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# Nano particle transport across the Blood Brain Barrier

Andreas M Grabrucker<sup>1</sup>, Barbara Ruozi<sup>2</sup>, Daniela Belletti<sup>2</sup>, Francesca Pederzoli<sup>2</sup>, Flavio Forni<sup>2</sup>, Maria Angela Vandelli<sup>2</sup>, and Giovanni Tosi<sup>2</sup>

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**Keywords:** BBB, drug delivery, drug targeting, liposome, nanoparticle, nanocarrier

**Abbreviations:** BBB, Blood brain barrier; BCB, Blood CSF barrier; CBB, CSF-brain barrier; CNS, Central Nervous System; CSF, Cerebrospinal fluid; GLUT1, Glucose transporter-1; LAT1, Large neutral amino-acid transporter type 1; NP, Nanoparticle; P-gp, P-glycoprotein; Tf, Transferrin; TJ, Tight junction

While the role of the blood-brain barrier (BBB) is increasingly recognized in the (development of treatments targeting neurodegenerative disorders, to date, few strategies exist that enable drug delivery of non-BBB crossing molecules directly to their site of action, the brain. However, the recent advent of Nanomedicines may provide a potent tool to implement CNS targeted delivery of active compounds. Approaches for BBB crossing are deeply investigated in relation to the pathology: among the main important diseases of the CNS, this review focuses on the application of nanomedicines to neurodegenerative disorders (Alzheimer, Parkinson and Huntington's Disease) and to other brain pathologies as epilepsy, infectious diseases, multiple sclerosis, lysosomal storage disorders, strokes.

neurotoxic substances. Together, the BBB creates the neural micro-environment that is crucial for healthy brain function<sup>2</sup>.

To fulfill this function, the brain has a very close-packed microvasculature.<sup>74,76</sup> This enables very short distances between the nearest blood capillary and neuronal cells,<sup>99</sup> which allows a fast delivery of substances after crossing the BBB. Further, the capillaries in the brain are quite different from capillaries found in the peripheral organs. Microvessels of the BBB are formed by non-fenestrated endothelial cells that contact each other forming tight junctions (TJs) (or zonula occludens). TJs prevent the passive diffusion of hydrophilic solutes via paracellular transport, the transfer of substances from the blood into brain parenchyma by passing through the intercellular space between the cells. Thus, water-soluble substances have to cross the BBB by mechanisms of transcellular transport that involve active transport via specific carriers.

## Introduction

### The blood brain barrier (BBB)

The central nervous system (CNS) is protected from toxins and metabolic fluctuations by barriers, among which the blood-brain barrier (BBB) plays a key role in maintaining homeostasis. The BBB is composed of four major cellular components, the endothelial cells of the brain capillaries, pericytes, astrocytes, and the basement membrane. Together, they build a functional unit, the 'Neurovascular unit', mediating exchange between neurons, capillaries and glia. That way, the BBB supplies the brain with necessary nutrients, glucose and oxygen to sustain normal neural functioning. At the same time, the BBB plays a protective role against

In addition, brain capillaries are surrounded by pericytes protruding into the basement membrane, and astrocytic end feet encapsulating the surface (Grabrucker et al., 2014). Astrocytes release inductive signals that regulate the BBB<sup>43</sup> and play a role in maintaining water, ionic, and amino acid homeostasis within the CNS.<sup>1</sup> With an electrical resistance estimated to be  $\approx 1500\text{--}2000 \Omega\text{cm}^2$ , brain endothelium possesses a much higher electrical resistance than other systemic endothelia with  $\approx 3\text{--}33 \Omega\text{cm}^2$ <sup>17</sup> further limiting diffusion of certain molecules across the BBB. However, the BBB is a highly dynamic structure and its formation, maturity and integrity controlled by a collective action of all of its constituents. In addition, neurons have been shown to release growth or nutritional factors maintaining BBB functioning.

The consequence of this intricate interplay is a controlled and restricted transport of molecules across the BBB. Only very small (<400 Da) and lipophilic molecules can diffuse through the BBB, while the crossing of lipid insoluble or larger hydrophilic molecules is very limited. However, additionally to lipophilicity, other factors such as the affinity toward available transport system further control BBB crossing. For example, the transport of glucose through the BBB is mediated by glucose transporter-1 (GLUT-1).<sup>60</sup>

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The effectiveness of the BBB function depends on several factors such as the controlled expression of the major constituents of TJs, such as occluding proteins and proteins of the claudin family.<sup>2</sup> Further, molecules released during neural activity may act on receptors and transporters of endothelial cells and/or contacting astrocytes. Examples include neurotransmitters such as glutamate and serotonin, but also calcium, and adrenaline.<sup>75</sup> Through these modulatory agents, several parameters of the BBB such as capillary diameter (and thus local blood flow), expression of specific transporters, and the tightness of TJs can be regulated (Pepiatt et al., 2006;<sup>14,27,47</sup>; Zlokovic, 2008). Therefore, disrupted signaling between endothelial cells, pericytes and/or astrocytes may affect the integrity of the BBB.<sup>9,13,28</sup>

In addition to regulating exchange of molecules between the peripheral blood circulation and the brain by its anatomical properties, the BBB is a metabolic barrier. A variety of degrading and metabolizing enzymes can be found at the membrane of brain capillary endothelial cells<sup>6,32</sup> along with a large number of transporter proteins that strictly regulate the influx and efflux of substances.

Taken together, these unique features of the BBB control the homeostasis of a large variety of molecules in the brain. However, it is the same features that impose a major obstacle to successful delivery of drugs into the brain. For example, various phase I and phase II enzymes of the drug metabolism system have been found in endothelial cells. The major drug efflux transporters include P-glycoprotein (P-gp),<sup>24</sup> ATP binding cassette (ABC), multidrug resistance-associated proteins (MRPs) and Breast Cancer resistance proteins (BCRP). Although in some conditions such as trauma, multiple sclerosis and Alzheimer Disease, the anatomical and functional state of the BBB may be altered, the delivery of active compounds into the CNS needs effective mechanisms to facilitate BBB crossing.

Although other barriers such as the blood-cerebrospinal fluid (CSF) barrier (BCB) and CSF-brain barrier (CBB) exist, the surface area of the BBB is far greater than the surface area of the BCB. Thus, the BBB is considered the main site for crossing of endogenous substances into the CNS. In addition, even despite the entrance of a drug into the CSF, it does not necessarily result in its penetration into the brain parenchyma. The penetration of substances from CSF to brain parenchyma is facilitated through diffusion. However, the large distances create a diffusion barrier that can be referred to as the CSF-brain barrier (CBB). In addition to the aforementioned external barriers, after crossing the BBB, the presence of further internal barriers creates another obstacle for effective drug delivery, such as fast clearing mechanisms by glial cells.

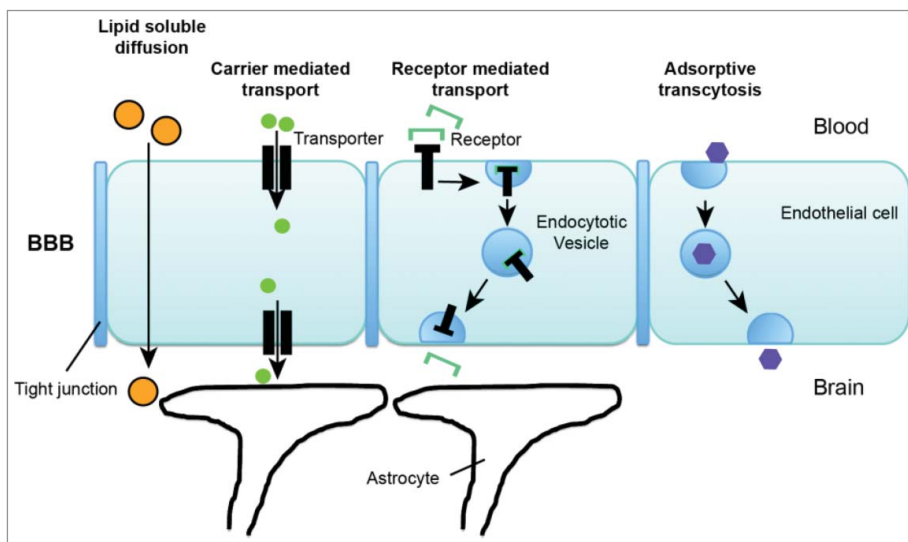
### Mechanism of BBB crossing by endogenous substances

Despite the restricted transport of molecules across the BBB, the brain has high demand for energy and nutrients. Therefore, multiple mechanisms exist that mediate the uptake of specific endogenous substances. Some of these provide a useful basis also for the development of strategies for drug delivery. In general, under physiological conditions, substances may cross the BBB by passive diffusion, carrier mediated transport, receptor mediated transport, and adsorptive transcytosis (Fig. 1).

