DIAGNOSTIC AND THERAPEUTIC USES OF NANOMATERIALS IN THE BRAIN

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ABSTRACT

Nanomedicine has recently emerged as an exciting tool able to improve the early diagnosis and treatment of a variety of intractable or age-related brain disorders. The most relevant properties of nanomaterials are that they can be engineered in such a way that they can cross the blood brain barrier, with the final aim of targeting specific cells and molecules and to act as vehicles for drugs. Potentially beneficial properties of nanotherapeutics derived from its unique characteristics include improved efficacy, safety, sensitivity and personalization compared to conventional medicines.

In this review, recent advances in available nanostructures and nanomaterials for brain applications will be described. Then, the latest nanotechnological applications for the treatment and diagnosis of neurological disorders, mainly brain tumors and neurodegenerative diseases, will be reviewed. Recent investigations of the neurotoxicity of the nanomaterial both *in vitro* and *in vivo* will be summarized. Finally, the ongoing challenges that have to be meet if new nanomedical products are to be put on the market will be discussed and some future directions will be outlined.

Keywords: Alzheimer's disease, brain tumors, diagnosis, engineered nanomaterials, nanoscience, nanotechnology, Parkinson's disease

1 INTRODUCTION

Nanomedicine can be defined as the use of nanostructured materials in medicine that have some exceptional medical effects due to their structure,like passive targeting to tissues or the capability to cross some biological barriers, for instance [1]. Approaches to nanomedicine range from the medical use of nanomaterials to nanoelectronic biosensors, and even possible future applications of molecular nanotechnology. Besides the established therapeutic modes of action, nanomaterials are opening up new options in cancer therapy, such as photodynamic and hyperthermia treatments. Furthermore, nanosized carriers are also capable of avoiding some drug delivery problems, that could not be effectively solved in the past and which include overcoming multidrug-resistance phenomenon and penetrating cellular barriers that limit drug accessibility to intended targets, such as the blood–brain barrier (BBB), among others [2].

One of the most promising aspects of nanotechnology is that it has the potential to change the way brain drug delivery is approached. Thus, nanomedicines might be advantageous for the treatment and diagnosis of a number of central nervous system (CNS) disorders including brain tumors or neurodegenerative disorders that are nowadays a major medical challenge (Figure 1) [2]. However, due to the physicochemical properties that these nanomaterials present, namely their large surface area, they may cause neurotoxicity after entering into the brain. As a result, there is an important need to assess their potential neurotoxic effects on the CNS function, as specific pathways and mechanisms through which these nanomaterials may produce their toxicity remain unknown. In this review, current advances in available nanostructures and nanomaterials for brain applications will be described.

Then, the latest nanotechnological applications for the treatment and diagnosis of neurological disorders, mainly brain tumors and neurodegenerative diseases, will be reviewed. Furthermore, recent investigations of the nanomaterial neurotoxicity both *in vitro* and *in vivo* will be summarized. Finally, the ongoing challenges facing those who aim to put nanomedical products on the market will be discussed and some future directions will be outlined.

1.1 ADVANCES IN AVAILABLE NANOSTRUCTURES AND NANOMATERIALS FOR NEUROSCIENCE

Various nanomedicines can be used in targeted delivery of drugs across the BBB, neuroprotection and neural regeneration. This section provides a summary of nanostructures and nanomaterials that are able to show progress in the diagnosis and treatment of brain disorders. The variety of structures and materials discussed below allows the selection of the better nanosystem for a specific CNS disorder.

Nanostructures used for the development of nanomedicines for brain disorders include (Figure 2):

Nanoparticles (NP) NP for pharmaceutical purposes are solid particles ranging in size from 1 to 1000 nm made of macromolecular materials in which the active principle (drug or bioactive material) is encapsulated, or to which the active principle is attached or adsorbed [3]. NP can be prepared using several materials such as natural and synthetic polymers, metals or lipids. They can be functionalized with targeting ligands or antibodies to cross the BBB and selectively target specific cells. Among lipid NP, different types can be found. Solid lipid nanoparticles (SLN) are colloidal carriers constituted by a solid lipid matrix at room and body temperature, composed of physiological lipids (lipid acids, mono-, di-, or triglycerides, glycerine mixtures, and

waxes), and stabilized by biocompatible surfactants (nonionic or ionic) [4]. Nanostructured lipid carriers (NLC) are developed by the creation of a lipid particle matrix as imperfect as possible in order to accommodate the active molecules in its core. To achieve this aim, a mix of solid and liquid lipids is used to produce NP that remain solid at temperatures up to 40 ºC. NLC present considerable crystal disorder, translated into a higher drug loading and less drug expulsion during storage [5].

Nanoliposomes. These are biocompatible nanoscale lipid vesicles composed by double phospholipid layers which may entrap aqueous solutions. They have structural flexibility in size, composition and bilayer fluidity as well as capability to entrap both hydrophilic and hydrophobic compounds [6, 7]. Furthermore, they provide an exclusive chance to transport actives into cells or even inside individual compartments. Inherent problems include poor stability. Polyethylene glycol (PEG) is commonly used to modify their surface, reducing opsonization in plasma and decreasing its recognition and removal. Targeted therapy through the brain can also be achieved using PEGylated nanoliposomes vectorized with monoclonal antibody to glial fibrillary acidic proteins, TfR (OX26) or human insulin receptor.

Lipid-polymer hybrid NP. These new NP combine the positive features of liposomes and polymeric NP while avoiding some of their drawbacks. They consist of a hydrophobic polymeric core, a lipid shell surrounding the polymeric core, and a hydrophilic polymer stealth layer outside the lipid shell [8].

Nanomicelles. Nanomicelles are obtained when amphiphilic molecules spontaneously assemble in aqueous media to form core-shell vesicles [9]. In addition to surfactants, amphiphilic block copolymers are generally used for preparing nanomicelles. Notably, they can solubilize poorly water-soluble drugs and their surface can be functionalized

for targeted delivery. However, due to their fragile structure, preparing long-circulating nanomicelles and sustained-release nanomicelles is challenging.

Dendrimers. These are highly organized nanoscale sized 3D structures with repeatedly branched polymers that arise from a central core that provides a high degree of surface functionality and versatility and that can also be loaded with drugs [10]. Targeted delivery is also possible via targeting ligands conjugated to the dendrimer surface.

Carbon nanotubes. Carbon nanotubes (CNT) are biodegradable nanometer-diameter cylinders consisting of a single graphene sheet wrapped up to form a tube [11]. CNT behave like nano-needles and pass through the cell membrane through a spontaneous and still unclear mechanism [12]. Therapeutic and diagnostic agents can be encapsulated, covalently attached or absorbed on the surface of CNT. However, their biomedical applications arise serious concerns and CNT toxicity remains a topic of debate. CNT may cause pulmonary inflammation and fibrosis [13]. Another common hurdle when working with this nanosystem is their low dispersibility due to their tendency to aggregate.

Nanogels. Nanogels are nanosized networks of physically or chemically cross-linked polymers that swell in a appropriate solvent. They have high drug loading capacity [14].

Up to now, liposomes and polymeric NP have been the most generally exploited nanostructures for brain applications. Most FDA-approved nanomedicines were developed using these two nanosystems.

Regarding materials, an important major requirement for brain delivery systems is a rapid biodegradability. A degradation time frame from a few days to a few months is preferable. Thus, non-degradable particles such as fullerenes, metal particles, quantum

dots or potentially risky CNT would not be the first option. Most of the FDA approved systems are liposomes or lipid-based systems. Lipids, due to their resemblance to *in vivo* components, are well tolerated in the organism and are less toxic than other materials. Among polymers, three types of polymer materials in particular appear to be the materials of choice: (1) poly(alkyl cyanoacrylates) (PACA) such as poly(butyl cyanocrylate) (PBCA) or poly(isohexyl cyanoacrylate) (PIHCA), (2) poly(lactic acid) (PLA) or its copolymer (lactide-co-glycolide) (PLGA), (3) chitosan. PLGA remains the most widely used material for NP development for brain treatment because of its biodegradability, biocompatibility, ease of processing and FDA-approval [15]. Other polymers extensively studied in nanotechnology applied to the CNS delivery of drugs are PACA [15]. Among them, PBCA is the fastest biodegrading material. Although some of these polymers have been described to be devoid of toxicity, they are not currently approved by the FDA for i.v. administration. Chitosan is one of the most widely used polysaccharides in the design of nose to brain drug delivery systems due to its special mucoadhesive and absorption enhancer properties and its great safety [16]. Concerning metals, principally iron, gold and silver are being investigated as magnetic resonance imaging (MRI) contrast enhancers and photosensitizers for diagnosis of brain disorders [17]. A concern with metallic NP is the possible toxicity due to the risk of retention from repeated exposure [17]. Studies addressing these issues are discussed in section 3. On the other hand, there has been a lot of research done in the field of materials proposing new candidates for biomedical applications. Novel polymers investigated for brain disorder therapy include block copolymers such as Pluronic block polymers based on ethylene oxide and propylene oxide which are able to improve the delivery of a wide spectrum of drugs across the BBB [18, 19] or polymer drug conjugates such as PEG-proteins or N-(2-hydroxypropyl)methacrylamide (HPMA)-

drugs [20, 21]. An innovative approach is the use of natural polymers or recombinant protein-based polymers (silk like proteins [22] or elastomer like proteins [23]) to obtain nanocarriers with excellent biocompatibility and biodegradability and low immunogenicity. New improvements will certainly come from stimuli-responsive polymers that allow targeting the drug to its site of action followed by on-demand drug delivery [24]. Finally, multifunctional materials able to perform *in vivo* diagnosis and release the targeted drug according to the correct time schedule might also be expected [25].

1.2 PROBLEMS ASSOCIATED WITH BRAIN DELIVERY: BLOOD BRAIN BARRIER

The penetration of the CNS by drugs remains a key issue to improve, in order to treat CNS disorders. . According to pharmacokinetic data estimated by different authors , drugs employed for diagnostic or therapeutic purposes are characterized for exhibiting high non-specific binding, and low residence time in the blood plasma [26]. As a result, the percentage of the administered drug that reaches the brain is quite low. Furthermore, the incorporation of drugs into the CNS is further hampered by the presence of the BBB [27].

The BBB is a structure composed by a complex system of endothelial cells, pericytes, astroglia and perivascular mast cells, which prevents the passage of most circulating cells and molecules (Figure 3) [28]. The compact network of interconnections confers a transelectrical resistance >1500 Ωcm^2 on the endothelial layer of the BBB, which is the highest among all endothelial districts [29]. This complex structure prevents the brain uptake of most drugs, except for highly hydrophobic compounds with a mass lower than 400–600 Da and small hydrophilic compounds with a mass lower than 150 Da, which are able to get across the membrane by passive diffusion [30]. Opiates, anxiolytics,

selective serotonin reuptake inhibitors and antipsychotics are some of the drugs that can cross the BBB. However, most antitumor agents and antibiotics cannot. As a result, the tightness of the BBB prevents pharmacological therapy in the case of many neurological disorders. Furthermore, it should be also taken into account that the existence of the P-glycoprotein (P-gp) pump in the BBB represents a further obstacle for drugs when it comes to crossing the cerebral capillary endothelium and enter the brain parenchyma. This P-gp complex allows the recognition of molecules necessary to be incorporated in the brain and the exclusion of other molecules, drugs included [31].

The BBB protects the CNS from molecules circulating in the blood that may be neurotoxic. These substances may be xenobiotics acquired from the environment, taken in the diet or endogenous metabolites or proteins. The key feature of the BBB are the 'tight junctions' (zonulae occludentes) which significantly reduce permeation of polar solutes between the endothelial cells from the blood plasma to the brain extracellular fluid through paracellular diffusional pathways [28, 32]. The tight junctions are responsible for the restriction of the paracellular diffusional pathway between the endothelial cells to ions and other polar solutes, and effectively block penetration of macromolecules by this route. This is of great importance as the adult CNS has been observed not to have regenerative capacity if it gets damaged and, therefore, fully differentiated neurons are not capable of dividing and replacing themselves under normal circumstances [33]. As a result, any increased entry of neurotoxins into the brain might increase the rate in the natural speed of cell death, which would be rather negative. The maintenance of many BBB properties depends on a narrow association with astrocytes. Furthermore, ABC energy-dependent efflux transporters (ATP-binding cassette transporters) dynamically pump many of the neurotoxic agents out of the brain

[34, 35]. These transporters are oriented in such a way that favor transport of molecules into or across the endothelial cell from blood to brain or viceversa.

