. This degradative pathway plays an essential role in cellular homeostasis maintenance, pathogen clearance and antigen presentation, and is strictly linked with cancer and neurodegenerative disease. Several studies on non-neuronal cells reported that different NPs induced autophagy, accumulated in lysosomes and caused impairment of the lysosome-degradation capacity (Cengelli et al., 2010; Gu et al., 2011; Li et al., 2010; Ma et al., 2011). In the model of human BBB using hCMEC/D3 endothelial cells connected by tight junction and exposed to 50 nm $SiO_2$ NPs (100 μg/ml; 1 h), electron microscopy showed the presence of these NPs in endosomes and lysosomes (Fig. 3B) (Ragnaill et al., 2011); however, in the same BBB model the carboxylated polystyrene NPs (100 nm, 100–300 μg/ml, up to 48 h exposure) accumulated within lysosomes without degradation (Raghnaill et al., 2013). The accumulation of NPs in endosomes and lysosomes was also confirmed in murin primary microglial cells treated with iron oxide NPs Resovist® (58.7 nm, 1–50 μg Fe/ml, 30 min) (Wu et al., 2013). In mice treated with micro Ag particles (<20 μm; 1.18 mg/mouse; observation after 7, 14 days and 9 months), heavy accumulations of Ag-sulphur nanocrystals was observed in endothelial cells, neurons and glial cells, in particular astroglia,

within lysosome-like organelles (Locht et al., 2011). It was observed that, in human brain-derived endothelial cells (hCEC) treated for 24 h with uncoated and oleic acid-coated iron oxide (core 8 nm; 15 μg/cm$^2$), $TiO_2$ (21 nm; 15 μg/cm$^2$) and $SiO_2$ (25–50 nm; 75 μg/cm$^2$) NPs, following their uptake the NPs were transported in small or large endosomes and in lysosomes (Halamoda Kenzaoui et al., 2012). In these cells, the features of autophagic response, such as double membrane vacuoles containing cellular material and LC3-II induction, together with autolysosomes containing NPs were observed, in particular in the cells exposed to 50 nm $SiO_2$ NPs. Here the activation of lysosomal proteases, such as procathepsin-D, were particularly enhanced in cells exposed to uncoated ultrasmall superparamagnetic iron oxide NPs and $TiO_2$ NPs (Halamoda Kenzaoui et al., 2012). Ag NPs in mouse's fibroblast cells (L929) have been also found in endosomes and endoplasmic reticulum (Wei et al., 2010).

The lysosome-degradative pathway has been commonly addressed as the catabolic pathway for different NPs, but the fate of NPs in the lysosome is still unclear. Are the cellular degradation systems able to completely dissolve or re-cycle NPs? Another question is how neuronal degradation systems react to NPs. In fact, neurons are long-living cells and accumulate "cellular garbage" during lifespan. The lysosomal system is not completely efficient, *i.e.*, it is unable to totally digest the worn-out organelles, proteins and other cellular components. Since ageing processes further decrease the lysosomal degrading efficiency (Rajawat et al., 2009), an accumulation of undegradable material in lysosomes can occur. Moreover, lysosome-like organelles in neurons accumulate reactive/toxic material that would otherwise cause neurotoxicity. This is the case of neuromelanin, a physiological neuronal pigments of melanic type identified in different brain areas, which is composed of 30 nm nanoparticulate subunits that accumulate in organelles together with lipid droplets (Zecca et al., 2008a; Zucca et al., 2014). Neuromelanin accumulates during ageing in autophagic vacuoles, possibly interfering with physiological degradation pathways (Sulzer et al., 2008). Considering that some NPs, such as carboxylated polystyrene NPs, accumulate within lysosomes and are not degraded (Raghnaill et al., 2013), the consequences of long term exposure to NPs on lysosomal-degradation capacity need to be deeply evaluated.

NPs may also interact with several cytoplasmic proteins of neurons, for example those of the cytoskeleton thus affecting its structure and functionality. $TiO_2$ NPs (20 nm; 0–50 μg/ml; 30 min, 4 °C) in microtubules from sheep brain have an inhibitory effect on tubulin polymerization (Gheshlaghi et al., 2008) with potentially dramatic consequence on neurotransmitters transport. Ag NPs (20 nm, 1, 5, 10 and 50 μg/ml) on primary rat cortical neurons cultures induced beta-tubulin and filamentous actin loss, disturbing the assembly/disassembly of cytoskeletal components in a dose dependent manner. Consequently, this results in disturbances of synaptic structures and functions: in fact, Ag NPs were able to dramatically reduce the number of synaptic clusters of the presynaptic vesicle protein synaptophysin, and the postsynaptic receptor density protein PSD-95 (Xu et al., 2013).