The possibility for substances to passively cross the BBB by diffusion is very limited (see above) due to the BBB physiology. The exception are lipid soluble small molecules with an molecular weight of less than 400 Da. Uptake of such substances is possible by lipid-mediated free diffusion across the BBB. In contrast to diffusion through water, diffusion through lipid membranes is highly dependent on the correlating features of molecular volume, molecular weight, and surface area of a substance.<sup>84</sup>

High lipid solubility in turn is correlated to low hydrogen bonding. It has been shown that the ability of a substance to permeate membranes is decreased by 10-fold with each pair of hydrogen bonds.<sup>112</sup> This rule has been confirmed in multiple studies i.e. investigating BBB transport of steroid hormones and oligopeptides.<sup>84</sup>

Therefore, the majority of substances is taken up into the brain by carrier mediated transport and receptor mediated transport. Here, the structure of the molecule possesses high affinity for specific carriers or receptor found at the BBB. For example, water-soluble metabolic substrates such as glucose, are bound by specific transporters, here the glucose transporter type 1 (GLUT1) that, however, has also affinity for other hexoses, including mannose and galactose.<sup>79</sup> Another example is the large neutral amino-acid transporter type 1 (LAT1), which is a carrier for



**Figure 1. Transport pathways across blood brain barrier.** Under physiological conditions, substances may cross the BBB by passive diffusion, carrier mediated transport, receptor mediated transport, and adsorptive transcytosis.

165 phenylalanine, but also transports over 10 other large neutral  
amino acids and, although with lower affinity, small neutral  
amino acids.<sup>82</sup> To date, many specific transporters have been  
identified. Their exact cellular location at the brain microvascula-  
170 ture and expression level, as well as their selectivity largely deter-  
mine the kinetics and turnover of the transported substances.

For example, the supply of Glucose for the brain is realized  
only by GLUT1. GLUT1 is enriched at the brain microvascula-  
ture only at the BBB on both luminal and abluminal membranes.  
This expression patterns enables not only uptake of Glucose from  
175 the blood into epithelial cells but indeed transport across the cells  
of the BBB. Due to the high energy - demand of the brain, the  
abundance of GLUT1 at the microvasculature is high, relative to  
other transporters, which enables rates of glycolysis in the brain  
that are over 100-fold greater than rates of amino-acid incorpo-  
180 ration into brain proteins.<sup>83</sup> However, due to the demand of car-  
riers, the process of carrier mediated transport of a substance  
across the BBB is saturable.

Carrier mediated transport is not only responsible for uptake  
of substances into the brain, but a process that regulates homeo-  
185 stasis in bi-directional manner. To that end, active efflux trans-  
porters can be found at the BBB that mediate the asymmetric  
efflux of metabolites from the brain back to the blood circulation.  
It is also these transporters that impose an obstacle to drug deliv-  
ery into the brain. The classical active efflux transporter system  
190 within the BBB is P-glycoprotein (P-gp). This protein is encoded  
by the multidrug resistance gene 1 (MDR1) and its functional  
complex is not only modified by internal factors but also environ-  
mental clues such as the presence of toxins.<sup>30</sup>

Receptor mediated transport processes additionally occur at  
195 the BBB as mechanism regulating the crossing of endogenous  
substances. These peptide receptors may mediate processes such  
as transcytosis of ligands from blood to brain or from brain to  
blood, or only uptake into the brain capillary endothelium with-  
out further export into the brain parenchyma. Well investigated  
200 examples include the transport of insulin or transferrin. Insulin  
is transported across the BBB by binding to an endogenous BBB  
insulin receptor.<sup>81</sup> Similarly, for transferrin (Tf) a specific re-  
ceptor, the Tf receptor (TfR) can be found at the BBB.<sup>80</sup> Similar  
to carrier mediated transport, the cellular location and expression  
205 levels of the receptors determine the kinetics and turnover of the  
transported substances. For example, the TfR mediates influx of  
holo-transferrin from blood to brain but also reverse transcytosis  
of apotransferrin from brain to blood due to expression on the  
luminal and abluminal membrane of the capillary  
210 endothelium.<sup>108,130</sup>

Finally, a mechanism of BBB crossing called adsorptive endo-  
cytosis has been described. This process requires an interaction  
between a ligand, such as a macromolecule or protein in the  
bloodstream and the cell surface. This interaction may be trig-  
215 gered by an electrostatic interaction between the positively  
charged ligand and negatively charged plasma membrane. Ulti-  
mately, this may result in adsorptive transcytosis of the ligand,  
which is mediated by clathrin-dependent endocytosis in most  
cases. This mode of BBB crossing seems to occur only unidirec-  
220 tional, from blood to brain. Adsorptive transcytosis has been

described for some compounds and Nanoparticles (NPs). How-  
ever, whether adsorptive transcytosis contributes significantly to  
the transport of endogenous substances across the BBB is still  
under investigation.<sup>119</sup>

Several of the mechanisms involved in BBB crossing of endog- 225  
enous substances can be used for the development of strategies for  
drug delivery. For example, delivery of drugs that have structures  
that mimic the endogenous substrate of transporters at the BBB  
may be facilitated. The vast majority of carrier mediated trans-  
port systems has been identified and transporters can be expressed 230  
in *in vitro* systems to screen for drugs that have an acceptable  
affinity for a given BBB transporter. A drug that shows such an  
affinity for a carrier mediated transport system is L-DOPA. L-  
DOPA is used in the treatment of Parkinson Disease and is a  
dopamine precursor that is transported across the BBB via the 235  
LAT1 carrier.<sup>55</sup> Alternatively, drugs might be conjugated to sub-  
stances that normally cross the BBB by carrier mediated pro-  
cesses. Further, the presence of endogenous receptors with  
specific ligands triggering BBB crossing mechanisms, such as the  
insulin receptor or the TfR, enables the engineering of peptides 240  
that attached to compounds or drugs mimic these ligands.

However, after successful crossing of the BBB, most drugs are  
taken up by cells. A major pathway mediating this uptake is cla-  
thrin-dependent endocytosis. This mode of uptake leads to the  
eventual delivery of substances into the endo-lysosomal compart- 245  
ments. Due to the low pH and the presence of degrading  
enzymes in lysosomal compartments, delivered biomolecule may  
be degraded creating a major obstacle in the delivery of active  
compounds, in particular of therapeutic proteins.

Thus, taken together, there is an urgent need to develop new 250  
strategies for the effective delivery of substances across the BBB.  
Therapeutic compounds need to be protected from degrading,  
metabolizing and inhibiting processes within the blood stream,  
need to be taken up into the CNS via the BBB with high effi- 255  
ciency, and need to be targeted to specific sites of action prevent-  
ing lysosomal degradation. Here, Nanomedicines provide major  
advantages compared to common drug delivery systems

## Nanomedicines

In this review, we would like to summarize the most interest- 260  
ing applications of the last 20 y of nanoparticles and liposomes  
in *in vivo* Brain experiments. Considering the variability of *in*  
*in vivo* models, we skip to review the “proof-of-concept,” but  
mainly focusing on “*In vivo* disease models.” The “*In vivo* disease  
models” describes the application of nanoparticles and liposomes  
265 in brain pathology, pointing the critical attention on the animal  
models of pathology and on the possible mechanism involvement  
in the treatments. This section is divided into two main under-  
chapter: “Neurodegenerative Diseases” and “Other Brain Patho-  
logies.” In “Neurodegenerative pathologies” section, we include  
270 Alzheimer, Parkinson and Multiple Sclerosis diseases. Finally, in  
“Other Brain Pathologies” section we include some of the most  
relevant papers on convulsive models, stroke, brain infectious dis-  
eases and prion diseases.

275 Firstly, we have to stress the difficulty to identify a “standard”  
in *in vivo* model for each disease, thus the possible meta-analysis of  
the data, which could help the neuroscientist in the basic  
research, is virtually impossible. In particular, to our best knowl-  
edge, in almost all neuro-pathologies conditions (such as Parkin-  
son disease, HIV-dementia and some infectious brain diseases),  
280 the BBB permeability is seriously compromised, while in other  
brain diseases (stroke and brain tumors, as examples), the BBB  
integrity appears to be seriously damaged. As example, we must  
consider that the use of brain tumors (namely, gliomas) *in vivo*  
application must be thought as “glioma” delivery and not as  
285 brain targeting or BBB crossing exploitation, since over the pro-  
gression of the pathology, it has been demonstrated that BBB is  
losing its integrity.<sup>10</sup>

Secondly, since this field of science (namely,  
“nanoneuroscience” or “neuro-nanomedicine”) is relative new  
290 and young, the most of papers are based on “Proof-of-Concept,”  
while the application on brain diseases models appears in con-  
stant increase, but still lower than the basic research. This evi-  
dence could be explained by the fact that a unique carrier for  
brain delivery does not exist, since it possible to take advantage of  
295 different ligands, receptors and mechanisms for BBB crossing  
and brain targeting. Then, the use of different starting polymer  
(poly-lactide-co-glycolide, poly-buthyl/heaxil-cyano-acrylate,  
albumin, chitosan, and many others) for nanoparticle prepara-  
tion or lipid composition (cationic/anionic/neutral, pegylated  
300 lipids and many others) for liposome preparation, results in dif-  
ferent brain targeting mechanisms, different biodistribution pro-  
files, different brain delivery efficacies and overall toxicities.