However, most therapeutic molecules are delivered across the BBB via the receptor-mediated transcytosis system. This procedure involves receptor-mediated endocytosis at the blood side followed by intracellular movement and exocytosis at the brain side of brain endothelial cells [36]. Several receptors on the BBB, such as the transferrin (Tf) receptor (TfR), low-density lipoprotein receptor (LDLR), insulin-like growth factor receptors 1 and 2 (IGFR1 and 2) and the insulin receptor (IR) , among others, have been widely studied as part of the transcytosis system. This receptor-mediated transcytosis allows large molecules to be transported across the BBB and, therefore, it is a useful method for the delivery of proteins, peptides and certain peptidomimetic monoclonal antibodies into the brain. This is why biopharmaceuticals, like recombinant proteins, have gained interest as potential agents for the treatment of CNS diseases over the last decades. However, in order to become applicable, they need a brain targeting moiety because, as it has been observed for other drugs, they cannot effectively reach the brain [37].

1.3 CENTRAL NERVOUS SYSTEM DELIVERY APPROACHES

Over the past decades, there have been many important achievements in drug discovery, from small molecules to biopharmaceuticals like recombinant proteins or antisense medicines. These molecules have gained interest as future possible agents for the treatment of different CNS diseases [38]. Nevertheless, these potential drugs are not able to get to the brain in effective amounts, due to the BBB as described above, so they need a brain targeting modality that will enable their use for such therapies [39]. Current existing approaches to deliver drugs to the brain are commonly divided into either invasive or non-invasive methods.

1.3.1 Invasive methods

A) Disruption of the BBB

Several invasive methods have been used in the past, like the direct intracerebral infusion of the drug [40] or the hyper-osmotic opening of the BBB by the use of a hypertonic solution of mannitol or urea, which it is known to open the tight junction network momentarily, as the capillary endothelial cells shrink by the induction of water efflux. As a result, drug compounds could cross the BBB as the paracellular flow was considerably increased. This procedure has been successfully applied to increase the BBB permeability for CNS active drugs in animals [41]. However, we have to take into account that the defense mechanism of the brain is altered due to these procedures, that increase its vulnerability to circulating chemicals or toxins. On the other hand, BBB can also be disrupted by the use of drugs. Cytotoxic agents like etoposide and cisplatin have been found to create openings between endothelial cells by disrupting tight junctions [42]. In a similar way, vasoactive agents like angiotensin II, peptidase inhibitors or bradykinin can also affect BBB permeability temporarily. These techniques might be frequently accompanied by some systemic side effects as the enhancement of the penetration of drugs into the CNS via the circulatory system will also increase the penetration of drugs throughout the entire body [43].

B) Direct implantation

The problems linked with the side effects of systemic drug delivery and the necessity to modify the surface of the delivery vehicle to make it able to cross the BBB can be avoided with the use of implantable local nanomaterials. Furthermore, these nanomaterials allow to reach much higher local drug concentrations compared to traditional approaches as drug is delivered directly to the targeted tissue. Gliadel®,

which is at present used in the clinic, consists of polyanhydride polymer wafers impregnated with bischloroethylnitrosourea (BCNU, carmustine) that are located in the resection cavity after the excision of the tumor [44]. This technology is considered the gold standard for intra-cerebral drug therapy [45]. Clinical trials have shown that the combination of Gliadel® with surgery and radiation increases survival of GBM patients up to fifteen months. Furthermore, as paracrine administration of interleukin-2 produces a potent antitumor immune response and improves survival in animal brain tumor models, Rhines *et al.* observed a synergistic antitumor effect in the combination of microspheres containing interleukin-2 and Gliadel® biodegradable polymer wafers, when they were both directly implanted at the site of an intracranial rat glioma [46].

Convection-enhanced delivery (CED) is a novel approach to deliver drugs directly into brain tissue and is defined as the continuous injection of a therapeutic fluid agent under positive pressure. In order to deliver drugs that would be too large to diffuse over required distances and would not cross the BBB, this technique was introduced by researchers from the US National Institutes of Health (NIH) by the early 1990s [47]. Using this approach, compounds employed for CNS disorders including chemotherapeutic agents, nanomaterials and macromolecules can be easily delivered with dose adjustment and minimal invasiveness [48-51]. Some nanocarriers that have already been injected by CED are nanoparticles (lipidic, polymeric or magnetic), liposomes, polymeric micelles and dendrimers (see Table 1) [50, 52-56]. Huynh *et al.* observed that the treatment by CED with ferrociphenol-loaded lipid nanocapsules significantly increased the survival time of intracranial 9L rat gliosarcoma tumor-bearing rats in comparison with an untreated group [52]. Similar results were obtained by Bernal *et al.* with temozolomide-loaded polymeric nanoparticles [50]. Dickinson *et al.* studied the infusion of liposomes by CED in canine healthy brains [56]. A mixture of

liposomes loaded with Gd and with CPT-11 was injected as a potential treatment strategy by CED. Results showed that liposomes presented a robust distribution volume in both gray and white matter, with minimal adverse effects.

Stereotaxy, or stereotactic surgery, is a type of minimally invasive brain surgery that uses a system of three dimensional coordinates to locate a site within the brain. It requires only a small incision and a hole less than 12.5 mm in diameter to be made in the skull, which is usually performed under local anesthesia. The stereotactic operation has been commonly employed in the field of neurosurgery to perform injections, implantations, stimulation and biopsies [57, 58]. Traditionally, frame-based techniques were the standard method used and more recently, frameless stereotaxy or neuronavigation has been introduced [59, 60]. One relevant aspect of stereotactic surgery is that drugs can be easily administered in precise, discreet and functional areas in the brain, without causing any damage in the surrounding tissue. Injections can be repeated if necessary. Most probably, the main disadvantage of local drug delivery administration is that the dosage cannot be adjusted after brain implantation [61].

All these methods present advantages, but on the other hand we have to take into account the degree of invasion of the techniques, which make them less patient friendly and more laborious, and which requires skill to avoid possible permanent damage to the brain. As a result, some alternative non-invasive methods have been proposed.

1.3.2 Non-invasive methods

A) Nasal delivery

Some macromolecular drugs like peptides and proteins, also called “biologics”, are too hydrophilic and large to move across the BBB from the systemic circulation [62, 63].

Furthermore, if these drugs are taken orally, they would be quickly degraded by GI enzymes before they were absorbed or by liver cytochromes. For these particular cases, a non-invasive therapy, like the intranasal route, is desirable for chronic patients suffering from PD or AD [64-66]. The extensive interest in intranasal route for therapeutic purposes arises from the particular anatomical, physiological and histological characteristics of the nasal cavity, which provides potential for rapid systemic drug absorption and quick onset of action via the unique connection of olfactory and the trigeminal nervous system between the brain and the external environment [67, 68]. Although less than 5% of the nasal cavity is occupied by olfactory epithelium, this route is direct, bypassing the BBB, since the olfactory neurons do not present a synapse between the receptive element and the afferent path [69, 70]. Therefore, drugs across olfactory epithelial cells may simply move slowly through the tight interstitial space of cells, or across the cell membrane by endocytosis, or transported by vesicle carriers and neurons [71]. Besides, intranasal absorption enhances drug bioavailability in comparison with that obtained after GI absorption, as the GI and hepatic presystemic metabolism is avoided [72, 73]. The delivery of a drug directly into the CNS is determined by a combination of biological and molecular characteristics of the drug. In animal models it has been observed that when the molecular weight (MW) (above 20 kDa), the degree of ionisation and the hydrophilicity of the drug is increased the drug transport into the CNS after intranasal administration can be reduced [67]. Furthermore, the enzymatic degradation in the olfactory epithelium or the P-gp pumps at the apical membrane surface also affect small MW drugs [74]. However, when nanomaterials are used for the delivery of actives across barriers, the transport is no longer dependent on the drug properties, but in the properties of the delivery system [75]. The main mechanism of uptake of nanomaterials (when larger than about 20 nm)

in nose-to-brain drug delivery is thought to be transcellular; however, the transcellular and paracellular routes of cell transport are also present [76]. The incorporation of mucoadhesive polymers into nasal formulation can increase the mucosal contact time and prolong the residence time of the dosage forms in the nasal cavity [73]. As we have previously commented, chitosan has been extensively studied for nose-to-brain delivery due to the non-toxic nature and its absorption enhancing and mucoadhesive properties of the delivery systems [77]. Chitosan can be formulated in combination with other polymers, like hydroxylpropylmethyl cellulose, as a mucoadhesive temperature-mediated *in situ* gel to enhance intranasal delivery of drugs like ropinirole, the dopamine (DA) D2 agonist, to the brain for the treatment of PD [78]. Similarly, Pardeshi *et al.* observed that when the same drug was loaded into polymer-lipid hybrid NP and administered intranasally, the therapeutic activity obtained with this formulation was comparable to that with the marketed oral formulation of ropirinole [79]. Along with the mucoadhesive polymers, lectins have been conjugated to bioadhesive systems in order to improve drug absorption through nasal mucosa. Lectins can specifically recognize carbohydrates and, therefore, bind to the glycosylated nasal mucosa. [80]. Gao *et al.* developed a PEG-PLA based system coupled with the lectin wheat germ agglutinin (WGA), which specifically binds to N-acetyl-D-glucosamine and sialic acid, both abundantly observed in the nasal cavity [81]. They showed that the brain uptake of a fluorescent marker-coumarin carried by WGA functionalized nanoparticles was about 2 fold in different brain tissues compared with that of coumarin incorporated in the unmodified ones. Recent studies have shown that the lipophilicity of the nanosystem also plays an important role in the success of the delivery of the drug to the brain through the nose-to-brain barrier in the treatment of a great variety of CNS diseases [65, 66, 72, 79, 82-84]. Yang *et al.* confirmed that rivastigmine liposomes improve the brain

delivery and enhance pharmacodynamics which respect to BBB penetration and nasal olfactory pathway into the brain after intranasal administration [66]. Li *et al.* observed that the efficiency of acetylcholinesterase inhibition of galanthamine when loaded into liposomes was greatly enhanced by intranasal administration compared with oral administration [65].

B) Cell penetrating peptides (CPP)

Cell-penetrating peptides (CPPs) are a class of short amphipathic and cationic peptides, typically with 5–30 amino acids that, unlike most peptides, are rapidly internalized across cell membranes [85]. Two decades have passed since the first CPP was discoveredand ever since CPPs have been used for a variety of applications, including the delivery of molecular cargoes such as liposomes, nanoparticles, imaging agents (fluorescent dyes and quantum dots), drugs, oligonucleotide/DNA/RNA and peptides/proteins into cells [86-93]. CPP-based delivery systems show a great ability in carrying these macromolecules across cellular membranes, combining a low cellular toxicity with high efficiency [94].

Among the CPPs, TAT, the transactivating protein of the human immunodeficiency virus type-1 essential for viral replication, is maybe one of the most frequently used CPPs for DDS modification. Its interaction with the negatively charged BBB is favored thanks to its cationic charges. As a result, the sequence is endocytosed after the permeabilization of the cell membrane via a receptor/transporter independent pathway. TAT has been used to improve the delivery of small chemotherapeutic molecules, like ciprofloxacin, across the BBB to the brain [95, 96]. Furthermore, CPPs can also be attached to nanomaterials in order to enhance the penetration of these across the BBB. Qin *et al.* prepared a TAT-modified liposomal formulation loaded with doxorubicin, showing a stronger inhibitory effect against C6 cell lines, higher efficiency of brain

delivery, longer survival time of brain glioma bearing animals and lower cardiotoxic risk than the free drug [97].

Beside TAT, SynB peptides and Angiopeps are the most extensively studied vehicles for the delivery of different drugs to the brain [98-100]. Many drugs have been conjugated to members of the SynB family of peptides, showing an increase of their *in vivo* activity in the brain [100-103]. Rousselle *et al.* described that the brain penetration of poor brain-penetrating drugs, like doxorubicin, dalargin or benzylpenicillin, was significantly increased when the drugs were conjugated to SynB vectors and intravenously administered to mice [102, 104, 105]. Angiopeps, a family of Kunitz domain-derived peptides, have also been used in both *in vitro* and *in vivo* studies to transport drugs to the brain in a highly efficient way. Regina *et al.* reported that a member of the angiopep family, Angiopep-2, can transport paclitaxel across the BBB to treat brain cancer [106]. This effect was also seen in a study by Che *et al.,* where Angiopep-2 bound to doxorubicin and etoposide killed cancer cell lines *in vitro* with apparently similar cytotoxic mechanisms to unconjugated doxorubicin and etoposide, but crossing the BBB with a dramatically high influx rate.[107] In recent years, much effort has been made to use Angiopeps to deliver drugs or nanoparticles across the BBB to the CNS, showing that Angiopep-mediated targeting is one of the most promising ways to reach the CNS for treatment of different brain diseases [98] [99] [108-110].