Another issue is the influence of NPs on neuronal excitability. The capacity of neurons to propagate the action potential depends on ion current, and up to date only few data are available on NPs effects. NPs are able to induce changes in the electrical activity of the neuronal network. For instance, $TiO_2$ NPs (10 μg/cm$^2$, as described above) caused severe inhibition of the general electrical network activity in primary cell cultures of mice frontal cortex (Gramowski et al., 2010). More detailed studies revealed that Ag NPs (50–100 nm; 10 μg/ml; 2–5 min) altered the action potential of hippocampal CA1 neurons by depressing voltage-gated current (Liu et al., 2009b). A recent study showed that in hippocampal CA1 pyramidal neurons the amplitude of miniature excitatory postsynaptic currents was inhibited by Ag NPs (50–100 nm, 10 μg/ml and 100 μg/ml), while the amplitude and frequency of spontaneous excitatory postsynaptic currents were increased. Furthermore, the spontaneous network activity was increased (Liu et al., 2012b). ZnO NPs (20–80 nm, 100 ng/ml) affect the physiological function of isolated rat hippocampal CA3 pyramidal neurons enhancing the amplitude of sodium current and rectifier potassium current, leading to the intracellular accumulation of $Na^+$ and neuronal $K^+$ efflux (Zhao et al., 2009). Also CuO NPs may interfere with hippocampal impulse propagation. In isolated CA1 pyramidal neurons, CuO NPs (10–70 nm, 50 ng/ml) inhibited the rectifier potassium current but had no significant effect on transient outward potassium current (Xu et al., 2009). $MnO_2$ NPs (23 nm) instilled into the rat trachea (for 3, 6, and 9 weeks in daily doses of 2.63 and 5.26 mg/kg) induced a shift of the spontaneous cortical activity to higher frequencies, lengthened cortical evoked potential latency, and slowed nerve conduction. As a consequence, also the animal behaviour changed: the exposed rats showed decreased ambulation and increased local activity (Oszlánczi et al., 2010).

NPs deposition was also observed in the nucleus and nucleolus (Au et al., 2007) thus explaining the NPs influence on DNA as previously described. NPs may interfere with gene expression, and DNA synthesis can also be affected. In undifferentiated PC12 cells, a 1-h exposure to Ag NPs (10–63 nm; 10 μM) was able to inhibit DNA synthesis and impair protein synthesis with serious effects on cell replication. Prolonged exposure of these cells to Ag NPs (24 h) led to oxidative stress, loss of viability and reduction in the number of cells. In addition, in differentiating PC12 cells Ag NPs selectively impaired neurite formation without suppressing overall cell growth and preferentially suppressed the development into acetylcholine phenotype in favour of dopamine phenotype (Powers et al., 2011).

### 5.4. Effects on glial cells and inflammation in the brain

Once inside the brain parenchyma, NPs are apparently able to induce significant modifications in glial cells and have inflammatory effects in the brain. Experimental evidence showed that quantum dots (water-soluble ZnS capped CdSe streptavidin coated quantum dots, 0.5 nM, after 18 h of incubation) are selectively taken up by microglia in primary cortical cultures containing neurons, astrocytes and microglia; moreover, in a mouse model, quantum dots stereotaxically injected in the hippocampus (3 μl at 100 nM) are also taken up by microglia with high efficiency (Minami et al., 2012). In primary cerebellar neuronal/glial co-cultures anionic magnetic NPs suspended in serum containing medium (50 μg/ml, after 24 h incubation) were predominantly taken up by microglia and induced microglia proliferation (Pinkernelle et al., 2012). This evidence supports the hypothesis that NPs, *per se* or due to the opsonization, could activate microglia.