Notwithstanding all of these differences in animal models and  
carriers, with this review, we aimed to produce a useful tool for  
305 all the neuroscientist to access a plenary of *in vivo* evidences.

### Nanoparticles and liposomes

Generally talking, polymeric nanoparticles (NPs) are solid col-  
loid matrix-like particles sizing from 1 to 1000 nm, made of pol-  
ymers of different nature, and encapsulating molecules during  
310 the preparation process, chosen depending on both the polymers  
and the drug to be delivered. There are several natural or syn-  
thetic polymers often mixed during the preparation of NPs, but  
considering the stringent request of FDA guidelines in terms of  
biodegradability and biocompatibility for *in vivo* administration,  
315 the list of polymers, that could be used to prepare NPs systems,  
results very short with few polymers available for drug delivery  
system preparation and approved for systemic administration.  
Among them, natural (e.g. Albumin) and poly-ester (poly-lactide  
and derivatives) polymers are the most accepted. In particular, it  
320 is a common opinion that the toxicology of a nanodevice should  
be related both to morphological and structural parameters of  
the systems (size, surface charge, shape) and the polymers used.

Liposome (LPs) are surely the most studied and old *smart* sys-  
tems, applied in different fields of medicine research, represent-  
325 ing the first innovative approach for the treatment of challenging  
pathologies, like cancer, HIV, strokes and many other diseases  
which require selective therapies.<sup>42,68</sup> As described for NPs, LPs  
need a surface modification in order to promote specific delivery

and targeting of the embedded drugs. Generally speaking, good  
in vitro results on LPs experimentations do not reflect in vivo 330  
confirmations, leading to a very low applicability of the lipo-  
some-approach in the treatment protocols. This event, connected  
with a very quick elimination and degradation when LPs are  
injected in the bloodstream, along with the type and properties  
of phospholipids which are constituents of the liposomes, was 335  
faced with different strategies such as using stabilizers or modifi-  
cation of preparation procedures. Particularly, the conjugation  
with molecules, able to protect the LPs from the degradation  
(GM1, poloamine, polaxamer and PEG) or able to induce an  
increased affinity to the diseased cells, represented one of the 340  
most applied strategies. The pegylation technology offers a  
potential resolution of the problem of liposome’s bio-stability,  
connected to the liposomal intravenous administration.<sup>50,91</sup>  
Finally, in order to increase the minimal affinity to the diseased 345  
cells and to avoid the toxic effect on non diseased tissues, an  
active targeting due to functionalization of the surface of the lipo-  
some is hardly needed.<sup>128</sup>

### In vivo disease models

If in the previous examples the integrity of the BBB was main-  
tained and the brain was considered as a “non-pathologic” brain, 350  
in the following examples, several researches have been reviewed  
concerning neuro-pathologies in which the BBB integrity and  
permeability is seriously compromised.<sup>12,51,103,135</sup> These pathol-  
ogies could vary from neurodegenerative diseases (Parkinson,  
Alzheimer and Huntigton’s) to epilepsy, infectious brain diseases, 355  
strokes and many others.

### Neurodegenerative disease

#### Alzheimer disease

Alzheimer disease (AD) is a chronic and progressive neurode-  
generative disorder that begins with cognitive and memory 360  
impairments, accompanied with behavioral disturbances such as  
aggression, depression, hallucination, delusion, anger and agita-  
tion and eventually progresses to dementia, physical impairment  
and death.<sup>26</sup> AD is the most common form of dementia, and the  
greatest risk factor for AD is age. It is estimated that 10% of people 365  
over the age of 65 are affected by AD<sup>4</sup> and by 2050, the  
number of people with AD in the US will increase threefold.<sup>45</sup>  
The definite diagnosis of AD is made upon histological verifica-  
tion, established by biopsy or at autopsy, of two main hallmarks:  
370 extracellular amyloid plaques and intracellular neurofibrillary  
tangles, which enclose hyperphosphorylated tau protein.<sup>26,102</sup>  
Furthermore, the neuropathology of AD is characterized by a  
progressive loss of synapses and neurons in a region- and cell-  
type-specific manner.<sup>95</sup> In Alzheimer disease, microglia and 375  
astrocytes are activated by  $\beta$ -amyloid protein and related oligo-  
peptides, leading to a cascade of events producing toxic mole-  
cules, neuronal damage, synaptic dysfunction and alteration of  
the permeability of the BBB.<sup>10</sup>

To study this pathology, a model of acute amnesia is induced in rats or mice by subcutaneous injection of scopolamine.<sup>86</sup> These animals are submitted to the passive avoidance reflex (PAR) test<sup>118</sup> to determine the memory changes; the PAR is based on the measurement of the retention latency times, necessities for the animals to avoid a painful stimulus; these latencies are directly proportional to the decrease of amnesia, thus representing a useful tool to describe the effectiveness of anti-amnesia drug injected. Kurakhamaeva and colleagues<sup>59</sup> used Nerve Growth Factor (NGF) loaded in PBCA NPs covered with PS-80 to reduce amnesic effect. With this study, they demonstrated that brain-targeted NPs loaded with NGF allowed an improvement of amnesia with a significant increase of the latent period of 2-fold, if compared to animals treated with NGF-free solution. In another paper, Abdel-Wahab<sup>3</sup> demonstrated the efficacy of 2 different systems: PBCA Np covered with polysorbate-80 and PBCA Np covered with PEG5000, both loaded with tacrine (reversible inhibitor of acetylcholinesterase) able to improve cognitive function. These NPs formulations increased the latency period in treated animals of 1,2-fold and 1,4-fold, respectively, compared to the injection of tacrine-free solution (Table 1).

In our opinion, the greater effect obtained with drug loaded PBCA NPs covered with PEG5000 as compared to the drug-free solution and to the drug loaded PBCA-NPs covered with Polisorbate-80 could be due to the alteration of the BBB permeability rather than a specific targeting; in fact, the presence of PEG onto the NPs surface confers stealth properties to the NPs, allowing them to remain over a longer time in the systemic circulation, thus improving the chances to reach the pathological site.

Moreover, the role of surfactants (polysorbate 80 as well as polaxamers or PEG) as possible pathways for BBB permeability increase was deeply debated by several authors, without any effective and clear result.<sup>7,87</sup> The only evidence was achieved by 2-D electrophoresis (2-D PAGE) showing that PBCA NPs coated with polaxamer 188 (Pluronic F68<sup>®</sup>) and Ps80 showed a considerable adsorption of apolipoprotein A-I (ApoA-I), leading to a possible interaction with BBB receptors. This finding could induce to propose a general pathway for BBB crossing of PBCA coated Ps80 (or Pluronic), based on a great absorption of APO A-I allowing a receptor-mediated transcytosis. This hypothesis cannot be generalized, since it should be taken into consideration the close interaction between polymer and surfactants as well as the whole NPs interaction with the BBB.

#### *Parkinson disease*

Parkinson disease (PD) is a multicentric neurodegenerative disease. Estimates state that 1% of people over age 60 are affected by PD, the leading cause of the movement disorder called Parkinsonism.<sup>4</sup> Clinical features of PD include tremor at rest, bradykinesia, rigidity and flexed posture.<sup>48</sup>

Pathologically, the key deficit in PD has been considered to be the loss of dopaminergic neurons in the substantia nigra, which causes consequent reduction of dopamine level.<sup>98</sup> Cell loss and Lewy body formation (abnormal intraneuronal aggregates of protein, predominantly  $\alpha$ -synuclein) occur in the locus coeruleus, dorsal motor nucleus, and substantia innominata.<sup>15</sup>

Consequently, nor-adrenergic, serotonergic and cholinergic neurons are also lost and this widespread neurodegeneration leads to the emergence of a variety of features known as “non-motor” symptoms in PD that include cognitive decline, apathy, depression, anxiety disorders, hallucinations, gait and balance disturbances, sleep disorders, sexual dysfunction, bowel problems, drenching sweats and pain.<sup>46</sup> At present, for this pathology no significant evidences of BBB permeability alteration are described.

As there is currently no permanent rescue and cure for Parkinson disease, and there are no known agents to slow the progression of PD, the current treatments are used to decrease the symptoms of PD.<sup>64,122</sup> Although symptomatic therapy of PD is effective (Levo-Dopa), novel therapeutics are required as well as advanced delivery methods, as the active substances will need to enter the CNS to exploit their effects (Table 1).