C) Drug Delivery Systems

Drug delivery systems (DDS) have the potential to overcome limitations of drugs such as poor solubility, lack of selectivity, toxic side effects and development of multidrug resistance [111]. They have been widely studied for the delivery of actives to many regions of the body, including the brain [112]. The aim when using nanosized drug

carriers in brain delivery is to increase the specificity toward diseased neurons, to protect the drugs against enzyme inactivation, and/or to improve their bioavailability by increasing their diffusion through the BBB [113]. This can be achieved by an adequate engineering to provide tailored functionalities using ordinary procedures in nanotechnology. Nanoparticle size and the nature and number of linkers on the NP surface can be optimized to control not only both the loading and the release of the entrapped or covalently linked drug components, but also the crossing through the BBB [114]. DDS can also deliver contrast agents in effective concentrations to the brain, improving the efficacy of existing CNS imaging and treatment regimens [115]. Furthermore, nanosystems can decrease the overall systemic toxicity and, therefore, increase the maximum tolerated dose of the drug by using biocompatible materials that avoid the release of the therapeutic agent within non-target tissues [116]. Eventually, the drug delivery across the BBB to the desired site of action in the CNS can also be manipulated by modifying the surface of the nanosized system, for instance, with CPPs [85]. This modification leads to increased therapeutic efficacy due to an increased accumulation of the either therapeutic or diagnostic agent in the CNS [117].

The uptake of these DDS into the CNS can be attributed to the combination of many factors [118]. For instance, the efficacy of polysorbate 80 used as coating agent in inhibiting the efflux systems that are present in the BBB, especially P-gp has been widely studied in the past [119, 120]. In *in vitro* studies, Estella-Hermoso de Mendoza *et al.,* observed that lipid NP coated with polysorbate 80 were able to reduce the P-gp activity, as compared to the same lipid NP without the polysorbate 80 coating. As a result of this, polysorbate 80 coated lipid NP showed a significantly higher uptake by the rat glioma C6 cell line which is naturally overexpressing P-gp [121]. Another hypothesis for the NP uptake was demonstrated with some *in vitro* experiments by

different authors, who observed that apolipoproteins E and/or A-I (apo E or apo A-I) were adsorbed on the surface of PBCA nanoparticles coated with polysorbate 80 or poloxamer 188 after their incubation in blood plasma [122, 123]. Therefore, they concluded that polysorbates and poloxamer 188 act as an anchoring point for apolipoproteins, so that they can then interact with lipoprotein receptors on the brain capillary endothelial cells.

As it will be shown in the following sections, many delivery systems have been developed along these last years for the diagnosis and treatment of CNS disorders. The most representative examples of DDS that have been used to deliver active compounds to the brain *in vivo* are shown in Table 1-4.

2 NANOMEDICINE FOR THE DIAGNOSIS OF NEUROLOGICAL DISORDERS

The task of evaluating and diagnosing damage to the nervous system is complicated and complex. Detection of neurological signs and neuroimaging abnormalities, which appear at relatively late stages in the disease, play a major role in current clinical diagnosis. Unfortunately, for many CNS diseases, successful treatment mainly depends on early detection. Finding potential targets and improving the sensitivity and specificity of currently available diagnostic tests are an important topic of current research. An overview of recent progress in the field of nanotechnology-based diagnosis for brain tumors and neurodegenerative diseases is provided in this section. The most relevant examples are included in Tables 1 to 5. The cases below show that nanomaterials have potential to overcome the low sensitivity problems faced by current diagnostic tools.

2.1 Nanotechnology for improving brain cancer diagnosis

The World Health Organization (WHO) identifies more than 100 brain tumor types classified according to histopathological features, genetics, clinical presentation, and malignancy [124]. Malignant brain tumors consist of high-grade primary brain tumors such as malignant gliomas and metastatic lesions to the brain from peripheral cancers [124, 125]. It is estimated that in the US alone, more than 23,000 men and women will be diagnosed with and 14,000 men and women will die of cancer of the brain and other nervous system in 2013 [126]. Among all brain tumors, the most common high-grade primary brain tumor in adults is glioblastoma [127, 128]. Overall, metastatic brain tumors are the most frequently occurring form of brain tumors in adults, as 10% to 20% malignant peripheral tumor patients develop brain metastases [125, 129]. Brain tumor diagnosis and grading follow the WHO classification [124]. Glioma diagnosis is based on neuroimaging with MRI confirmed by neurological examination or encephalography. Neuroimaging has become increasingly important in assessing brain tumors. Novel contrast agents allow tumor microvasculature scanning and delineation of areas with increased cellularity and vascular proliferation. On the other hand, there is no sensitive biomarker for brain cancer diagnosis in plasma at present.

There are several reports of successful use of nanotechnology to diagnose brain cancers (reviewed in Orringer *et al.* [130] and in Meyers *et al.* [131]). Major benefits in this area include the enhancement of the analytical sensitivity of brain imaging technologies improving the detection and delineation of tumor margins, among others. NP have the potential to improve both preoperative and intraoperative brain tumor detection.

Chelated gadolinium (Gd) is the standard T1 MRI contrast agent due to its paramagnetic properties [132]. However, it suffers from short blood half-life requiring repeated injections, high dosages and false-positive contrast enhancement. Nanotechnology strategies used so far for improved cell uptake and retention are the following: (1) Oxide NP have shown to be the best at increasing properties of Gd. For instance, Park et al. reported high contrast *in vivo* T1

MR images of the rat brain tumor using ultrasmall Gd oxide NP with a diameter of approximately 1 nm with lack of toxicity *in vitro* [133]. Faucher et al. used ultrasmall Gd oxide NP to label GL-261 glioblastoma multiforme cells, in order to localize and visualize them *in vivo* using MRI [134]. More recently, Zhou et al. showed that small-sized zwitterion-coated Gd-embedded iron oxide (GdIO) NP exhibited a strong T1 contrast effect for imaging of tumors through the EPR effect. Zwitterion coating may reduce nonspecific protein adsorption and inter-particle agglomeration increasing their circulation half-life *in vivo* [135]. (2) Gold NP delivering simultaneously Gd, photoacoustic and raman imaging agents have demonstrated picomolar sensitivity in the delineation of tumor margins both *in vitro* and in living mice. NP intravenous (i.v) injection into orthotopic glioblastome-bearing mice led to specific NP accumulation and retention by the tumors for an extended period of time allowing for non-invasive pre-and intraoperative tumor delineation using MRI, photoacustic and raman imaging through the intact skull [136]. (3) The use of Gd loaded liposomes administered by CED [137, 138] and (4) Gd loaded PAMAM dendrimers for MRI contrast enhancement have similarly been reported [139, 140].

Superparamagnetic iron oxide-NP (SPIO) are a novel T2 MRI contrast agent developed over the past decade that tend to persist longer in the brain parenchyma and delineate tumor margins more accurately than other contrast molecules [130]. The non-toxicity of biodegradable iron based-NP has also been demonstrated. The capacity for highly selective tumor targeting is a major advantage of iron oxide-NP over Gd. Ultrasmall superparamagnetic iron oxide (USPIO) are taken up by reactive phagocytic cells that are commonly found at infiltrating tumor margins [141] and long circulating dextran coated iron oxide NP are internalized by dividing tumor cells [142]. Iron-oxide NP surface allows chemical linkage of functional groups or ligands to improve diagnostic specificity. Thus, over the past few years, iron oxide NP have been linked to specific brain tumor ligands for imaging. In this context,

amphiphilic blocked polymer coated iron oxide NP have been conjugated to EGFRvIII antibody present in human glioblastoma multiforme for MRI guided CED and targeted glioblastoma therapy [54]. Bioconjugated NP locally administered allowed MRI contrast enhancement in a mouse glioma model. Effective intratumoral and peritumoral distribution of NP in the brain together with a significant increase in animal survival were found after EGFRvIII–conjugated NP administration [54]. This study provides the proof that monoclonal antibodies conjugated to iron oxide NP may provide specific brain cancer diagnosis with the use of MRI. Chlorotoxin 4, a highly specific marker for glioma cells has also been attached to USPIO [143]. In this sense, Veishe et al. developed a multifunctional nanoprobe capable of targeting glioma cells detectable by both MRI and fluorescence microscopy. Iron oxide NP were coated with PEG and then functionalized with chlorotoxin and with the fluorescent molecule Cy5.5 [143]. This nanoprobe was further validated in a transgenic mouse model of human medulloblastoma, the most common malignant childhood brain tumor, demonstrating its ability to cross the BBB without causing BBB damage and specifically target brain tumors. MRI and NIRF imaging demonstrated NP specific targeting to tumors *in vivo* and validated the nanoprobe as MRI and optical contrast agent [144]. The peptide F3, a tumor specific peptide that binds to nucleolin overexpressed on proliferating tumor endothelial cells was conjugated to SPIO NP and i.v. administered to the rat 9L glioma model. F3-coated NP provide a significant magnetic resonance imaging contrast enhancement compared to non-coated F3 NP [145].

A different approach is proposed by Nie et al., who studied F3-targeted hydrogel NP with covalently linked coomasie blue for delineation of brain tumors [146]. The nanosystem allowed direct brain tumor visualization with no need for extra equipment or special lighting conditions.

2.2 Nanotechnology for improving AD diagnosis

Alzheimer disease (AD) is the leading cause of dementia worldwide. It is estimated that more than 35 million people worldwide have AD [155]. The loss of memory and other cognitive domains cause death within 3 to 9 years after diagnosis. The pathological hallmarks include two distinct types of protein aggregates: the extracellular amyloid-β (Aβ) plaque and the intracellular hyperphosphorylated tau neurofibrillar tangles [155]. Both aggregates are neurotoxic and can produce cognitive impairment. The pathological changes are accompanied by increased oxidative stress, elevated metal ion levels and a widespread degeneration of cholinergic neurons in the cortex, hippocampus, basal forebrain and ventral striatum, which results in lower acetylcholine (ACH) levels and a reduction of cholinergic transmission of cortical neurons in the brain [155]. The diagnostic guidelines of AD include brain imaging and cerebral fluid biomarkers. Although blood is more accessible than cerebral fluid, protein concentration in the blood is lower making the detection more difficult to perform. Ideally, biomarkers may provide means of early AD detection that is very interesting for disease modifying treatments. In addition, biomarkers may be preferably stage-specific. Current biomarkers include phosphorylated tau indicating the tangle pathology, total tau amount that correlates with neuro-axonal degeneration, and the 42 amino acid Aβ isoform (Aβ42) which correlates inversely with plaque pathology [156]. Recently, amyloid-derived diffusible ligands (ADDLs) have been proposed as early AD indicators [157].

Nanotechnology appears to be a useful and promising tool in AD diagnosis (reviewed in Brambilla *et al.* [158]). Nanotechnology may improve the analytical sensitivity of both imaging and cerebral fluid biomarkers. This may result in early disease detection leading to less costly therapeutic demands and improved clinical results. Remarkably, many of the examples described below have not been validated *in vivo*. Hence, attention should be paid to the potential toxicity of nano-based diagnostic medicines.

2.2.1 Brain imaging biomarkers based on nanotechnology

The use of iron oxide NP as MRI contrast agents has been extensively investigated The most frequent iron oxide NP conjugated to Aβ peptide for amyloid plaque detection are monocrystalline iron oxide NP (MIONs) [151], small SPIONP [159] and USPIONP [152]. The main inconvenience of these biomarkers is that they detect Aβ plaque that is observed in later more advanced stages of the disease. Thus, the technique would not be useful for very early AD diagnosis. More innovative is the approach of Skaat *et al.,* who prepare a hybrid system that combines magnetic and fluorescence imaging into one nanostructure system. For that purpose, the authors prepare fluorescent magnetic γFe2O3-rhodamine and γFe2O3-congo red NP. The system might enable early detection using both MRI and fluorescence microscopy and plaque removal using a magnetic field [160]. Only *in vitro* studies have been published with this technology until now.

Thioflavin T selectively recognizes B-sheet structures of Aβ both *in vitro* and *in vivo*. However, thioflavin T is unable to cross the BBB. Siegemund reported that Aβ can be selectively targeted by Thioflavin T after its release from core-shell polystyrene polysorbate-80 PBCA NP. Aβ deposits in the hippocampus were observed by fluorescent microscopy in transgenic mice with age-dependent β-amyloidosis after thioflavin T loaded NP intrahippocampal injection [161]. No targeting of Aβ was observed after iv infusion of the particles.