Systemic or intracerebroventricular administration in rats and mice (intraperitoneal, 50 mg/kg; intravenous, 30 mg/kg; intracarotid, 2.5 mg/kg; intracerebroventricular 20 μg) of metallic NPs (Al, Ag and Cu, 50–60 nm) induced, 24 h after treatment, glial cell activation and heat shock protein upregulation, in addition to neuronal cell injuries (Sharma et al., 2009). When injected in rats, Ag NPs (62.8 mg/kg) induced astrocyte swelling (Tang et al., 2009). Aluminum oxide NPs (less than 100 nm), intraperitoneally injected (1 mg/kg, once every second day), after 30 days of exposure did not induce glia activation, while after 60 days of exposure induced local activation of glial cells, in particular astrocytes, in the rat brain (Li et al., 2009). Astrocytes cell cultures treated with iron oxide superparamagnetic NPs (10 μg/ml) lost their adhesion capacity and 4 days after treatment their viability was compromised (Au et al., 2007). It is worth to re-iterate here the key importance of NPs contaminants characterization, since all the



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inflammatory effects observed *in vivo* and *in vitro* may indeed be caused by LPS or other bioactive contaminants rather than by the NPs. Thus, all the alleged inflammatory and toxic effects attributed to NPs should be confirmed with rigorously clean NPs preparations before they could be considered as due to NPs. As cited before, the inflammatory effects of NPs may also be due to the proteins which may aspecifically bind to the NPs surface: depending on the route of administration, NPs get in contact with different proteins (*e.g.*, blood circulating proteins such as immunglobulins, lipoproteins and complement factors, etc.) that bind to NPs surface and affect pharmacokinetics, biodistribution, targeting and immunogenicity. Moreover NPs, once inside the cell, may adsorb cytosolic proteins on their surface with consequent effects on protein functions at intracellular level.

Shape also seems to have an important role in the inflammation induced by NPs. In mice intranasal administration of Au NPs (5 μl; $10^9$ particles/ml) of different shapes revealed kinetical differences in their effects. Spherical Au NPs (23 nm) caused small but detectable microglia activation after 24 h that persisted for a week. In contrast, rod Au NPs (43 nm long, 12 nm wide) caused biphasic activation with an early peak at 24 h and another after one week. PEGylated urchin Au NPs (77 nm) did not significantly activate microglia at any time, while cetyl trimethylammonium bromide-coated urchin Au NPs (77 nm) caused a transient activation that disappeared after a week (Hutter et al., 2010).

In all cases, both *in vitro* and *in vivo*, it is mandatory in the evaluation of the observed "toxic" effects the correlation with the dose of NPs to which cells/animals are exposed. Indeed, in most experiments the NPs concentrations used are unrealistic as compared to possible exposure in real life, which make the toxicity assessment hardly representative of real risk.

Not only engineered NPs are able to induce neuroinflammation. The injection of human neuromelanin NPs into rat cerebral cortex and substantia nigra induced microglia activation with release of neurotoxic factors; in addition to microgliosis, neuromelanin induced neuronal loss in these *in vivo* models and in primary ventral midbrain neuronal-glia co-cultures (Zecca et al., 2008b; Zhang et al., 2011).

The ability of NPs to induce neuroinflammation depends not only on their chemical–physical properties but also on the time of exposure and previous health conditions of the subjects. A single exposure to $TiO_2$ NPs is not sufficient to evoke an inflammatory response in the brain of healthy mice, but cumulative exposure to $TiO_2$ NPs induces a pathological response in the normal brain. For example, Ti accumulation in the brain, reduction in brain weight and brain damage were observed in mice injected daily for 14 days with nano-anatase $TiO_2$ NPs (5 nm; 50, 100, 150 mg/kg, i.p.) (Liu et al., 2009a). However, the acute treatment with $TiO_2$ NPs (21 nm; 40 mg/kg, in a volume of 5 ml/kg) aggravated the neuroinflammation induced by LPS in mice, enhancing the production of inflammatory cytokines and ROS and increasing the activation of microglia (Shin et al., 2010). In mice the intranasal instillation of $Fe_2O_3$ NPs (17–48 nm; 130 μg/day; every other day, for 40 days) led to activation of microglia in olfactory bulb, hippocampus and striatum. Moreover in murine immortalized BV2 microglial cells the exposure to $Fe_2O_3$ NPs (0.02, 0.2, 2 mmol Fe/l of $Fe_2O_3$ NPs suspensions; 6 h) induced cell proliferation, phagocytosis and generation of ROS and nitric oxide, but did not cause a significant release of inflammatory factors such as IL-1β, IL-6 and tumour necrosis factor-α (Wang et al., 2011). Altogether these results suggest that the activation of microglial cells induced by NPs may act as an alarm signal that can initiate a defensive reaction in response to the entry of exogenous NPs but also cause an abnormal microglia activation leading to neurodegeneration.