Animal PD model is obtained through injection of neurotoxins: the most used toxin is 6-hydroxydopamine (6-OHDA stereotaxical injected),<sup>16</sup> but also 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP i.p. injected) is used<sup>18</sup>: both agents destroy neurons by generating active oxygen species such as superoxide radicals. Kurakhamaeva and colleagues<sup>59</sup> used mice treated with MPTP to test the efficacy of NGF entrapped in PBCA NPs covered with polysorbate-80. This mice were submitted to many tests to evaluate the improvement in Parkinson symptoms: i) open field test, that allows to check the orientation-research reaction; ii) locomotor activity in the actometer, to value the quality and the quantity of movement; iii) rigidity, through the registration of the alteration of gain dynamic; iv) tremor, through the registration of intensity and duration of tremor. With all these tests, the researchers observed that the administration of targeted NPs allowed a significant improvement in PD symptoms already after 90 minute and maintained for 21 day.

The research group of Pardridge performed several studies using rodents 6-OHDA treated with apomorphine or amphetamine to induce rotation: the reduction in the number of rotations, promoted by administration of anti-parkinson drugs, demonstrated a improvement in PD induced syndrome. In fact, Xia and co-worker<sup>126</sup> prepared a LPs formulation loaded with GDNF plasmid (Glial-derived neurotrophic factor) and targeted to the brain with OX-26 (mAb against the mouse TfR). The single administration of that system to 6-OHDA rat treated with apomorphine, showed a general improvement of PD symptoms, as they observed a decrease in rotational behavior of 58% and a great increase of Tyrosine Hydroxylase (TH) activity as compared with saline control. In another paper, Zhang and colleagues improved the results obtained by Xia, using a multiple injection of the LPs,<sup>132</sup> achieving a decrease in the rotational behavior until 90% and an increase in the TH activity until 77%, compared with saline control. Besides, Zhang performed another test, namely the Vibrissae-Elicited Forelimb Placing Test, aiming to evaluate the forelimb response to ipsilateral facial whisker stimulation<sup>8</sup> This test showed an improvement of response up to 4-fold, if compared to saline control, thus allowing the authors to corroborate the results obtained before. In another article,<sup>131</sup> Zhang showed the results obtained with the same kind of

**Table 1.** Neurodegenerative diseases models and Nanotech applications.

<b>PATHOLOGY</b>	<b>TARGETING (BBB/tumor)</b>	<b>APPROACH</b>	<b>DRUG</b>	<b>MODEL</b>	<b>TEST</b>	<b>RESULTS</b>	<b>REF</b>
Alzheimer	LDL Receptor	PBCA-NPs-Ps80	NGF	Mice treated with scopolamine. <sup>86</sup>	PAR test. <sup>118</sup>	Increase of retention latency (2,12-fold vs drug free)	Kurakhmaeva et al., 2009
	LDL Receptor	PBCA-NPs-Ps80	Tacrine	Rats injected with scopolamine	PAR test and HPLC biodistribution	Increase of retention latency (1,20-fold vs drug free; increase brain delivery (1,43 fold vs drug free)	Abdel-Wahab et al 2009
	/	PBCA-NPs-PEG500	Tacrine	Rats injected with scopolamine	PAR test and HPLC biodistribution	Increase of retention latency (1,37 fold vs drug free; increase brain delivery (1,75 fold vs drug free)	
Parkinson	LDL Receptor	PBCA-NPs-Ps80	NGF	C57Bl/6 mice treated with MPTP. <sup>18</sup>	Open field test (Schmidt et al., 2001); actophotometer test; rigidity test (Amende et al., 2005); tremor test. Rotarod test and actophotometer test	Significant reduction of Parkinson symptoms; reduction of rigidity; improvement of locomotor activity	Kurakhmaeva et al., 2009
	/	LPs (CHOL:LEC: Span 80:Tween 20 1:9:2:1)	Dopamine	Rats treated with Haloperidol		Increase of muscular coordination activity (3,5-fold); increase of locomotor activity (3,4-fold)	<sup>88</sup>
	Transferrin Receptor	LPs (POPC:DDAB: DSPE-PEG 47:1:2)-OX26	877 cDna (TH gene)	Rats treated with 6-OHDA (RMFB) (16) injected with apomorphine to induce rotation	Count of contralateral rotation	After 3 day post i.v. decrease of rotational behavior (> 70%); decrease of TH enzyme activity during time (after 9 day is like control); TH enzyme activity is $\alpha$ to injected dose.	Zhang et al., 2003
	/	LPs (PC:Chol: SA 6:3:1)	Dopamine HCl	Chlorpromazine-induced catatonia in rats	Reduction in degree of catatonia	83% of animal obtain complete reduction of catatonia until 150 minute after injection. (0% with dopamine solution)	<sup>53</sup>
	Transferrin Receptor	LPs (POPC:DDAB: DSPE-PEG 47:1:2)-OX26	GDNF plasmid DNA	Rats treated with 6-OHDA (RMFB) then injected with apomorphine or amphetamine to induce rotation	Count of contralateral and ipsilateral rotation; Vibrissae-Elicited Forelimb Placing Test ( <sup>6</sup> )	Decrease of rotational behavior (> 87-90%); increase of TH enzyme activity (>77 %); improvement of response to Vibrissae test (4-fold vs saline control)	Zhang et al., 2008
	Transferrin Receptor	LPs (POPC:DDAB: DSPE-PEG 47:1:2)-OX26	GDNF plasmid DNA	Rats treated with 6-OHDA (RMFB) then injected with apomorphine	Count of contralateral rotation induced by apoM	Decrease of rotational behavior after 4 week post i.v. (>58 %); increase of TH enzyme activity (> 19 fold vs saline control)	<sup>126</sup>



Transferrin Receptor	LPs (POPC:DDAB:DSPE-PEG 47:1:2)-OX26	877 cDna (TH gene) (10 ug/rat) PrgTH3 (TH plasmid) (10 ug/rat)	Rats treated with 6-OHDA (RMFB) then injected with apomorphine.	Count of contralateral rotation induced by apoM	127
Huntington's Disease	BBB-crossing glycopeptides PLGA	877 cDna (TH gene) (10 ug/rat) and PrgTH3 (TH plasmid) (10 ug/rat)	KO HD mice	Pharmacology Behavior Pathological evidences Confocal Microscopy	116
Multiple Sclerosis	PEG-LPs (DPPC: DSPE-PEG <sub>2000</sub> : CHOL 1.85:0.15:1)	Prednisolone	Rats with EAE induced injection of freshly activated, MBP-specific T cells (Gold et al., 1995)	Immunohistochemistry	<sup>100</sup> , Schmidt et al., 2003b
	PEG-LPs (EPC:CHOL: DSPE- PEG <sub>2000</sub> 54:41:5)	TMN	Mice with acute EAE (Pollak et al 2003) and chronic EAE (Offen et al 2000)	PCR-Real time and expression of HPRT mRNA (proinflammatory cytokines)	56

LDL, low-density lipoprotein; NP-s, nanoparticles; PBCA, poly(butylcyanoacrylate); Ps80, polysorbate80; NGF, nerve growth factor; PAR, passive avoidance reflex test; PEG, polyethylene glycol; HPLC, high performance liquid chromatography; LPs, liposomes; CHOL, cholesterol; LEC, lecithine; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; DDAB, dimethyldioctadecylammonium bromide; DSPE-PEG, distearyl phosphatidylethanolamine polyethylene glycol; OX26, murine monoclonal antibody against the mouse transferring receptor; TH: tyrosine hydroxylase; 6-OHDA, 6-hydroxydopamine; RMFB, right medial forebrain bundle; PC, phosphatidylcholine; SA, stearylamine; GDNF, glial-derived neurotrophic factor; apoM, apomorphine. DPPC, dipalmitoyl phosphatidylcholine; EAE, experimental autoimmune encephalomyelitis; CNS, central nervous system; EPC, egg phosphatidylcholine; TMN, tempamine. PCR, polymerase chain reaction.

490 targeted LPs loaded with 877 cDNA (a cDNA of TH gene). It was seen that the LPs, administered at a different dose of loaded drugs, allowed a decrease in rotational behavior of 70% until 3 day post injection, but after 6 d the TH enzyme activity decreased and after 9 d it was comparable to the control. Xia and colleagues, in a following article,<sup>127</sup> demonstrated that the incorporation of a TH plasmid in the same targeted systems can allow a prolongation of the neuroprotective effect but, unfortunately, without showing the effect 3 d after the injection.

Haloperidol and chlorpromazine were also used to induce extra pyramidal effects in rat or mice. In particular, Pichandy and colleagues<sup>88</sup> treated rats with haloperidol to test the anti-PD effect of Dopamine loaded in LPs formulated with the use of span 80 and tween 80. Both formulations allowed an increase in muscular coordination activity, tested with the rotarod test, along with in locomotor activity, tested with actophotometer. On the contrary, Jain and coworkers,<sup>53</sup> took advantage of rats, injected with chlorpromazine (inducing catatonia) to test non targeted LPs loaded with dopamine, showing a complete reduction of catatonia state in the 83% of animals treated 150 min after injection.