Choi et al. described the use of a novel contrast agent based on gold NP to improve the MRI sensitivity. A cobalt (II) magnetic core and a platinum shell directly fused onto gold NP and stabilized by a coating of lipoic acid-PEG were prepared. The terminal carboxyl groups of the PEG chains allowed covalent binding with neutravidin lysine residues at the NP surface. NP were used together with MRI to monitor Aβ assemblies structural evolution, especially Aβ protofibrils in the early reversible stages [162].

Quantum dots (QD) are nanoscale semiconductor crystals with special fluorescent properties. However, possible health hazards associated with the use of semiconductor materials have limited their general application. In order to reduce toxicity, some authors have encapsulated QD in polymers or have coated them with PEG. Tokuraku *et al.* developed PEG-coated QD-crosslinked with Aβ peptide able to examine Aβ fibril oligomer formation *in vitro* and in an intact cell system. Remarkably, QD-Aβ nanoprobes successfully pass through the BBB [163]. Recently it was reported that transferrine conjugated QD-Aβ were able to traverse through an *in vitro* BBB model via receptor mediated transport [164].

Härtig labeled hippocampal Aβ with the fluorescent acetylcholinesterase inhibitor PE154 released from two types of NP, carboxylated polyglycidylmethacrylate NP and polystyrene-PBCA NP, in triple transgenic mice. Targeting of Aβ, but not phospho-tau, by PE154 was shown by confocal-laser scanning after NP intrahippocampal injection [165].

2.2.2 Fluid biomarkers based on nanotechnology

Georganopoulou *et al.* have demonstrated that bio-barcode assays based on DNA-NP conjugates are capable of measuring subfemtomolar concentration of ADDL level in CSF. The bio-barcode assay is a ultrasensitive diagnostic tool used for the enzyme-free detection of proteins and nucleic acids. In the case of proteins they are 10^6 times more sensitive than ELISA because carrier gold NP match the specific antibody of the target biomarker with hundreds of DNA barcodes [166].

Noble metal NP, such as silver or gold NP, have been explored to develop ultrasensitive fluid biomarkers for AD. For instance, a nanosensor based on silver NP optical properties to detect low ADDL concentration in CSF using localized surface plasmon resonance (SPR) was developed. Modifications in the NP external environment produce changes in the surrounding magnetic field refractive index that result in variations in the silver NP λ max detectable via

spectroscopy. Since the solution concentration directly changes the refractive index, the biosensor is sensitive to different ADDL concentrations [167, 168].

Regarding bioassays using gold NP, Chikae *et al.* proposed an electrochemical Aβ sensor based on saccharide-protein interactions whose detection sensitivity was improved by immobilizing the saccharide sialic acid on gold NP. The detection of Aβ peptide down to submicromolar concentration was demonstrated [169]. Lee *et al.* used an ultrasensitive immunosensor for Aβ (1-40) detection based on SPR. Gold nanoparticle functionalized with an antibody fragment able to specifically recognize Aβ was used as a way to enhance SPR detection [170]. The immunosensor developed proved to be highly sensitive in the detection of Aβ and enhanced the detection limit from 10 ng/mL to 1 fg/mL compared to a bare gold substrate. Another approach was the development of an electrical method for Aβ immunodetection based on gold NP using scanning tunneling microscopy (STM). The vertical detection immunoassay that comprises corresponding antibody fragments, Aβ, and gold NP-antibody conjugates was combined with STM for electrical detection. The current proposed method successfully detected 1 fg/mL of Aβ [171]. It is also possible to use a Rayleigh scattering assay based on gold NP coated with a monoclonal antibody against tau for the selective detection of tau protein at a concentration of 1 pg/mL. The two-photon Rayleigh scattering assay showed a strong sensitivity for tau protein. It was able to discriminate other proteins such as bovine serum albumin which is one of the most abundant protein components in CSF [172].

2.3 Nanotechnology for improving PD diagnosis

PD is a complex and heterogeneous neurodegenerative disease characterized by the progressive nigrostriatal dopaminergic system degeneration, which causes DA loss in the brain. This disease affects approximately 5 million people globally and its etiology is still unknown [173]. Clinically, it is characterized by four motor symptoms that are bradykinesia,

resting tremor, rigidity and a marked difficulty to perform coordinated movements. It is generally accepted that Parkinsonian motor signs appear when 70–80% of striatal dopaminergic nerve terminals and 50–60% of substantia nigra compacta dopaminergic neurons are death [174]. Regarding PD diagnosis, there is an urgent need for biomarkers, preferably at early premotor stages when neuroprotective drugs have been shown to slow disease progression. However, there is no validated, reliable, inexpensive and simple biofluid or imaging marker available yet [175]. A promising potential premotor biomarker recently proposed by Shannon *et al.* is α-synuclein pathology in the colon [176]. However, its sensitivity and specificity have not been established yet for a reliable cost-effective paradigm. In this context, nanotechnology could be particularly useful. Other novel nanotechnological approaches to diagnose PD include the work of Baron *et al.,* who described the use of a colorimetric assay to detect DA, L-DA, noradrenaline, adrenaline and tyrosinase activity based on the growth of Au-NP induced by the neurotransmitters and detected by plasmon absorbance [177].

Aptamers are functional nucleic acid sequences able to bind specific targets. Nanoparticle-aptamer bioconjugates have been recently used for targeted delivery and diagnosis of several cancers. An aptamer with a high specificity and binding affinity for α-synuclein could be used to test α-synuclein levels in the blood of patients with PD. With this idea, Tsukakoshi *et al.* reported the identification of DNA aptamers that bind to soluble α-synuclein oligomers. A competitive screening method based on aptamer blotting was used to isolate 8 DNA aptamers that specifically bind to α-synuclein oligomers [178].

Concerning the use of nanotechnology for *in vivo* PD diagnosis few data are available. Especially remarkable is the work of Tisch *et al.* who detect asymptomatic nigrostriatal dopaminergic lesion in rats using carbon nanotube sensors to analyze exhaled air. This approach relies on the principle that the volatile organic compound pattern is different in the

exhaled breath of healthy and PD patients. Changes were observed in the chemical composition of the breath samples from 6-OHDA-lesioned rats and sham-treated animals, which led to the sensor-array breath-print. This study demonstrates that breath testing could improve neurodegenerative disease early detection [179].

3 NANOMEDICINE FOR THE TREATMENT OF NEUROLOGICAL DISORDERS

3.1 Nanotechnololgy for brain tumor treatment

Malignant gliomas are generally treated with a combination of surgery, radiotherapy and systemic chemotherapy [180, 181] and metastatic brain tumors with a combination of surgery and radiotherapy [126, 182-185]. However, patient median survival times are low [127, 186, 187]. The point has already been made that the ineffectiveness of chemotherapeutic drugs in treating brain tumors is mainly ascribed to the BBB, which hampers the delivery of the drugs to the extravascular compartment of the tumor. However, it has been shown that when the tumor cell cluster reached a volume large enough (> 0.2 mm^3) the BBB will be damaged and the blood-brain tumor barrier (BBTB) will be formed [128]. This BBTB exists between the brain tumor tissues and capillary vessels and prevents the delivery of most hydrophilic molecules and antitumor agents to brain tumor [188]. In contrast to this, it has also been observed that BBB could remain intact in the case of infiltrative gliomas or micrometastases [189]. Brain tumor neovasculature is functionally different from both normal brain capillaries and the neovasculature of peripheral tumors. In this case, the gap size in the vascular endothelium of the BBB was found to be up to 600 nm in diameter, [190, 191] while the size of those that can be found in brain tumors is significantly smaller (≈12 nm) [190]. Researchers have taken this characteristic into account in the design of brain-tumor targeting nanostructured carriers with smaller diameters that can access the brain tumor through these 12 nm gaps, but are not able to extravasate across the normal BBB [192]. Even though

treating tumors in the CNS is difficult, the development of nanotechnological drug delivery systems can play importantroles in overcoming the hurdles of current therapies against brain tumors. These nanomaterials can be formulated with chemotherapeutic drugs to produce drug-polymer assemblies that can beinjected or implanted and, as a result, allow a localized and sustained delivery of the entrapped drug [114].

3.1.1 Non-invasive approaches

One of the clearest advantages of systemic drug delivery is its non-invasive nature. Nevertheless, in order to achieve therapeutic concentrations in the brain, larger systemic drug doses would be required. In this case, the properties of nanomaterials may offer solutions to this drawback. The best treatment for brain tumor-targeted drug delivery should be able to transport the therapeutic agents at the brain tumor foci minimizing the involvement of healthy brain tissue as well as of peripheral tissues [115, 193].

A) Polymeric nanoparticles

Polymeric NP are probably the most widely used DDS to deliver chemotherapeutic drugs to the brain (Table 2) and Tf is one of the most frequently studied receptors for the targeting of nanomedicines by receptor mediated transcytosis, because of its high expression on the BBB [194, 195]. For instance, Liu *et al.* conjugated Tf to the surface of doxorubicin loaded PEG-PLA NP to specifically target the NP to glioma. They observed that intravenously administered NP could deliver doxorubicin into the tumor sites, leading to a reduction of the tumor growth and prolonged survival of the animals, compared to controls [196]. Cui *et al.* designed a Tf-conjugated magnetic silica PLGA NP loaded with doxorubicin and paclitaxel to overcome the BBB. After their iv administration, these NP exhibited the strongest anti-glioma activity as compared to the control formulations [197]. Similar or even better anti-tumor effects were obtained by Wohlfart *et al.,* with doxorubicin bound to PLGA NP coated with

poloxamer 188 [198]. Increased brain tumor concentrations also were observed with polysorbate 80-coated PBCA NP loaded with temozolomide, methotrexate or doxorubicin after their i.v. injection to rats [122, 199-201]. Wang *et al.* investigated the anti-tumor activity of gemcitabine bound to the polysorbate 80-coated PBCA NP in rats after C6 glioblastoma implantation into the brain [202]. They observed a 20% increase in medium survival time from day 21 to 25. In another study by Xin *et al.,* paclitaxel loaded into mPEG-PCL NP was intravenously administered to C6 glioblastoma bearing mice [203]. The mean survival times with this formulation increased by 40% compared to a paclitaxel administration and by 20% compared to non-PEGylated NP.

Polymeric materials can be used to deliver nucleic acids such as oligonucleotides, but often have low efficacy in human cells. However, while the simple attachment to NP alone results only in a slight improvement of intracerebral uptake of oligonucleotides, an additional coating with polysorbate 80 can lead to a 20-fold higher amount of cellular uptake in an in vitro model of the BBB [204, 205]. This was observed by Schneider *et al.,* who concluded that polysorbate 80 coated NP provide a non-viral method of gene delivery to brain cells and brain tumors [206]. Lu *et al.* achieved a significantly delayed tumor growth and induced apoptosis *in vivo* after repeated i.v. injections of cationic albumin-conjugated pegylated NP loaded with plasmid pORF-hTRAIL as a nonviral vector for gene therapy of gliomas [207].

B) Lipid nanoparticles

Many studies have been performed in order to deliver chemotherapeutic drugs to the brain by means of lipid nanoparticles (Table 3). *In vivo* studies in rats by Martins *et al.* showed that fluorescently labeled SLN containing camptothecin were detected in the brain after i.v. administration [228]. Our group observed that there was an increased amount of drug in brain tissue when the antitumor lipid edelfosine loaded into lipid nanoparticles was orally administered, compared to the drug solution [121]. However, there are still few efficacy

studies performed *in vivo*. Jin *et al.* proved that the i.v. administration of c-Met siRNA-PEG/cationic SLN complex in orthotopic U-87MG xenograft tumor model significantly inhibited c-Met expression at the tumor tissue and suppressed tumor growth without showing any systemic toxicity in mice [229]. Huynh *et al.* observed that the treatment with DSPE-mPEG2000-FcdiOH-LNCs and FcdiOH-LNCs statistically improved median survival time (28 and 27.5 days, respectively) compared to the control (25 days) in a 9L intracranial gliosarcoma model.