## 6. Conclusions and perspectives

### 6.1. The massive environmental invasion of NPs and related safety issues

Over the last years NPs, due to their exciting and promising properties, have been widely employed in industries, everyday life products and medicine (Etheridge et al., 2013). Consequent to the increased production and use of NPs, the number of workers and consumers exposed to NPs has increased. However, up to date the beneficial applications of NPs have been extensively investigated, while information about their toxicity and implementation of specific nanosafety regulations have not followed the nanotechnology development at the same pace. Classical toxicological assessment is often not applicable to investigating nanosafety, due to the peculiar physical–chemical characteristics of NPs, thus specific nanosafety assays need to be designed and validated.

Among nanosafety issues, of special interest is the interaction of NPs with the brain. If such interaction may be among the contributing factors in the initiation and/or progression of neurodegenerative and neuroinflammatory pathologies, it is of key interest to implement effective preventive and therapeutic measures. NPs can enter the human body through several routes (skin, digestive tract, airways, blood in the case of intravenous nanomedicines) and only a small part of these NPs may cross the BBB and reach the CNS. The route of exposure/administration, the chemical–physical properties, size and shape of NPs determine the features of their passage through the BBB and consequently their effects on CNS cells. Moreover the effects of NPs depend also on the species used in the experimental procedures (Sharma et al., 2009). Concurring factors, such as the individual health and metabolic conditions or the presence of other molecules/agents (either physically associated to NPs or co-present during exposure) are additionally modulating the eventual effects of NPs on CNS.

### 6.2. Limited reliability of the available data and inadequacy of experimental models used to investigate the biological effects of NPs

Most available studies and results on the adverse effects of NPs on CNS have been performed randomly, by using different models (*in vivo* or *in vitro*, with cell lines, animal cells or human cells), different exposure protocols (routes of administration, dosages, protocols of dispersion, solvents composition and volume, acute *vs.* chronic *vs.* cumulative exposure) and different kinds of NPs (chemical composition, size, shape), often not accurately characterized at the moment of the experiment (NP ageing is a critical issue) in terms of ion release, chemical contaminants and, of major importance, biological contaminants. Contamination with LPS (bacterial endotoxin) can be found in practically all engineered NPs preparations, even if sterilized for *in vitro* and *in vivo* use, at concentrations that can be responsible for the totality of the toxic, functional and inflammatory effects described in the literature. The only reliable way to obtain endotoxin-free NPs is to synthesize them in endotoxin-free conditions, a procedure that is never implemented in chemical laboratories or by NPs producers (Vallhov et al., 2006). The interpretation of the *in vivo* and *in vitro* tests is therefore difficult, not only because the enormous variability of the experimental plans does not allow accurate comparison of data, but also because the results obtained might have been affected by undetected contaminations of NPs. In the experimental plan, in addition to the size distribution of NPs the presence of any contaminant, such as LPS, should be tested, especially in the case of inflammatory response to NPs exposure evaluation.

The effects of NPs on CNS in *in vivo* animal models are inadequate to address the large quantities and different kinds of

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existing NPs for a series of reasons. In particular when addressing inflammatory effects, the differences between human and rodent reactivity is such that conclusions drawn from animal studies may hardly be transferred to the human situation (Davis, 2008). An example is IL-37, an anti-inflammatory cytokine and gene transcriptional regulator that plays a key role in down-regulating inflammation, whose isoform IL-37a is brain-specific. IL-37 is present in man but not in rats and mice, which use different mechanisms to regulate inflammation (Boraschi et al., 2011). Furthermore, in the case of brain diseases, the rodent models are all artificially built, thereby only partially resembling human conditions.

Moreover in some cases the problems of the experimental procedure may generate misleading results. For example, to verify the translocation of NPs to the CNS *via* nose-to-brain, in some experiments a huge dose of NPs was instilled (Moberly et al., 2012; Wang et al., 2008a, 2008b, 2007), most probably leading to damage of the olfactory epithelium and to non-physiological transport. Physiological transport would be better investigated by using inhalation models and realistic NP doses.

Furthermore the real quantity of NPs able to pass the BBB and translocate to the brain parenchyma have to be well estimated depending on route of administration, chemical–physical characteristics of NPs and patient's health condition. From *in vivo* biodistribution data, it appears that less than 1% of brain-targeting NPs actually enter the brain, while the majority is entrapped by the liver (Costantino and Boraschi, 2012).

Eventually it is of major importance, even in the case of clear damage, to evaluate the ability of CNS cells to recover from the damage and to re-establish tissue homeostasis or to progress into an irreversible pathological condition.