#### *Huntington's disease*

Huntington's disease (HD) is a genetic neurological disorder caused by a CAG expansion in the gene encoding the huntingtin (HTT) protein. HD is featured by motor, cognitive and psychiatric disturbances, neuronal dysfunction, atrophy of the striatum and other brain regions, and progressive loss of striatal medium-sized spiny neurons (MSNs) and of cortical pyramidal neurons (Vonsattel et al 1998). Several molecular and cellular dysfunctions have been identified (Zuccato et al 2010) and one affected pathway implicates brain cholesterol. Brain cholesterol biosynthesis and cholesterol level are reduced in Huntington's disease (HD) mice suggesting that locally synthesized, newly formed cholesterol is less available to neurons. This might be detrimental for neuronal function, especially given that locally synthesized cholesterol is implicated in synapse integrity and remodeling. Biodegradable and biocompatible polymeric nanoparticles (NPs) modified with glycopeptides (g7) and loaded with cholesterol (g7-NPs-Chol), which per se is not blood-brain barrier (BBB) permeable, were successfully apply in order to obtain high rate brain delivery after intraperitoneal injection in HD mice. g7-NPs, in contrast to unmodified NPs, efficiently crossed the BBB and localized in glial and neuronal cells in different brain regions. We also found that repeated systemic delivery of g7-NPs-Chol rescued synaptic and cognitive dysfunction and partially improved global activity in HD mice.<sup>116</sup>

#### *Multiple sclerosis*

Multiple Sclerosis (MS) is a disorder of the CNS with inflammatory and neurodegenerative components, and characterized by a variable clinical pattern, which ultimately leads to a progressive neurological dysfunction.<sup>63</sup> Its pathological hallmarks are demyelination and cellular infiltration of T cells and macrophages (Mf). Despite long-term immunotherapy, relapses occur, which are commonly treated by repeated i.v. injections of high dose (pulse

glucocorticosteroids (GS) as a potent antiinflammatory drug (Table 1).

The most applied in vivo experimental model of MS is the experimental autoimmune encephalomyelitis (EAE) with a high tissue inflammation and the partial disruption of the BBB.<sup>73,115</sup> Considering MS-diseased BBB, it has been shown that disease severity could be correlated with alteration of BBB integrity, as confirmed by loss and delocalization of brain endothelial junction proteins, like occluding, VE-cadherin and others.<sup>67,123</sup> Schmidt and co-workers, in two different works,<sup>100; 2003b</sup> demonstrated that long-circulating prednisolone LPs (PL) were able to localize the high tissue therapeutic levels of glucocorticosteroid in the inflamed cerebral area of autoimmune EAE rats model<sup>39</sup> as compared with an equivalent dose given as free drug with a following reduction of inflammation and a significantly improvement in the disease course of (AT)-EAE. Moreover, the drug targeting by LPs appears highly effective in restoring the BBB integrity, ameliorating the disease activity of active and adoptive transfer (AT)-EAE without detectable side effects. The authors hypothesized a selective targeting, but in our opinion the altered permeability of BBB surely increases the penetration and the localization of LPs in inflamed tissue. Similarly, Kizelsztejn et al.<sup>56</sup> demonstrated the ability of long-circulating LPs to transport a potent antioxidant (Tempamine, TMN) to the brain tissue in a concentration able to significantly attenuate clinical symptoms of EAE in the mouse model.<sup>89</sup> The authors hypothesized that the size and the long circulation properties of these LPs could promote their extravasation into a well permeable inflammation sites.

#### **Other brain pathologies**

##### *Epilepsy*

Epilepsy is defined as a condition characterized by recurrent (two or more) epileptic seizures, unrelated to any immediate identified cause<sup>44</sup> Epilepsy is one of the oldest known conditions to mankind and still the most common neurological condition affecting individuals of all ages. At any given time, it is estimated that 50 million individuals worldwide have a diagnosis of epilepsy<sup>124</sup> ; 2001b). Recently an excellent descriptive review concerning the epidemiology of epilepsy was published.<sup>11</sup> Several evidences demonstrated that the epileptic seizures alter blood-brain barrier (BBB) properties and permeability.<sup>31,54,117</sup> Particularly, a recently in vivo study demonstrated the dynamic changes of BBB permeability during epileptogenesis. The long-lasting increased permeability of the BBB indicated that it may contribute to increased excitability in the epileptogenic foci, leading to the progression of epilepsy and seizure development.<sup>117</sup> Considering the application of drug delivery systems as a treatment strategy in epilepsy (Table 2), Mori and coworkers, in two different papers, compared the anticonvulsant effect of valproic acid and  $\gamma$ -butyrolactone- $\gamma$ -carbonyl-L-histidyl-L-propionamide citrate (DN-1417) entrapped in LPs with that obtained after free drug i.v. administration in amygdaloid-kindled rats<sup>70</sup>; 1992b). The animal models were obtained implanting a bipolar electrodes in the right area amygdalaris anterior that are stimulated at a defined

**Table 2.** Other brain pathologies models and Nanotech applications.

<b>PATHOLOGY</b>	<b>TARGETING (BBB/tumor)</b>	<b>APPROCHES</b>	<b>DRUG</b>	<b>MODEL</b>	<b>TEST</b>	<b>Results</b>	<b>REF</b>
Epilepsy	/	LPs (PC:CHOL: Sterylamine 7:2:1)	PHT or HRP	Rats with status epilepticus	Anticonvulsivant effect Analysis of HRP reaction products in the brain	Suppression of AM discharges by two doses of L-PHT; accumulation of HRP (by L-HRP admin.) in the AM epileptogenic focus (2 of 5 animals tested)	71
	/	LPs (PC:CHOL: Sterylamine 7:2:1)	DN-1417	Amygdaloid kindled rats	Anticonvulsivant effect	Suppression of kindled seizure and prolonged anticonvulsivant effect after L-DN 1417 admin. than free DN 1417	70
	/	LPs (cationic)	VPA	Amygdaloid kindled rats	Anticonvulsivant effect	Prolonged anticonvulsivant effect after cationic L-VPA admin (2 days) than anionic L-VPA admin and free VPA (1 h)	72
	LDL Receptor	PBCA-NPs-Ps80	MRZ 2/576	Convulsion model in mice	Anticonvulsivant effect (MES test)	Longer duration in MES test after coated Np-MRZ 2/576 admin. (up to 210 min) than uncoated Np admin. (60 min)	36
Lysosomal storage disorders	Transferrin receptor (BBB)	LPs (POPC: DDAB : DSPE-PEG2000: DSPE-PEG2000 -maleimide + 8D3 mAb)	pCMV-GUSB plasmid DNA	MPS type VII GUSB null mice	GUSB enzyme activity measurement Protein concentration determination	GUSB enzyme activity in the brain of adult mice = $13.3 \pm 0.9$ nmol/hr/mg protein; GUSB enzyme activity in the brain of GUSB null mice after L/plasmid DNA treatment = $1.7 \pm 0.1$ nmol/hr/mg protein; GUSB enzyme activity in the brain of GUSB null mice after saline admin. < 0.2 nmol/hr/mg protein.	132
Cerebral Ischemia (Traumatic Injury)	/	LPs (DPPC:CHOL: Sterilamine 14:7:4)	CuZn-SOD	Rats cold- injury brain model (Chan et al 1983)	Determination of CuZn-SOD; determination of superoxide radicals; Evans Blue Permeability (BBB permeability)	Protection of drug; increase of CuZn-SOD brain level 8from 30 min to 2 h); reduction in brain level of superoxide radicals and water content and ameliorated BBB permeability	Chan et al 1987
	/	LPs (PC:CHOL: Sulfatides)	Calpain inhibitor (ALL NA1)	Gerbil model of forebrain ischemia (Yokota et al 1995)	Immunohistochemistry and immunoblot analysis	Dose dependent prevention after L- ALL Na1 of CA1 neuronal damage	Yokota et al., 1999
	/	LPs (DPPC:CHOL: Sterilamine 14:7:4)	CuZn-SOD	Rats model of focal cerebral ischemia (Chen et al., 1986)	CuZn-SOD activity and measure of infarct size	Increase of CuZn-SOD activities in the brain; reduction of infarct size (33%, 25% and 18% for the anterior, middle and posterior slices, respectively)	49
	/	NPs	MRZ 2/566		Quantification of infarct lesion	Increased neurological score vs placebo Reduction at 53%of	111

(Continued on next page)