C) Liposomes

Liposomes have also been used to deliver chemotherapeutic drugs to the brain (see Table 4). They have also been linked to Tf in order to achieve higher brain tumor delivery. For instance, Soni *et al.* conjugated Tf to the surface of liposomal vesicles to enhance the brain delivery of the anticancer drug 5-florouracil [240]. Biodistribution studies suggested a selective uptake of the Tf coupled liposomes from the brain capillary endothelial cells. Indeed, after the liposomal delivery of 5-fluorouracil researchers observed an average of 10-fold increase in the brain uptake of the drug, while the TfR-coupled liposomes caused a 17-fold increase in the brain uptake of 5-fluorouracil. In a similar way, Ying *et al.* attached Tf and aminophenyl-alpha-D-manno-pyranoside to the surface of their daunorubicin loaded liposomes to both target brain tumor tissue by the Tf and cross the BBB, due to the specific binding of the pyranoside molecule to the GLUT1 receptor in the BBB. They observed that the median survival time of tumor bearing rats after administering these targeted liposomes was significantly longer than that after giving free daunorubicin [241]. Topotecan and tamoxifen have been also delivered to the brain loaded into liposomes showing WGA on their surface. Du *et al.* showed that tamoxifen could inhibit the efflux of MDR proteins in the BBB, while the WGA would enhance the endocytosis of the liposomes in the BBB and in the brain tumor, correlating with an increased efficacy of the nanosystem [242].

D) Dendrimers

The branched architecture of dendrimers is an attractive feature for targeted delivery applications, as they can present targeting ligands in a manner favorable to promote multivalent binding to target brain receptors and eventually cross the BBB [252]. The most representative examples of drugs administered using dendrimers for brain tumor treatment are included in Table 5. Interferon beta was successfully linked to arginine surface modified PAMAM dendrimers. In this study, Bai *et al.* observed that U87MG tumor bearing mice treated with PAMAM-R/pORF-IFN-beta exhibited a significantly smaller tumor size than control mice and PAMAM-R/pORF treated mice [253]. In another experiment, Huang *et al.* suggested that chlorotoxin (CTX) could be exploited as a special glioma-targeting ligand, as PAMAM–PEG–CTX/DNA NP showed high gene delivery in a mouse glioma model via i.v. administration [254].

The cell adhesion molecule integrin $\alpha_v\beta_3$ plays an important role in cancer progression and is overexpressed in melanomas, glioblastoma, ovarian, breast, and prostate cancers. The Arg-Gly-Asp (RGD) containing peptides have been identified to have high affinity with integrin $\alpha_v\beta_3$ [255]. Zhang *et al.* observed that their RGD modified doxorubicin loaded PEG-PAMAM conjugates were able to increase the median survival time up to 50 days, compared to the 14 days achieved with the free doxorubicin [256].

E) Carbon nanotubes

So far there are few studies that show the efficacy of these nanomaterials *in vivo*. Ren *et al.* investigated the feasibility of Angiopep-2 linked PEGylated oxidized multi-walled CNT containing doxorubicin for the treatment of brain glioma in glioma bearing mice [110]. These nanosystems significantly prolonged mean survival time compared to the administration of saline or free doxorubicin.

3.1.2 Invasive approaches; local administration

Among the treatment approaches that were previously described, it seems that direct injection of therapeutic agents into the brain after tumor resection is not always the best option in brain cancer treatment, as the diffusion coefficient of compounds is rather limited [259]. As a result, new anticancer drug formulations should be developed in order to be useful for brain tumor treatment by direct injection in the CNS as this provides a higher drug concentration at the tumor site while systemic toxicity is decreased [114]. As we have already discussed, strategies for local drug delivery include intraventricular, intraparenchymal or intratecal delivery of the agent, the CED and locally implanted systems, all of which are invasive. However, the most significant advantage of all these systems is that they directly bypass the BBB, increasing the bioavailability of the therapeutic agent in the CNS [260]. Illustrative examples are included in Table 2, 4 and 5.

A) Polymeric nanoparticles

Few studies can be found for local delivery of polymeric nanomedicines to treat brain tumors (see Table 2). Polymeric micelles composed of polyaspartic acid and PEG have been used to deliver doxorubicin by CED to xenograft gliomas in rats, resulting in significantly longer survival rates of animals compared to the free drug [214]. Temozolomide was also incorporated into polymeric nanoparticles along with an MRI agent in order to obtain a multifunctional platform that can be used for image-guided treatment of malignant glioma [50]. PLGA has also been widely employed for brain DDS preparation. Sawyer *et al.* observed that camptothecin loaded PLGA NP stereotactically delivered by CED improved survival in rats with intracranial 9L tumors: the median survival for rats treated with these NP was significantly longer than that of unloaded NP and free camptothecin infusion [209].

B) Liposomes

In order to favor the association and interaction with the brain, liposome size, charge and surface properties can be easily modified by adding new components to the lipid mixture before liposome preparation, by varying liposome preparation methods or by selecting the appropriate administration route [261]. For instance, Chen *et al.* observed a significantly greater anti-tumor activity and survival benefit from CED of irinotecan-loaded liposomes, compared to the systemic administration of the same liposomes [55].

Monoclonal antibodies could also act as a "molecular Trojan horse" and allow delivery systems to cross BBB. MAb-conjugated liposomes, also known as immunoliposomes, have proved effective as brain drug delivery systems [262]. For instance, Zhang *et al.* developed immunoliposomes, carrying a plasmid DNA encoding the EGF receptor antisense mRNA, conjugated with two monoclonal antibodies directed to mouse Tf receptor, in order to get through the BBB, and to human insulin receptor for intratumor cell delivery [263]. This study showed that these immunoliposomes are effective after i.v. administration in mice bearing U87 brain tumors. Similarly, Gosk *et al.* observed that when OX26 monoclonal antibody was coupled to daunomycin containing PEG-immunoliposomes, the accumulation of drug was increased in the brain tissue after i.v. administration, compared to the PEG-liposomes without the monoclonal antibody [264].

C) Dendrimers

In the last few decades, various PAMAM-based drug carriers have been developed to investigate their potential use for cancer therapy (Table 5). Even though PAMAM dendrimers have shown potential as DDS in brain tumor cell lines *in vitro*, [265-269] so far the reports of dendrimers applied to brain tumor targeting and therapy *in vivo* are still limited and still need to be optimized [252]. Yang *et al.* studied the feasibility of using boronated PAMAM dendrimers with EGF as targeting moiety for the treatment of gliomas. They observed that the

CED of the EGF bound dendrimer was therapeutically more effective than the intratumor injection of the same nanosystem [258].

Nevertheless, we have to take into account that local delivery methods showed some limitations. The exponential decrease of the drug diffusion from the implanted nanostructure as the distance from resection cavity increases and the limitation of drug dosage by the size of the implant are two significant examples of these drawbacks [270]. These facts reduce the therapeutic drug concentration in cells that are a few centimeters away from the implant, presenting high risk of local neurotoxicity, cerebral edema or infection. Furthermore, the use of these invasive techniques requires the hospitalization of the patient and the need for highly experienced personnel in order to apply anesthesia for the local implantation of devices [260].

3.2. Nanotechnology for AD therapy

While there is no cure for AD, cholinesterase inhibitors are approved by the FDA to treat its symptoms and are so far the most effective therapeutic approach. These drugs provide symptomatic short-term relief without affecting disease progression, though a neuroprotective potential has also been proposed (revised in Salomone *et al.*[271]). Results reviewed by Salomone *et al.* demonstrate that there is an urgent need to develop disease modifying treatments able to counteract the progression of AD since none of the current available strategies have demonstrated efficacy in phase III clinical trials. Advances in nanotechnologies hold great promise to exert a significant impact on AD treatment. The most thoroughly investigated nanotechnology-based approaches have been directed to combat amyloid cluster toxicity enhancing their clearance or modifying their aggregation kinetics in the brain or in the blood with the idea of reducing their brain levels (the so-called “sink effect”). Other strategies have been focused on the encapsulation of several drugs with anti-oxidant, neuroprotectant or cholinesterase inhibitor properties into NP for their targeted delivery to the brain (reviewed in Garbayo *et al.*[61] and in Brambilla *et al.*[158]). Although

promising, these findings should be considered preliminary since few of them have demonstrated their efficacy using *in vivo* AD models or in clinical practice.

3.2.1 In vitro studies

A) Nanoparticle-mediated protein aggregation manipulation

Curcumin, a bioactive component of the golden spice turmeric (Curcuma longa), has anti-amyloid aggregation, anti-tau hyperphosphorylation, anti-oxidant and anti-inflammatory properties. However, it shows poor bioavailability due to its instability, low solubility and rapid metabolism. Mathew *et al,* evaluated the anti-amyloid and anti-oxidant properties of curcumin PLGA NP conjugated with a targeting moiety-Tet 1 peptide and their *in vitro* uptake by GI-1 glioma cells showing that they can be a potential tool to treat AD. Tet-1 peptide, which has affinity to neurons and possesses retrograde transportation properties, was effective in neuronal targeting. In addition, NP were able to destroy amyloid aggregates and showed free-radical scavenging activity and no cytotoxicity *in vitro* [272].

Markedly elevated zinc, copper and iron concentrations in amyloid deposits on the human AD brain are well documented in the literature [273]. Thus, chelating agents provide another tactic to reverse Aβ plaque formation. In this regard, the copper chelator D-penicillamine covalently conjugated to lipidic particles was able to dissolve pre-existing Aβ aggregates *in vitro* [274]. Further studies are needed to evaluate its *in vivo* efficacy and to demonstrate that this strategy is a viable alternative to traditional chelating agents.

Another strategy to reduce protein aggregation is the use of gold NP. Recently, Hiesh *et al.* explored the gold NP inhibitory effect on the fibrillogenesis process of insulin fibrils demonstrating that when gold NP were co-incubated with insulin, an amyloidogenic protein model, the structural transformation into amyloid-like fibrils was delayed about a week in

vitro [275]. Similarly, Liao et al. overall showed that negatively charged gold NP inhibited Aβ-fibrillization [276].

Gobbi *et al.* prepared and characterized liposomes and SLN functionalized with the amphipathic lipid dimyristoylphosphatidic acid that showed high *in vitro* affinity for Aβ peptide. The ability of the lipid-based nanosystems to bind the peptide was assessed *in vitro* by using SPR technology. These nanovectors are very promising for the targeted delivery of diagnostic and therapeutic molecules [277].

Particularly innovative is the use of biocompatible poly-aminoacid-based polymer NP containing hydrophobic dipeptides in the polymer side chains, proposed very recently by Skaat *et al.,* to inhibit Aβ-aggregation. Two dipeptide residues were designed similarly to the hydrophobic core sequence of Aβ and included in the polymer side chains for NP preparation. Thus poly(N-acryloyl-L-phenylalanyl-L-phenylalanine methyl ester) (polyA-FF-ME) NP and poly(N-acryloyl- L-alanyl-L-alanine methyl ester) (polyA-AA-ME) were synthetized and characterized. A significant inhibition of the Aβ40 fibrillation process in vitro in the presence of these NP was observed together with no significant toxicity on different cell lines [278]

B) Nanogel-assisted protein refolding

Several authors have investigated the potential application of biocompatible nanogels as artificial chaperones for controlling Aβ fibril aggregation and cytotoxicity. In this view, Ikeda *et al.* demonstrated that biocompatible cholesterol-bearing pullulan (CHP) nanogels inhibited amyloid fibrin from forming and released monomeric Aβ molecules on addition of methyl-β-cyclodextrine [279]. CHP nanogels prevented AB oligomerization and protected PC12 and primary cortical and microglial cells from Aβ neurotoxicity [279-281]. CHP nanogels could be a valid approach to treat AD, but further experiments demonstrating in vivo BBB surpass and efficacy are required.

C) Nanoliposomes with high affinity for amyloid ß peptide.

Mourtas *et al.* prepared two types of nanoliposomes functionalized with curcumin derivatives with high affinity for Aβ peptide by a conventional synthetic method or with a click chemistry method. Curcumin-decorated nanoliposomes prepared with the click chemistry showed the highest affinity for Aβ fibrils reported to date and sufficient integrity and stability for *in vivo* applications [282]. Taylor *et al.,* who studied the effect of nanoliposomes associated with different ligands on Aβ aggregation obtained similar results. Ligands evaluated were curcumin, phosphatidic acid, cardiolipin, or GM1 ganglioside, the click-curcumin type being by far the most effective [283]. Unfortunately, no *in vivo* experiments have been performed.

D) PEGylated nanomicelles that inhibit protein aggregation

Pai et al. proposed for the first time the potential use of PEGylated phospholipid nanomicelles as therapeutic agents against AD. The work demonstrated that nanomicelles were effective in mitigating the Aβ capacity to aggregate into plaques and to moderate its *in vitro* neurotoxicity using the human neuroblastoma cell line SHSY-5Y [284]. If further *in vivo* studies confirm these results, the authors suggest that PEGylated phospholipid nanomicelles could be effective to slow down AD progression since they mitigate Aβ aggregation by accommodating Aβ molecules in α-helical conformation that results in reduced aggregation and amyloidogenicity [284].