### 6.3. Potential of new models using induced pluripotent stem cells to study NPs biology and health risk

Thus, there is an urgent need of reliable models for the accurate investigation of the interaction of NPs with brain cells in health and disease, in terms of the NP potentially harmful effects (toxicity, alteration of functions, induction or exacerbation of inflammation, etc.). These models, given the above considerations, should be *in vitro* because human cells have to be preferentially used. In addition, the use of primary cells should be privileged above employing cell lines, which are mostly transformed or tumour cells and do not reproduce accurately the reactivity of normal cells. In addition to cells isolated from surgical or post-mortem samples, a novel source of brain cells comes from induced pluripotent stem (iPS) cells. Very briefly, the possibility of re-programming human somatic cells to pluripotency allows us to generate human iPS cell lines that can be stably and indefinitely propagated in culture, and which can be differentiated into any cell type of the organism. One key advantage of iPS cells is that they capture the genetic complexity of the donor, so it is possible to assemble a collection of iPS cell lines (or cells differentiated from them) representing a cross-section of a given population. For instance, a range of iPS cell lines can be generated widely representing healthy individuals (with a good coverage of age and gender), as well as cells from individuals with selected disease conditions (*e.g.*, Parkinson's disease). Dopaminergic neurons can be differentiated from iPS cell lines that are electrophysiologically active and release dopamine (Sánchez-Danés et al., 2012a,b). These neurons form synapses and, when co-cultured with astrocytes, can be maintained in good conditions *in vitro* for up to 75 days, thus representing an excellent model for studying the interactions with NPs and the subsequent reactivity of cells from healthy *vs.* diseased individuals, young *vs.* old, males *vs.* females.

Once representative models are selected and validated, it will be possible to carry out detailed mechanistic studies on the interaction of NPs with the different brain cells, starting from interaction with plasma membrane and mechanisms of uptake, intracellular trafficking and fate (degradation, accumulation and storage in lysosomes, expulsion from the cell).

### 6.4. Need to investigate neuroinflammatory, neurodegenerative and carcinogenic effect of NPs

Once NPs enter neurons or glial cells, depending on the mechanism of entry they may be immediately channelled to the lysosomal compartment, but they could also persist in the cytoplasm and interact with organelles such as mitochondria, endoplasmic reticulum, Golgi, nucleus, and they can also react with other cellular components like proteins. The interaction of NPs with proteins may change the protein structure, aggregation and modulate their proteasomal degradation, thereby altering the cellular functions that depend on such proteins (Shemetov et al., 2012). Several types of NPs have been found able, probably through lysosomal destabilization and cathepsin B release, to activate the assembly and activation of the NLRP3 inflammasome, an intracytoplasmic protein complex that is responsible for the cleavage/activation of caspase 1 and, consequently, of the caspase 1-dependent cleavage/activation of the inflammatory cytokines IL-1β and IL-18 (Demento et al., 2009; Gross et al., 2011; Re, 2011). Inflammasome activation is almost invariably accompanied by pyroptosis (inflammation-induced death), thereby leading to neuroinflammation and neurodegeneration. The interaction with NPs may also change the properties of membranes, leading to changes in the permeability or transport mechanisms with possible consequences also on the nerve impulse transmission.

The possibility that NPs could upregulate gene expression in brain cells has been suggested by *in vitro* studies in rat tumour cells, a model hardly representative of the behaviour of human primary cells (Choi et al., 2010), thus leaving the issue practically unexplored. The direct interaction of NPs with DNA and the mechanisms by which NPs may act on transcription are unknown. Also, studies on the possible effects of NPs on epigenetic regulatory mechanisms (methylation, miRNA, ceRNA, etc.) are missing.

Moreover, it is unknown if NPs effects can be truly attributed to NPs or they may be due to the aspecifically bound proteins or contaminants. This issue requires more attention of the scientific community, to better define the realistic potential neurotoxicity of the NPs.

A critical issue for assessing the effects of NPs on the CNS is that chronic prolonged exposures, as they may occur *in vivo* in man, are extremely difficult to reproduce in experimental systems. Animal models are required here, in order to assess the role of NPs in the development and progression of neurodegenerative diseases or tumours, or the effects on neurodevelopment upon *in utero* exposure of foetuses.

The understanding of the potential toxic effects of NPs and knowledge of the cellular and molecular mechanisms involved will be extremely useful to prevent risk, and will also be helpful for therapeutic purposes. Controlled toxicity of NPs (either intrinsic or engineered) may be exploited for targeted destruction of brain tumour cells. Controlled modulation of barrier functional activation may induce therapeutic effects on the tissue behind the barrier without needing to breach it. The safe use of NPs is the key notion in nanomedicine, as opposed to the rather unrealistic option of synthesizing "safe by design" NPs.

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