**Table 2.** Other brain pathologies models and Nanotech applications. (Continued)

<b>PATHOLOGY</b>	<b>TARGETING (BBB/tumor)</b>	<b>APPROCHES</b>	<b>DRUG</b>	<b>MODEL</b>	<b>TEST</b>	<b>Results</b>	<b>REF</b>
	/	PLGA-NPs	SOD	Rats models of focal cerebral ischemia Rats models of focal cerebral ischemia - perfusion injury model	and neurological severity score Quantification of infarct lesion and neurological severity score	total infarct size (60% of cortical and 42% of striatal infarction) 5% of survival after SOD-Np admin. at 28 d (infarct volume after SOD-Np = 20%, saline control=56%, SOD- SOL=75%. Ischemic lesion area after SOD-Np=25%, saline control =50%, SOD-SOL =80%. Neurological severity score after SOD-Np=3.5, saline control=10, SOD-SOL=11)	90
	/	PLA-NPs	QC	Rats model of cerebral ischemia	Diene level, index of lipid peroxidation and GSSG/GSH ratio	Significant protection to endogenous antioxidant enzymes against ischemia induced oxidative damage in neuronal cells	29
Arsenic induced oxidative damage	/	PLGA-NPs	QC	Rats with neuronal cell damage after NaASO <sub>2</sub> injection	Intracellular GSH, GPx, GR, GST and G6PDH activity in the brain	QC-Np prevent the antioxidant depletion in brain cells (levels of GPx from Np 4.98 ± 0.31 vs 2.17±0.09 μmol NADPH oxidation/min/mg protein; G6PDH nmol NADPH reduced/min/mg protein 6.07 ±0.39 vs animal model 3.61 ±0.29; GR μmol NADPH oxidation/min/mg protein 17.92 ±2.33 vs 9.92 ±0.87;GST nmol product/min/mg 97.86 ±7.22 vs 69.26 ±9.88)	38
Traumatic Brain Injury	/	PANAM dendrimers	N-acetyl-cysteine, valproic acid	Traumatic brain injury, rats	In vivo administration, biodistribution and pharmacological test	PANAM localize in the injured neurons and microglia in the brain. -loading with N-acetyl cysteine and valproic acid	69
	/	self-assembling peptide nanofiber scaffold	/	Traumatic brain injury, rats	In vivo administration	Reduction of acute brain injury and brain cavity formation	96
	/	pegylated PLGA NP (100-200-800 nm)	/	Traumatic brain injury animal model	In vivo administration, brain localization	Smaller NPs are facilitated in BBB crossing	25
	/	PLGA NPs	Cerebrolysin	Traumatic brain injury, rats	In vivo administration, brain edema formation, rescue tests	Ability in delivering cerebrolysin to site of action; - reduction of brain pathology features	94
Infectious diseases	/	LPs (PC:CHOL:DSPG -AmBisome)	AMB	Candida Albicans infected rabbits	CFU in the brain tissue	Reduction of infection in the brain	41

/	LPs (PC;CHOL:DSPG-Ambisome)	AMB	Murine model of cryptococcal meningitis (Cryptococcus neoformans)	CFU in the brain tissue and therapeutic activity	20 mg/kg and 30 mg/kg of Ambisome were able to clear the yeast from the brains of the mice (44 and 78% clearance, respectively)	5
/	LPs (PC;CHOL:DSPG-Ambisome)	AMB	Murine model of cryptococcosis (Cryptococcus neoformans)	Therapeutic activity in the brain tissue	10 mg/kg AmBisome = 5.69±0.38 log <sub>10</sub> CFU/brain; 1 mg/kg fungizone = 6.92±0.50 log <sub>10</sub> CFU/brain. Ten mg/kg AmBisome=50–60% survival; 1 mg/kg fungizone = 12–30%.	113
/	LPs (PC;CHOL:DSPG-Ambisome)	AMB	Immuno-suppressed rabbits with Coccidioides immitis	CFU in the brain tissue and therapeutic activity	3x15mg/kg Ambisome for 3 weeks clears the fungus from the brains (3/8 rabbits). None with other treatments. CFU reduction vs control	23
/	LPs (PC;CHOL:DSPG-Ambisome) Association with other VCZ, CAS, MICA	AMB	Murine models of CNS aspergillosis	CFU in the brain tissue and therapeutic activity of animals	1 mg/kg AmBisome from 1 to 3 d with VCZ from days 4 to 10 demonstrated higher efficacy than other combinations	22
LDLr (?)	PLA-b-PEG-NPs-Ps-80	AMB	Cryptococcal Meningitis Bearing Mice	Fungal growth and therapeutic activity	Survival: twice than controls	92
	CG <sub>3</sub> R <sub>6</sub> TAT NPs	CG <sub>3</sub> R <sub>6</sub> TAT	Staphylococcus Aureus induced meningitis rabbit model	CFU in the brain tissue	Np 2 Ig CFU/g tissue; control 3.7 Ig CFU/g tissue; Suppression of bacterial growth in the brain, decreasing the degree of the infection	61
	CG <sub>3</sub> R <sub>6</sub> TAT NPs	CG <sub>3</sub> R <sub>6</sub> TAT	Cryptococcal neoformans meningitis rabbit model	CFU in the brain tissue and biodistribution studies (FITC-loaded Np)	Suppression of yeast growth in brain tissue (Np treatment < 2CFU/g; control ≥ 15/g).	121

LPs, liposomes; PC, phosphatidylcholine; CHOL, cholesterol; PHT, phenytoin; HRP, horseradish peroxidase; AM, amygdala; DN-1417,  $\gamma$ -butyrolactone- $\gamma$ -carbonyl-L-histidyl-L-proprionamide citrate; VPA, valproic acid; PBCA, poly(butylcyanoacrylate); NPs, nanoparticles; MRZ 2/576, 8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino[4,5-b]quinoline-5-oxide choline salt; MES, prevention of maximal electroshock; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; DDAB, dimethyldioctadecylammonium bromide; DSPE-PEG<sub>2000</sub> distearylphosphatidylethanolamine polyethylene glycol; 8D3 mAb, monoclonal antibody against the mouse transferring receptor; GUSB, b-glucuronidase; MPS, mucopolysaccharidosis; CuZn-SOD, CuZn superoxide dismutase; DPPC, dipalmitoyl phosphatidylcholine; DPPS dipalmitoyl phosphatidylserine; MRZ 2/566, (8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino[4,5-b]quinolin-5-oxide choline salt; PANAM, polyamidoamine; PLGA, poly(D,L-lactide co-glycolide); SOD, superoxide dismutase; QC, Quercetin; GSSG, oxidized glutathione; GSH, reduced glutathione; PLA, Polylactide; NaASO<sub>2</sub>, sodium arsenite; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; G6PDH, glucose-6-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; DSPG, distearylphosphatidylglycerol; CFU, colony forming units; VCZ, voriconazole; CAS, caspofungin; MICA, micofungin; Ps80, Polysorbate 80; AMB, amphotericin B; CG<sub>3</sub>R<sub>6</sub>TAT, cholesterol conjugated 3 glycine, six arginine and TAT peptide; FITC, fluorescein isothiocyanate.

time intervals and frequency as described by Löscher and co-workers.<sup>62</sup> In all cases, while the administration of free drug suppressed amygdaloid kindled seizure for few time, the drug encapsulated in positive charged LPs showed a prolonged anticonvulsant effect. The authors hypothesized that LPs protected the drug from the enzymatic degradation and that LPs surface charge played an important role in allowing the rapidly cross of the BBB, by taking advantage of adsorptive-mediated endocytosis. Recently, the same group of research evaluated the effect of phenytoin (PHT), entrapped in LPs (PHT-LPs), on the status epilepticus, induced in rats by injecting a combination of dibutyltyryl-cAMP (db-cAMP) and ethylenediaminetetraacetic acid (EDTA) into the amygdala (AM).

The researchers observed the suppression of AM discharges by two injections of PHT-L. The authors hypothesized that the AM epileptogenic focus created by db-cAMP/EDTA has a high affinity for LPs, and this factor participates in the local suppression of AM discharges by PHT-L. Even if, the mechanism underlying the selective augmentation of LPs in the epileptogenic AM is not known, the amount of LPs in the circulation may be important for the understanding of this finding, since two injections of PHT-L caused preferential suppression of AM discharges as well as two injections of horseradish peroxidase (HRP; to which the blood-brain barrier is impermeable) loaded LPs increased their augmentation in the AM. Since, the blood flow is reliably increased in the epileptogenic focus, it is our opinion that the possible cause of LPs local augmentation could lie in the blood-flow increase, along with the permeability of BBB increasing over the disease progression, consequently promoting the brain localization of LPs (Mori et al., 1995).

Friese and coworkers evaluated the prevention of maximal electroshock induced convulsions in mice model after i.v. administration of a novel non-competitive NMDA receptor antagonist MRZ 2/576 (potent but rather short-acting anticonvulsant, rapidly discharged from the central nervous system by transport processes that are sensitive to probenecid) bound to PBCA NPs coated with PS-80; this formulation prolonged the length of the anticonvulsive effect up to 210 min and after probenecid pre-treatment up to 270 min compared to 150 min with probenecid and MRZ 2/576 alone.<sup>36</sup> Reasonably, the NPs operated as drug delivery systems, able to protect and to prolong the release of drugs to the CNS. Moreover, the coating of NPs with PS-80 seems to be necessary to target and to achieve an uptake of the particles into the brain. It is also important to consider that the BBB properties can be altered by the treatment performed to achieve animal model, thus promoting and enhancing NPs BBB crossing.