E) Nanoparticles for cholinesterase inhibitors

Pagar explored a novel L-lactide-depsipeptide copolymer for rivastigmine-loaded polymeric NP preparation. The effects of excipients and formulation variables on the NP were analyzed in detail [285]. More recently, Luppi *et al,* investigated intranasal formulations of tacrine based on albumin NP carrying different cyclodextrins and some of their hydrophilic derivatives. NP carrying cyclodextrins showed mucoadhesion *in vitro* and *ex-vivo* and in

particular HP-β cyclodextrin showed the highest mucoadhesive properties. Intranasal absorption studies in AD animal models are now needed to further validate this strategy [286].

F) Carbon nanotubes for acetylcholine administration

Yang *et al.* proposed for the first time the use of CNT as drug carriers for the treatment of CNS diseases [287]. CNT are able to enter the brain via nerve axons. Single-walled CNT were loaded with ACH and their efficacy and toxicological profile after oral administration were examined in an experimentally-induced mouse AD model. Since CNT have generated serious concerns about their safety profile, toxicological experiments were of great importance to address whether CNT could be used as drug carriers. CNT successfully delivered ACH to the brain and improved learning and memory capabilities whereas free ACH or CNT alone did not elicit any effect. These positive effects showed good dose-effect relation. Regarding toxicity, CNT were highly safe at low doses and only high doses caused pathological changes in the ultrastructure of mitochondria and lysosomes [287]. Since not much is known regarding chronic CNT toxicity, further experiments are required to address/elucidate the possible health risk and hazards.

3.2.2 Non invasive approaches

Examples of nanomedicines for AD treatment already tested *in vivo* are provided in Tables 2, 3 and 4.

A) Nanoparticle-mediated protein aggregation manipulation

Chen *et al.* tested the efficacy of curcumin PEG-PLGA-polyvinylpyrrolidone NP freeze dried with β-cyclodextrin orally administered in AD Tg2576 mice [210]. Curcumin nanovector-treated animals showed significantly better cue memory in the contextual fear conditioning

test compared to placebo, and better working memory in the contextual fear maze test than with free curcumin and placebo after a three-month-treatment [210].

B) Nanoparticle-mediated neuroprotection

Quercetin, a natural flavonoid with antioxidant activity, was nanoencapsulated into polysorbate 80-coated solid lipid NP and intravenously administered in rats with aluminium-induced dementia [234]. Animals improved memory retention in the spatial navigation task and in the elevated plus maze paradigm compared to free quercetin administration. Moreover, quercetin-loaded NP significantly reversed the increase in malondialdehyde and nitrite levels and the depletion of reduced GSH induced by the aluminium chloride chronic administration demonstrating the potential of solid lipid NP as a platform technology.

The octapeptide derived from the activity-dependent neuroprotective protein (NAP) is a promising neuroprotective agent for AD. In order to enhance its brain delivery and to protect the neuropeptide from degradation, Liu *et al.* proposed its nanoencapsulation in B6 peptide-modified PEG-PLGA NP [219]. *In vivo* biodistribution experiments after i.v. administration of the nanovector through the tail vein demonstrated that B6 peptide mediated brain targeting and allowed a higher NP brain accumulation. B6-NP-NAP significantly ameliorated the loss of hippocampal neurons, the spatial learning deficit and the cholinergic dysfunction in mice stereotaxically coinjected with Aβ-1-40 and ibotenic acid.

C) Nanoparticles for cholinesterase inhibitors

Rivastigmine, an established non-competitive and reversible cholinesterase inhibitor, improves or maintains cognitive function, global function and behavior in patients with AD. However, its oral therapy includes limited entry into the brain due to its hydrophobicity, frequent administration and cholinergic side effects. With the idea of improving rivastigmine treatment, Wilson prepared polysorbate 80 PBCA NP and investigated if they enable the

transport of rivastigmine across the BBB and the effect of polysorbate 80 on drug brain delivery. Biodistribution studies after i.v. NP administration in rats demonstrated a 3.8 fold increase in rivastigmine brain uptake compared to free drug [221]. Apo E adsorbed from the blood to the particle surface after i.v. administration mediates NP internalization through BBB low-density lipoprotein receptors. Similar results were obtained by the same author when using chitosan polysorbate 80 coated NP [222]. In another study, Joshi *et al.* reported memory improvement in scopolamine-induced amnesic mice after treatment with PLGA or polysorbate 80-PBCA rivastigmine loaded NP [223]. Piperine has shown significant anti-acetylcholinesterase activity. However this drug has first pass-effect and high doses are required to exert its neuropharmacological effect. In order to overcome this limitation piperine NP could be prepared. Yusuf *et al.* studied the therapeutic effect of polysorbate 80 coated lipid NP encapsulating piperine intraperitoneally administered in an experimentally induced AD model in rats [235]. Piperine delivered by SLN at a dose 2.5-fold lower than the control donepezil reduced the amyloidal content and tangles of the nucleus basalis magnocellularis through reduced oxidative stress and cholinergic degradation [235].

Tacrine is another cholinesterase inhibitor with potential significance in AD. With the idea of increasing tacrine brain delivery and to reduce its side effects Wilson *et al.,* prepared polysorbate 80-coated PBCA NP and polysorbate 80-coated chitosan NP and studied NP biodistribution in rats after its i.v. application into the tail vein [224, 225]. The polysorbate 80 significantly increases tacrine uptake into the brain in comparison with the free drug alone and the drug bound to NP for both formulations confirming the specific role of polysorbate 80 in brain targeting.

Md S *et al.* showed a high concentration of donepezil in brain after i.v. administration of the drug nanoencapulated in PLGA NP. Biodistribution studies using gamma scintigraphy techniques revealed a significantly higher percentage of NP formulation in the brain as

compared with the drug solution, demonstrating the potential of the nanosystem to enhance drug delivery to the brain [212].

Especially remarkable are very recent studies from Zhang *et al.,* which reported dual-functional NP based on a PEGylated PLA polymer targeting amyloid plaques in AD mice brains [288]. Two targeting peptides were conjugated to the NP surface: one specifically targets ligands at the BBB while the other has good affinity for Aβ. Brain distribution studies in mice and *ex-vivo* imaging confirmed that dual-functional NP achieved enhanced and precise Aβ targeting *in vitro* and *in vivo*. In addition, no cytotoxicity in PC12 cells and bEnd 3 cells was found after 24 h of treatment with the particles. Ideally, multiple target NP will allow for better specifity and selectivity, thereby reducing the needed drug dose, as well as the potential harmful side effects [288].

D) Liposomes for cholinesterase inhibitors

Liposomes have been investigated to deliver rivastigmine to the CNS via the intranasal route [245]. The pharmacokinetic of the drug intranasally administered using liposomes in rat plasma and brain was studied. Significantly greater levels of rivastigmine were found in the brain compared to the administration of the free drug through the intranasal route or orally administered. No efficacy studies in AD models have so far been reported using this delivery system [245].

E) Liposomes for antioxidants

Huang *et al.* investigated if the oral bioavailability and brain distribution of (+)-catechin could be improved using polysorbate-80 coated liposomes [243]. This drug improves brain atrophy and learning memory functions and ameliorates PD and AD progression [289]. However, its oral bioavailability is low. *In vitro* studies demonstrated that (+)-catechin loaded liposomes remained stable in the presence of gastrointestinal fluids. A significant increase in (+)-

catechin blood levels was observed 6 and 8 hours after the oral administration of the drug-loaded liposomes in rats. Brain distribution studies showed higher levels of the drug in the cerebral cortex, hippocampus, striatum and thalamus [243]. Efficacy studies using AD animal models are needed to further validate this novel strategy.

3.2.3 Invasive methods; local administration

A) Nanoparticle-mediated neuroprotection

Vascular endothelial growth factor (VEGF) is a growth factor implicated in angiogenesis with specific roles in axonal outgrowth and neuroprotection. Like other proteins, VEGF clinic application is hampered because of formulation and delivery problems. A novel nanotechnology-based strategy was recently proposed by Herran *et al.* to deliver VEGF locally to the brain [227] (see Table 2). In order to avoid adverse effects related to neurotrophic factor systemic administration, a local drug delivery approach was proposed to deliver VEGF to target areas. VEGF-loaded PLGA nanospheres improved behavioral deficits, decreased Aβ deposits and promoted angiogenesis when administered through minimally invasive craniotomy in double transgenic amyloid precursor/presenilin 1 mice [227].

3.3 Nanotechnology for PD therapy

Current treatments for PD are largely aimed at addressing motor symptoms enhancing DA levels in the brain and far fewer are focused on alleviating non-motor symptoms or on modifying disease progression (Revised in Meissner *et al.* [290] and in Garbayo *et al.* [291]) Regarding symptomatic therapies, both levodopa (L-DOPA), which exhibits low oral bioavailability and very low brain uptake due to its high peripheral degradation, and DA agonists are currently used in the management of PD patients. However, both treatments do not stop or slow PD progression and can potentially cause long-term motor complications such as the “wearing-off” effect, the “on–off” phenomenon, and dyskinesias. Thus, although

DA replacement is efficacious in the early stage of the disease, new agents that can extend the length of the treatment or ideally reverse the degenerative process are needed. Concerning neuroprotective and neurorestaurative treatments for PD, most of them are based on the use of protein or peptides that are easily degraded by enzymatic and body fluids. Thus, brain administration of these molecules constitutes a challenge as well. Currently, various nanoscale systems are being explored to deliver all of these drugs to the brain (reviewed in Garbayo *et al.* [61, 291])

3.3.1 In vitro studies

A) Nanoparticles for antioxidants, dopamine and dopamine agonist delivery

Carrol *et al.* encapsulated the antioxidant Tempol in PLGA NP conjugated with the TfR OX26 antibody to increase the delivery to the brain by bypassing the BBB. *In vitro* studies demonstrated that antibody addition increased NP uptake by primary neuronal cells and by RG2 rat glioma cells. Cell viability studies showed that Tempol-OX-NP were more effective in preventing cell death by resveratrol in RG2 cells than Tempol-NP or than the free drug in solution [292].

An innovative multifunctional nanoplatform with both imaging and therapeutic purposes was proposed by Malvindi *et al.* Highly fluorescent quantum dots were functionalized with 2 biomolecules: (1) succinyl DA which can be hydrolyzed by the enzymes cellular esterase to release the prodrug within the cells and (2) a galactose shell that can be recognized by the transporters of GLUT-1. Human nasopharyngeal epidermal carcinoma (KB) cells overexpressing the GLUT transported internalized the nanosystem through GLUT-1 on the outer cellular membrane. MTT cytotoxicity assay showed that the galactose core shell enhanced NP biocompatibility in comparison with the original nanocrystals [293].

The dopamine agonist ropinirole shows hepatic first pass metabolism. With the aim of impoving its therapeutic efficacy in PD Patil *et al.,* prepared ropinirole loaded-PLGA NP whose surface was engineered using vitamin E for naso-brain delivery of the drug [294]. The nanovector showed good retention of the formulation with no signs of damage on nasal mucosa.

3.3.2 Non invasive approaches

Currently available nanotechnology tools for PD treatment tested *in vivo* are included in Table 2, 3, and 4.

A) Nanoparticles for dopamine replacement

Chitosan-based NP are currently one of the most widely studied nanosystems for PD. A vehicle for DA delivery based on chitosan NP was prepared by De Giglio *et al.* Quartz cristal microbalance with dissipation monitoring (QCM-D) and X-ray photoelectron spectroscopy showed a predominant location of DA on NP surface suggesting a rapid availability of the neurotransmitter in the brain [295]. Evaluation of the toxic effect of DA-NP and the free neurotransmitter on the in vitro BBB model MDCKII-MDR1 cell line, fthrough MTT assay showed that DA-NP were less toxic than the neurotransmitter after 3 hours of incubation. Measurement of oxygen reactive species suggested low neurotoxicity of DA-NP. Transport studies using the same cell line showed an improvement in DA transport through the *in vitro* BBB model using the nanovector. *In vivo* microdialysis studies in rats with intraperitoneal DA-NP injection demonstrated that the nanosystem was able to transport the neurotransmitter through the brain. In addition, it was observed a dose-and time-dependent striatal DA level increase [213]. Chitosan NP have also been used to encapsulate L-DOPA [218]. NP were combined with a thermo-reversible gel of pluronic for intranasal delivery. Chitosan NP suspension in saline elicited higher L-DOPA brain levels compared to NP dispersed in

pluronic gel. Pluronic gel was able to increase the residence time of NP in the nasal cavity but decreased the migration of NP to the brain due to gel viscosity [218]. Chitosan NP have been also investigated as a delivery system to enhance bromocriptine brain targeting efficiency following intranasal administration. Bromocriptine NP were able to reverse haloperidol-induced catalepsy and akinesia in mice, the nanoencapsulated drug being more effective than bromocriptine in solution. Moreover, a significant increase in bromocriptine brain uptake was observed after the intranasal administration of the radiolabeled drug nanoencapsulated in the mucoadhesive NP suggesting a direct nose to brain transport bypassing the BBB [208].

Yang *et al.* prepared PLGA NP loaded with L-DOPA methyl ester/benserazide and tested its efficacy after subcutaneous administration in the 6-OHDA toxic lesion PD model in rats. NP significantly reduced the axial, limb, orolingual and locomotive dyskinesias compared to the free drug [217].

Tsai *et al.* prepared tripalmitin and hydrogenated soybean phosphatidylcholine solid lipid NP to improve apomorphine oral bioavailability and brain distribution [230]. Glyceryl monostearate or polyethylene glycol monostearate were used as emulsifiers in SLN preparation. Pharmacokinetic studies comparing the oral formulation administration with the i.v. drug injection were done in rats. Both systems increased 12- to 13-fold apomorphine oral bioavailability compared to the control. Drug brain distribution studies after oral administration of the formulations indicated detectable apomorphine concentration in the cerebellum, brainstem and striatum. Moreover, both formulations improved motor behavior of 6-OHDA rats the polyethylene glycol monostearate NP being more efficient than the glyceryl monostearate ones [230].

Solid lipid nanoparticles [236] and polymer-lipid hybrid NP [79], both with modified surface, have recently been proposed for Pardeshi as intranasal nanocarriers for ropinirole hydrochloride. Nanovectors demonstrated good retention of the formulations with no signs of

damage to nasal mucosa. *In vivo* pharmacodynamics studies comparing the nanosystems with the commercial oral formulation demonstrated the efficacy of the nanovectors.

B) Nanoparticles for growth factor and peptides delivery

A novel biodegradable brain drug delivery system was proposed by Hu et al., who developed lactoferrin conjugated polyethylene glycol PLGA (PEG-PLGA) NP encapsulating the fluorescent coumarin-6 [226]. In vitro studies showed that clathrin-related endocytosis mediated NP incorporation by bEnd.3 cells. Following i.v. administration of lactoferrin-NP, 3 times more fluorescent probe was found in the straiatum and substantia nigra than with NP administration. To explore the utility of the delivery system in PD, the cytoprotectant peptide urocortin was incorporated into the Lactoferrin-NP. I.v. administration of the nanosystem significantly attenuated the 6-OHDA-induced lesion improving rotational behavior, striatal DA content and TH-immunoreactivity [226].

The lectin, odorranalectin was conjugated to PEG-PLGA NP to improve nose to brain drug delivery [73]. Odorranalectin bioactivity was maintained after NP preparation as confirmed by an *in vitro* haemagglutination test using red blood cells. DiR fluorescent tracer was incorporated to the odorranalectin-NP to investigate the nose-to-brain delivery of the system by in vivo fluorescence imaging. The brain uptake of DiR loaded NP was effectively increased by odorranalectin. In order to study the efficacy of this nanomedicine in PD, urocortin was used as drug model and nanoencapsulated in OL-NP. The intranasal administration of the system enhanced urocortin neuroprotective effect in hemiparkinsonian 6-OHDA rats [73].

Nerve growth factor (NGF) is a potential disease modifying therapeutic protein for AD due to its neurotrophic activities on basal forebrain cholinergic neurons. However, its clinical application is hindered by major problems associated with effective CNS delivery and adverse

effects. In this connection, Kurakhmaeva *et al.* investigated NGF brain delivery after iv administration using PBCA NP coated with P80 and the pharmacological efficacy of this delivery system in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model [216]. NGF transport through the BBB was analyzed by direct NGF measurement in the mouse brain. In addition, the nano-formulation significantly reduced basic PD symptoms such as oligokinesia, rigidity or tremor [216].

C) Nanoparticle-based gene therapy

Gene therapy has been extensively explored in the PD context and many clinical trials using gene therapy are under investigation. In parallel to existing viral vectors, NP can be used as non-viral vectors for brain gene delivery. With this purpose, Huang *et al.* examined the neuroprotective effects of lactoferring conjugated-PANAM/PEG NP encapsulating hGDNF gene using a multiple-dosing regimen i.v. administered in two different rat PD models. NP significantly improved locomotor activity, reduced dopaminergic neuronal loss and enhanced monoamine neurotransmitter levels in both animal PD models [296, 297]. Recently, the same author prepared NP conjugated to Angiopep, a ligand that specifically bind to low-density lipoprotein receptor-related protein which is overexpressed on the BBB. Angiopep was conjugated to dendrigraft poly-L-lysine, a poly-L-lysine-based dendrimer, via PEG. Angiopep conjugated NP exhibited higher cellular uptake and gene expression in brain cells compared to unmodified counterpart. Best improved locomotor activity and apparent dopaminergic neuron recovery was observed after five i.v. injections of hGDNF-NP in the rotenone-induced PD model [98].

F) Nanoliposome-based gene therapy; the Trojan horse nanoliposome technology

The group of Pardridge *et al.* has great experience in transgene delivery to the brain following i.v. administration of PEGylated immunonanoliposomes. This technology has been validated

in multiple animal models in mice, rats and monkeys demonstrating that Trojan horses can be administered chronically without toxicity or immune reactions. Regarding PD, they worked with TH and GDNF plasmids. These authors demonstrated that it is possible to normalized TH activity in the 6-OHDA depleted striatum and to reverse motor impairment by i.v. administration of PEGylated immunoliposomes encapsulating TH plasmid targeted with the OX26 murine monoclonal antibody to the rat TfR [249]. This technology was further improved with the use of a TH plasmid engineered with a brain-specific promoter to avoid ectopic transgene expression [251]. When a GDNF plasmid was used, a near complete rescue of experimental PD in rats was observed. The GDNF transgene expression was under the influence of the rat tyrosine hydroxylase (TH) promoter to express the neurotrophic factor only in the regions of the brain that express TH gene. Trojan horse liposomes were able to reduced 87% apomorphine-induced contralateral rotation and 90% amphetamine-induced ipsilateral rotation. In addition, motor function improvement correlated with a 77% increase in striatal TH activity [250].This technology could soon be translated to humans.

E) Nanoemulsion gel for dopamine agonist delivery

Transdermal nanoemulsion gel containing ropinirole has been designed for the efficient treatment of PD [298]. Pharmacokinetic studies revealed a greater and more extended ropinirole release from the nanoemulsion compared to the conventional gel and to the orally administered marketed drug tablet suspension. Drug bioavailability was enhanced more than two fold with the nanoemulsion gel formulation. Ropinirole loaded nanosystem efficacy following transdermal administration was evaluated in terms of oxidative stress marker levels in the 6-OHDA-lesioned striatum of rats. A significant increase in thiobarbituric acid reactive substances and in reduced glutathione and catalase activity were reported demonstrating its significant value in clinical PD treatment [298].

3.3.3 Invasive methods; local administration

GDNF is one of the most promising candidates for PD treatment given its well-known neuroprotective and neuroregenerative properties. In order to resolve the crucial delivery issues poses by neurotrophic factors, several groups, including ours, have administered GDNF locally to the striatum using biodegradable and biocompatible microparticles in different animal models of PD [299-301]. Functional recovery in addition to increase in striatal dopaminergic innervation has been reported [299-301]. Regarding nanotechnology strategies for local administration of GDNF, Yurek *et al.* published several studies using GDNF plasmid DNA compacted into NP using a polycation-like 10 kDa polyethylene glycol (PEG)-substituted lysine 30-mers (see Table 3). In the first paper the authors combine this nonviral gene therapy with neural grafts in order to improve the survival of the grafted cells and the recovery of parkinsonian rats [238]. Compacted DNA NP locally implanted into the striatum overexpressed GDNF in the lesioned striatum to levels able to provide support to grafted cells. Authors showed that survival of grafted cells was improved. In addition, a more extensive fiber outgrowth from the graft with more dopaminergic cells was found. This led to a better functional recovery by the animals [238]. The same group observed a sustained GDNF overexpression after single injections of rat GDNF DNA NP into the striatum [239]. Recently, in order to achieve a long-term transgene activity in the brain GDNF plasmids were optimized. GDNF plasmid were compacted into DNA NP and injected into the brain achieving a long-term expression in the brain [237].

The site-specific delivery of DA from an intracranial nano-enable scaffold device (NESD) implanted in the frontal lobe parenchyma was proposed for Pillay *et al.* [302]. The NESD is composed of a binary crosslinked alginate scaffold containing cellulose acetate phthalate NP loaded with DA. The in vivo evaluation of the device upon implantation into the rat brain demonstrated that the system was biocompatible, biodegradable and had a positive effect on DA concentration in the brain [302].

4 CURRENT CHALLENGES FOR THE CLINICAL DEVELOPMENT OF NANOMATERIALS

Nanotechnological application to diagnose and treat medical disorders shows great promise to provide powerful tools in medicine. However, after nearly twenty years of research, nanotechnology approaches to brain drug delivery remain under study. One of the reasons responsible for this is the BBB mentioned previously. But in addition to this there some more challenges that researchers need to face in order to make nanomaterials safe and effective.

4.1 Toxicity of nanomaterials

While the application of nanomaterials in biomedical field is a increasing, their potential hazard for human health is still under study, due to their special physicochemical properties, mainly their possible toxic effects on CNS [303]. Generally, the combination of various factors are responsible for the harmful effects of nanomaterials. Among them, the high surface area and the intrinsic toxicity of the surface are particularly important [304]. Therefore, the assessment of the neurotoxic effects of these nanomaterials on CNS function is a must, as the mechanisms and pathways through which nanomaterials may cause their toxic effects remain unidentified. When drugs are delivered to the brain, we have to bear in mind that many of the drugs that can be distributed in the CNS cause unwanted neurotoxicity by themselves [305, 306]. Also, recent investigations suggest that several nanomaterials, such as polysorbate 80-coated NP, are able to cross BBB through either oral or i.v. administration and accumulate in the brain [121, 200, 202]. As these NP penetrate the BBB, they could cause side effects after affecting the BBB function and brain physiology. So far, there are not so many reportsthat explain neurotoxicity of NP both *in vitro* and *in vivo* [303, 307].

4.1.1 In vitro toxicity of nanomaterials

Several research groups have reported potential toxicity of nanomaterials on different types of cells *in vitro* [202, 308-310]. Ever since Greene and Tischler used PC12 neuronal phenotype cells as a model for neurobiological and neurochemical studies, this cell line has become the most widely used cell model for nanoparticle neurotoxicity studies [311]. Wang *et al.* observed how the expression of dopaminergic system-related genes in PC12 cells induced by metallic nanoparticles made of Cu and Mn changed, inducing DA depletion in this cell line [312]. The results suggested that Mn and Cu NP could produce dopaminergic neurotoxicity and might share some common mechanisms associated with neurodegeneration. Hussain *et al.* observed similar results reporting that the exposure of PC12 cells to manganese oxide particles could deplete DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in a dose-dependent manner and increase the production of reactive oxygen species (ROS), while cytotoxic silver nanoparticles could produce cell shrinkage and irregular membrane borders [313]. In another study, Pisanic *et al.* showed that exposure to increasing concentrations of anionic magnetic nanoparticles (MNP) diminished the viability of PC12 cells [310]. Wu *et al.* carried out studies to elucidate the toxicity of SiO_2 nanoparticles, demonstrating that exposure to SiO_2 decreased cell viability, increased levels of lactate dehydrogenase, triggered oxidative stress, disturbed cell cycle, induced apoptosis, and activated the p53-mediated signaling pathway in the PC12 cell line. Zhang *et al.* investigated and compared the concentration-dependent cytotoxicity of single-walled CNT (SWCNTs) and SWCNTs functionalized with polyethylene glycol (SWCNT-PEGs) in neuronal PC12 cells [314]. They found that SWCNTs elicited cytotoxicity in a concentration-dependent manner, and SWCNT-PEGs exhibited less cytotoxic potency than uncoated SWCNTs. Reactive oxygen species (ROS) were generated in both a concentration- and surface coating-dependent manner after exposure to these nanomaterials, indicating different oxidative stress mechanisms, and therefore suggesting that surface functionalization of SWCNTs decreases ROS-mediated toxicological response *in vitro*. In recent studies, Xue *et al.* demonstrated that

microglia secretion levels of TNF-alpha, IL-1beta and IL-6 were variably increased by SiO_2, TiO_2, hydroxiapatite (HAP) and Fe_3O_4 inorganic NP. They also observed that microglia-derived soluble factors induced by TiO_2-NP suppressed Th gene expression, and those induced by TiO_2-NP and HAP-NP caused dysfunction and cytotoxicity in PC12 cells [315].