#### *Lysosomal storage disorders*

The lysosomal storage diseases (LSDs) are a heterogeneous group of disorders that affect 1/7,000 live-born infants, the majority of which develop CNS disease. Each LSD results from a deficiency of a single lysosomal enzyme, pivotal for degrading macromolecules that must be turned over in lysosomes.<sup>110</sup> More than 40 LSDs have been described. The neurological compromise is generally refractory to treatment, which will require long-

term correction of lesions dispersed throughout the central nervous system to be effective. A promising approach is enzyme replacement therapy (ERT) or gene therapy by gene transfer methods enable the treatment of these diseases by transferring a wild-type copy of the gene of interest to some target organ, where the production and export of the protein proceed for extended periods of time.<sup>109</sup> In this contest, Zhang and co-workers<sup>133</sup> treated a mice model for type VII mucopolysaccharidosis (MPS-VII), deficient for the  $\beta$ -glucuronidase (GUSB) lysosomal enzyme, with GUSB expression plasmid (p-CMV GUSB) encapsulated in LPs and superficially engineered with the rat 8D3 MAb to the mouse transferrin receptor (TfR) to achieve BBB crossing (Table 3). The results demonstrated that sub-normal, yet therapeutic levels of GUSB enzyme activity, are achieved in brain following the i.v. administration of the TfR mAb-targeted LPs encapsulating p-CMV GUSB plasmide. This kind of strategy, even if interesting, will required repeat administrations, considering the chronic progression of disease and the inability of the plasmid delivered by LPs to integrate into the host genome.<sup>21</sup>

#### *Stroke*

After heart disease and cancer within the developed world, stroke is the third largest killer, second most common cause of neurologic disability after AD.<sup>78</sup> There are over five million deaths/year from stroke and over nine million stroke survivors. Between the ages of 45 and 85, 20-25% of men and women, respectively, can expect to have at least one incident of stroke. Atherosclerosis, heart disease, hypertension, diabetes, and lifestyle habits are risk factors correlated with disease. Unlike AD and PD, many of the risk factors for developing this disease can be modified. The etiology of stroke is brain vascular occlusions (thrombotic stroke) or rupture (hemorrhagic stroke). The neuropathological hallmarks of stroke are necrotic infarcts of variable size with inflammatory gliosis.

The effect of hypoxia-ischemia on the BBB has been extensively investigated<sup>57</sup>; Bolton et al., 1998;<sup>34,65</sup> this disease sets in motion a series of events, which leads to disruption of TJ and increased BBB permeability, probably mediated by cytokines, VEGF, and NO. Moreover, elevated levels of proinflammatory cytokines, IL-1 $\beta$ , and TNF- $\alpha$  have been demonstrated in animal brains after focal and global ischemia<sup>33</sup> and in cerebrospinal fluid of stroke patients.<sup>114</sup>

The potential of LPs and NPs in protecting and modulating the delivery of un-stable drug during the ischemic insult was deeply investigated in several researches with interesting positive results (Table 2): the success of the use of nanocarriers is not to be correlated to the need of an active targeting, but better to the facilitate diffusion of these systems through the compromised and broken BBB. LPs and NPs were used to deliver drugs able to prevent ischemic neuronal damage. Several researchers used rats as animal models, in which the focal cerebral ischemia was produced by the method described by Chen and colleagues<sup>20</sup> based on an extensive, but reproducible infarct. In an old study, Chan and coworkers<sup>19</sup> described the ameliorated condition of animals manifesting the reduction in brain level of superoxide radicals when treated with superoxide dismutase (SOD) loaded into LPs. This success could

Q3

710 be firstly searched both in the protection exploited by LPs along  
with their ability to operate a prolonged delivery of drug. More  
recently, Imaizumi and co-workers, using the same animal model,  
demonstrated that LPs facilitate the delivery of SOD, thus elevat-  
ing SOD activities into the brain, not only in the infarct, but also  
715 in the non-infarcted subcortical areal. The authors try to justify  
these results hypothesizing the penetration of SOD loaded LPs  
across the leaky BBB in the ischemic zone and subsequently LPs  
uptake by adjacent neurons and microglia extending the area of  
action; otherwise, SOD loaded LPs could exert their action on  
720 extracellular superoxide on the luminal side of capillaries, without  
penetrating the BBB.<sup>49</sup> More recently, Reddy and coworkers dem-  
onstrated the ability of PLA NPs to encapsulate and to stabilize  
SOD from degradation and to provide a localized modulated brain  
delivery that assures the protective effect of the enzyme by neutral-  
izing the deleterious effect of reactive oxygen species which are the  
725 mediators of reperfusion injury.<sup>90</sup> By using focal cerebral ischemia  
model rats, Sopala and co-workers<sup>111</sup> evaluated the neuroprotec-  
tive activity of the novel glycine- $\beta$  site NMDA (N-methyl-D-  
aspartate) receptor antagonist MRZ 2/576, formulated in NPs  
that provide a system able to protect and to modulate the delivery  
730 of the drug to the CNS.

In another study, citicoline-loaded LPs were administered in  
ischemic reperfused mice; the LPs formulations produced an  
increase in rat survival rate of about 24% and a reduction of 60%  
in diene levels (index of lipid peroxidation) compared to the free  
735 drug.<sup>35</sup> Yokota and colleagues (Yokota et al., 1999) administered  
a calpain inhibitor to gerbils to rescue ischemic neuronal damage,  
but with a little beneficial effect due to BBB crossing inability of  
the inhibitor. On the contrary, the calpain inhibitor-LPs complex  
was able to produce a potently and selectively inhibition of cal-  
740 pain activation. LPs protect the drugs and maintain a higher con-  
centration in the brain than free drug.

Quercetin is an interesting herbal compound that exhibits anti-  
oxidant properties against many neurological diseases. Initially,  
Das and coworkers<sup>29,97</sup> proposed the use of LPs encapsulating  
745 quercetin, administered i.v. to rats (pre-treated with arsenic) as a  
good formulation to attenuate the oxidative damage induced by  
ischemia reperfusion injury in the rat brain. Recently, they pro-  
posed the use of PLGA NPs as quercetin carriers in combating  
arsenic induced oxidative damage in brain of rats. A singular dose  
750 of sodium arsenite was sub-cutaneously injected in rats to obtain a  
model of arsenic toxicity. Nanoencapsulated quercetin, orally  
administered before the arsenite injection, prevented the arsenic  
cerebral oxidative damage.<sup>38</sup> The authors hypothesized the ability  
of these NPs to cross the BBB via particle uptake mechanism  
755 based on an endocytotic pathway by the brain capillary endothelial  
cells. It is not clear if the treatment of rats with arsenic maintained  
the BBB integrity, thus it is difficult to give an absolute conclusion  
on the real efficacy and BBB crossing pathway. The increased effi-  
760 cacy of these formulations should be found in their ability to stabi-  
lize the drug and to prolong the brain delivery.

#### *Traumatic brain injury*

Traumatic brain injury (TBI), happens when an external force  
traumatically injures the brain. TBI can be classified on severity,

mechanism (closed or penetrating head injury) and area (specific  
765 or widespread area). TBI is now considered as one the major  
major causes of death and disability worldwide, especially in chil-  
dren and young adults.. It could vary on severity depending on  
the casues, but the common features are mainly consiting on  
alterations in cerebral blood flow and the pressure within the  
770 skull, contributing substantially to the damage from the initial  
injury. In this kind of pathology, blood-brain barrier features are  
strongly altered, as permeability and transport across the BBB are  
dramatically changed.

Several attempts in prevention and “quick” interventions were  
performed in the past years, also dealing with nanomedicines,  
775 able to transport and deliver efficiently in the traumatic areas  
those drugs needed to restore “altered” parameters.<sup>105,107</sup>

In particular, a recent study<sup>69</sup> demonstrates that the adminis-  
tration of polyamidoamine (PAMAM) dendrimers could be  
taken up in the brain of injured animals and selectively localize  
780 in the injured neurons and microglia in the brain. Besides, these  
dendrimers were also able to deliver two clinically approved  
drugs, N-acetyl cysteine (attenuating neuroinflammation) and  
valproic acid (attenuating excitotoxicity), building on positive  
outcomes in a rabbit model of perinatal brain injury.  
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In another elegant research,<sup>96</sup> self-assembling peptide nano-  
fiber scaffold were used to reduce acute brain injury and brain  
cavity formation, and to improve sensorimotor functional recov-  
ery. SAPNS serves as biocompatible material in the hemorrhagic  
790 brain cavity. Local delivery of this nanomaterial may facilitate the  
repair of ICH related brain injury and functional recovery.