In addition to PC12 cell lines, other primary culture cell lines have also been used to assess the neurotoxicity of NP. To examine the possible neurotoxicity of the photocytotoxic material TiO_2, Long *et al.* exposed brain cultures of immortalized mouse microglia (BV2), rat dopaminergic neurons (N27), and primary cultures of embryonic rat striatum to different concentrations (2.5 – 120 ppm) of the TiO_2. This compound did not produce cytotoxicity in N27 cell line after 72 h exposure. Primary cultures of rat striatum exposed to the nanomaterial showed a reduction of immunohistochemically stained neurons and microscopic evidence of neuronal apoptosis after 6 h exposure. Furthermore, BV2 microglia showed an immediate and prolonged release of ROS. Microarray analysis on these TiO_2-exposed BV2 microglia indicated up-regulation of inflammatory, apoptotic, and cell cycling pathways, and down-regulation of energy metabolism. These results indicate that TiO_2 is nontoxic to isolated N27 neurons, but stimulates BV2 microglia to produce ROS and damages neurons at low concentrations in cultures of brain striatum, probably through microglial generated ROS [316]. Similar results were found by Wang *et al.*, who observed that the proliferation rate of U87 glioma cell line was decreased when TiO_2 nanoparticles were combined with UVA irradiation. Results from their work suggested that TiO_2 induction of glioma cell apoptosis is associated with changes in the expression of genes encoding Bcl-2 family members [317]. Zinc oxide (ZnO) nanoparticles were also assessed for their neurotoxicity in mouse neural stem cells. Deng *et al.* found that ZnO nanoparticles induced cell apoptosis due to the dissolved Zn^{2+} in the culture medium or inside cells [308]. In another study, Locatelli *et al.* developed lipophilic Ag NP that were entrapped into PEG-based polymeric nanoparticles and

conjugated with the peptide chlorotoxin for the treatment of glioblastoma. Results from this study reveal that the uptake of Ag into the cells was improved up to 8.4 times with respect to the non-targeted NP. Furthermore, they also observed a greater cytotoxic effect on U87 glioma cell lines [318].

4.1.2 In vivo toxicity of nanomaterials

Cell cultures have been extensively employed to test the safety of nanomaterials [319]. However, these methods only explore some of the aspects of the biological system, whereas the *in vivo* machinery is far more complex with interdependent pathways that cannot be captured in a single *in vitro* experiment [320]. As a result, nanotoxicology is gaining a lot of interest in order to assess the unpredictable effects that these nanostructures might exert in biological systems. Even though we have described some of the *in vitro* studies that demonstrate adverse effects of NP on neuronal or glial cells, effects of NP on the CNS *in vivo* are still not well known.[321] Therefore, further *in vivo* studies are needed to provide vital information to assess the neurotoxic effects of NP [322, 323].

There are many biodegradable and biocompatible polymers which have been approved by FDA for clinical application. However, the brain targeting delivery of these polymer-based nanoparticles is still limited. Liu *et al.* evaluated the *in vivo* toxicity and immunogenicity of poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) NP conjugated to WGA after repeated intranasal administration [324]. These NP induced slight oxidative stress and excitotoxicity, a process by which nerve cells are damaged by excessive stimulation by neurotransmitters, as evidenced by increased glutamate levels in rat brain and enhanced LDH activity in the rat olfactory bulb.

NP made of noble metals have been also used in brain delivery for theranostic purposes [317], [325-327]. Neurotoxicity of silver in the brain has already been reported after systemic,

intracerebral and intranasal administration [321, 325, 328, 329]. In recent studies, Liu *et al.* studied the effects of the Ag-NP on hippocampal synaptic plasticity and spatial cognition in rats after two-week exposure to Ag-NP through nasal administration. They observed the formation of an elevated amount of ROS in the hippocampus, which might be the reason for the neural damage caused by silver nanoparticles [325]. Prasek *et al.* studied the neurotoxicity of Pt-NP as potential brain cancer treatment. After the administration of Pt-NP hydrocolloids at concentrations of 1 to 20 μg/ml to chicken embryos at the beginning of embryogenesis, they observed that there was no change in the number of cells in the brain cortex of the chicken embryo; however, analyses of brain tissue ultrastructure reported mitochondrial degradation [326].

Xu *et al.* recently observed that the i.v. administration of TiO_2 NP to mice could induce damage in the brain. More precisely, when mice were treated with a single dose of TiO_2 (1387 mg/kg BW) the brain tissue showed neuronal cell degeneration and vacuoles were observed in the hippocampus, which is indicative of fatty degeneration in the hippocampus [330].

To sum up, the above mentioned studies indicate that there are potentially harmful effects of nanomaterials to biological systems. Furthermore, the toxicity of these nanomaterials after their BBB crossing have not been fully studied. All these studies support the need for further research on the acute and long-term effects of nanomaterials both *in vitro* and *in vivo*, as their toxicity has been mostly studied in mice. More studies will determine if these results can be extrapolated to humans.

4.2 Fate of nanomaterials

The development of nanomaterials which specifically target the correct population of diseased cells sparing healthy ones is one of the most challenging tasks when aiming to treat disorders in the CNS, for example, to target toxic drugs at brain tumors.

With nanotechnology, intelligent drug delivery systems overcome the difficulty of these required tasks.

When NP are designed for clinical applications, we should bear in mind that they can undergo important modifications after their systemic administration. More precisely, the nonspecific interaction between the NP surface and proteins that circulate in the blood, that leads to the opsonization of their surface. This forms the "corona". The opsonization of the NP by these proteins significantly changes the original properties of the NP, determining their removal from the blood by the reticuloendothelial system (RES), mainly in liver and spleen. However, there are common approaches to escape RES and thus avoid premature clearance of the NP. Coating the NP surface with different hydrophilic surfactants, such as PEG and polysorbates, the formulation of NP with neutral surface charge, or the use of small sized nanoparticles (e.g., <80 nm) are some examples to achieve this goal [331]. NP that present these features, called "stealth" NP, circulate in the blood for a longer time , and their surface may be modified to cross the BBB [332].

The corona on the shell of NP determines not also their clearance but also their distribution in different compartments and their ability to cross from one to another or their successful uptake by cells [333]. The formation of the corona supports the idea that unmodified NP do not exist *in vivo*, because as soon as they are administered the adsorption of proteins present in the blood with more affinity for the particle surface will immediately modify them, thus forming a weak layer (soft corona) or a more or less tightly bound layer (hard corona) [334, 335]. The binding of different proteins to the NP shell not only influences their surface charge of the NP, but also modifies their total size and can hide functional groups. This means that the originally bound targeting groups for the crossing of the BBB may be covered. The stability of the corona attached to the surface of the NP is time-dependent because as long as the NP spend longer time travelling through the body, the protein shell will be exchanged as the particles will pass through different cell layers more often [336]. Furthermore, it has also

been observed that the corona may not play a role only in cellular uptake, but can also activate the complement and blood clotting processes, which might not be desired [337].

It is worth noting that among the *in vitro* studies performed to study how the NP translocate into the brain, there is no single one that studies the surface modification that NP undergo after their administration and how these modifications affect the crossing of the BBB. Moreover, once NP are crossing the BBB, the corona they show when they exit towards the brain might be different depending on the process that took place, namely, endocytosis, transcytosis and exocytosis, and, thus, produce additional either beneficial or toxic effects on neurons. As a result, there is much further research to be done in this field in order to understand the mechanisms underlying in this post-administration modification process.

4.3 Commercialization problems

Commercialization of highly innovative products has always represented a great challenge, particularly when it comes to high risk/high return products. In the case of nanomedicine, multiple barriers delay going on the market. So far, the process of NP-based therapeutics commercialization has been long and hard. The most important challenges and risks are summarized in Figure 4 and will be discussed below [2, 338-340].

Some of the problems that pharmaceutical companies are facing are associated with the NP manufacturing process. Issues to be solved are the lack of quality controls, the high manufacturing costs, scalability issues or problems related to the production rate enhancement among others [340]. Another important challenge is the insufficient evidence from *in vivo* studies, the relatively few clinical trials investigating NP that are currently under way and the few commercial products based on nanotechnology that are currently on the market [2, 338, 340]. Unfortunately, most of the results presented in this review, have been obtained in *in vitro* models and are still at the concept level. Therefore, the potential of many nanomedicines is yet to be determined. In addition, as mentioned earlier in this review, little is known about nanomaterial and nanoparticle safety. Scientists and regulators are struggling to characterize

these materials in an effort to create appropriate toxicological testing and assessment tools. Another obstacle to clinical translation is the FDA-approval process. Nanomedicines are the most heavily regulated consumer products throughout the pre-and post-marketing phases. The requirements set by the FDA for clinical trials with nanotherapeutics are extremely complex and demanding [338-340]. The approval procedure and regulations of medical products based on nanotechnology are different from those other industries using nanotechnology with no regulatory limitations. On top of that, reforms at the Patent and Trademark Office are needed to create a robust patent system that helps any commercialization effort and avoids confusion and delay [340]. Overlapping with patents can be also taken into consideration. Finally, attracting investment for nanomedicine research is also particularly challenging [338, 340]. Commercialization of nanomedicine is currently driven by small and medium-size companies and by startups. Universities are also pushing for funding to adapt basic nanomedical research into real products. One the other hand, big pharmaceutical companies are very cautious about making large investments in nanotherapeutics because positive returns occur only in the long term. They are also concerned about whether the FDA will be even stricter in regulating nanomedicines in the future.

In spite of all these obstacles to the growth of nanotechonology for medical applications, investment in nanomedicine is expected to increase. Doxil or Ambrasane success among others have made the risk/reward ratio more appealing and have impacted the healthcare system. Moreover, since nanoformulations of older therapeutics may be patentable, nanomedicine is expected to prolong the economic life of proprietary drugs creating more revenues. It is also estimated that novel or reformulated nanotherapeutics will disrupt the generic drug market as well [338, 340]. All of these have generated great expectations in big pharmaceutical companies. In summary, there are many problems that need to be overcome, but this is an area that still shows enormous potential.

5 CONCLUSIONS AND FUTURE DIRECTIONS

Nanotechnological application to medical problems aims to increase expectations for the delivery of drugs to diagnose and treat brain-related diseases like neurodegeneration and cerebral tumors. These diseases are nowadays a major medical challenge and they are becoming more prevalent in society as the population become older. Remarkably, as this paper has shown, nanotechnology has proven to make possible the transport of many drugs across the BBB in various models of neurodegenerative disorders and brain tumors, one of the current obstacles faced by conventional therapeutics. In addition, nanotechnology has demonstrated potential to enhance the sensitivity of current diagnostic tools and to be more effective than conventional therapies with fewer side effects. Although promising, these findings should be considered preliminary since few nanomedicine candidates have reached clinical practice and its potential is yet to be determined.

At the moment the mechanism of drug transfer into the brain mediated by NP appears to be characterized. However, the further fate of NP in the brain and how to target specific neuron populations still requires much more basic research. On the other hand, the amount of drug that enters the brain remains low (1-2% approximately). Although this is significant enough to exert a beneficial effect, the remaining question is how to maximize the amount of drug that reaches the brain in order to avoid NP accumulation in other organs. Moreover, even though nanotechnology is rapidly advancing rigorous safety studies to ensure public acceptance of nanotechnology are needed.

Finally, nanotechnology must overcome difficulties related to its commercialization process since this area is still in its infancy. Attracting investment by big pharmaceutical companies for nanomedicine research is also particularly challenging due to the risk associated to the commercialization of this highly innovative products. In this sense, cooperation between

doctors, patients, researchers, technologists, economists, investors, healthcare providers, , in order to reduce the high risk associated to investments in nano-based drugs with the final goal of providing many benefits for patients is crucial and must be facilitated.

Hopefully, nanomedicine will eventually bring hope for better diagnosis and management of brain disorders making therapies far more effective.

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