Besides, in another paper,<sup>25</sup> the impact on the size of nanome-  
dicines (pegylated PLGA NPs sizing 100nm, 200 nm and  
800nm) on distribution in TBI animal models was studied. The  
results highlighted that smaller NPs are facilitated in BBB cross-  
795 ing while bigger NPs may result in a lower permeation. This  
finding would be extremely important in order to understand the  
rationale in planning nanomedicine for this kind of interventions  
and may help in designing NPs with a higher performance in tar-  
geting traumatic brain areas depending on the size and on the  
800 BBB permeability under TBI events.

In the treatment of TBI, the use of mixture of peptides acti-  
vating growth factors was deeply studied in the last 5 y<sup>106</sup> Cere-  
brolysin is a peptide mixture able to ameliorate symptomatology  
and to delay progression of neurological disorders such as Alz-  
805 heimer disease and dementia. Cerebrolysin loaded PLGA NPs  
were therefore designed, formulated, tested on stability over time  
in plasma and finally in animal models, where they were able to  
deliver cerebrolysin to reduce brain pathology following trau-  
810 matic brain injury.<sup>94</sup>

#### *Infectious disease*

Brain inflammatory diseases such as meningitis and encephali-  
tis are among the top ten infection causes of death; they are  
caused by bacteria (such as Bacillus anthrax, Staphylococcus  
aureus), fungi (ex Candida albicans, Cryptococcus neoformans)  
815 or viruses (Jafari et al. 1994;<sup>77,93</sup> Despite the general efficacy of  
antibiotic treatment, high mortality and morbidity is frequently

recovered due to the difficulty of drugs to the BBB and access the brain (Table 3).

820 The LPs approach in brain infectious therapy could be mainly referred to the use of AmBisome<sup>®</sup>, a liposomal formulation of amphotericin B in which the drug is strongly associated with the bilayer structure of unilamellar LPs.

825 Several works, using different animal models, provided evidences of AmBisome effectiveness against brain infections. Groll and colleagues<sup>41</sup> evaluated groups of un-infected and *Candida albicans*-infected rabbits treated with AmBisome and the other commercially formulation of amphotericin B (Amphotec<sup>®</sup>), showing AmBisome treatment able to completely clear *Candida albicans* from the brain of infected animals. Several years ago, Albert and colleagues<sup>5</sup> described a mouse model of meningitis caused by *Cryptococcus neoformans*; also in this case, the AmBisome therapy (20 and 30 mg/kg) produced a disappearance of *Cryptococcus* in the brain. Several years later, Takemoto and co-workers<sup>13</sup> treated the same animal model with a lower dose of AmBisome (10 mg/kg) in comparison with other antifungal drugs as fungizone. AmBisome exhibited greater efficacy and, more importantly, it is distributed into the brain as the penetration enhanced by the progression of cryptococcal meningitis correlating with the *in vivo* activity. Clemons and colleagues administered AmBisome to treat different brain infections: in a first study, AmBisome was compared with other drugs (as fluconazole or amphotericin B alone) to treat coccidioidal meningitis in rabbits. The animal model was represented by immune-suppressed rabbits, challenged intra-cisternally with *Coccidioides immitis*<sup>23</sup> Recently, the authors studied the efficacy of AmBisome, micafungin (MICA), caspofungin (CAS), amphotericin B, voriconazole (VCZ), given alone or in combination, after administration in immune-suppressed mice, infected intracerebrally with *Aspergillus fumigatus*. In the first study, the brain infection was significantly reduced by AmBisome treatment if compared with fluconazole and amphotericin B treatments. On the contrary, only the combination therapy at suboptimal doses of AmBisome and VCZ improved the outcome in CNS aspergillosis.<sup>22</sup> Thus, AmBisome seems to have an important role in treating brain infections, in which the inflammation, due to pathogens, alters the permeability of BBB; in fact AmBisome could allow to deliver high and active concentrations of drug in the tissue, reducing or eradicating the fungal burden.

860 Only in the last two years, polymeric NPs were investigated as an alternative treatment in brain infection diseases. Liu and co-workers tested a new kind of NPs in rabbit models to treat the brain infections disease. Amphiphilic cholesterol-conjugated G3R6TAT peptide (CG3R6TAT), which contains TAT sequence, was designed and assembled into core/shell NPs. These NPs possessed a broad spectrum of strong antimicrobial activities, much stronger than the hydrophilic peptide, incapable of forming NPs. The NPs were firstly examined for *in vivo* activity against *Staphylococcus Aureus* in comparison with vancomycin, a therapeutic agent widely used for the prophylaxis, suppression, and treatment of *S. Aureus* meningitis. The NPs were found to be as efficient as vancomycin in treating the meningitis in rabbits (Liu et al., 2009). In another experiment, CG3R6TAT NPs

showed a good antimicrobial activity against the brain infections caused by *Cryptococcus neoformans* in rabbit model, in which the meningitis was induced inoculating intracisternally the yeast suspension. The authors demonstrated that the NPs crossed the BBB and suppressed the yeast growth in the brain tissues with similar efficiency as amphotericin B did.<sup>121</sup> They hypothesized that cationic peptide-decorated NPs may be taken up by the BBB through adsorptive endocytosis, diffusing into the brain tissue and suppressing bacterial growth in the brain.

880 Finally, starting from the hypothesis that<sup>37,58</sup> in NPs coated with polysorbate 80 seem to be uptaken by the brain capillary endothelial cells due to specific interaction with LDL receptors, Ren and co-workers prepared polysorbate 80 coated amphotericin B/PLA-b-PEG NPs to target cryptococcal meningitis-bearing mice. The results confirmed the therapeutic efficacy of these formulations in fungal infection in the brain, decreasing the speed of colony growth and the count of colony, thus increasing the survival time of animals.<sup>92</sup>

## Conclusion

The interest in developing targeted systems, able to reach the CNS, represented one of major section of neuroscience, over the last 20 y The pathologies affecting the “Brain” are surely the most difficult to be treated and the less favorable in term of rescue of the integrity of the neuronal structures. These pathologies, meaning gliomas, PD and AD, neurodegenerative diseases as MS, or infectious and stroke diseases, all have similar features, which are common and could represent a possible target for innovative therapies. Moreover, the presence of the BBB is usually a great limitation for drugs, which are normally unable to enter in the CNS.

905 Thus, the application of nanotechnology to CNS diseases (namely Nano-Neuroscience) is surely representing one of the most innovative strategy to improve the current treatments and, possibly, to find newer and, most of all, more efficacious therapies. Both NPs and LPs have been deeply investigated with several *in vitro* and *in vivo* models. In this review, we tried to summarize the most interesting and valuable *in vivo* evidences, since we do consider extremely risky to translate *in vitro* results (on BBB co-cultures) to *in vivo* outlooks of efficacy.

915 Moreover, in some of the cited pathologies, the integrity of BBB is seriously compromised, such as in glioma or stroke, as well as in other diseases, like in AD and PD, the permeability across the inflamed BBB is actually implemented. In these cases, a brain targeting has not to be considered as BBB crossing goal, but better as “pathology-targeted” aim.

920 Several experiments on polymeric NPs and LPs have been reviewed considering a general aim of understanding both the rate of brain delivery and the biodistribution; in particular, the last aim is pivotal for the scale-up of neuro-nanomedicine, since scientists must strongly assess both brain delivery efficiency and safety of nanocarriers. Moreover, beside a number of proof-of-concept experiments, relatively few pre-clinical data have been reached by using *in vivo* models (mice and rats, mainly), assessing



the efficacy against brain disease of active molecules (gene, drugs, enzyme, etc..) by means of nanocarrier-mediated delivery. These results pointed out a batch of data which would be useful for

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improving, developing and finalizing this new, non-invasive and efficacious strategy for the treatment of the brain diseases, such as AD and PD, gliomas, infectious disease, MS and LSD. A final remark should be given regarding the possible development of nanocarriers for CNS and hopefully to drive a complete

research project for neuro-nanomedicine. Firstly, the choice of biodegradable and biocompatible starting materials is pivotal in speeding up the acceptability and FDA approval of new nanocarriers; secondly, the targeting strategy (meaning surface engineering with CNS targeting ligands) should be chosen in function of the pathology, since the BBB integrity and permeability is varying from disease to disease. It could be possible that the need for BBB crossing would not be required,

especially in the case of stroke and gliomas, while it is fundamental in other BBB-healthy pathologies (such as AD or MS). Third, the choice of the carriers should be considered as function of the kind of drugs to be delivered: in particular, polymeric NPs could be useful in both hydrophilic and hydrophobic active substance encapsulation, while LPs would better contribute to an effective gene therapy. Finally, the overall fate of carriers and, most of all, their stability in the bloodstream should be accurately investigated a-priori and the proper corrections and tricks should be taken in great consideration, to definitively allow nanocarriers to exploit their job at their best.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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