Potential use of nanomedicine for drug delivery across the BBB in health and diseased brain

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Abstract: The research of efficacious non-invasive therapies for the treatment of brain diseases represents a huge challenge, as people affected by disorders of the Central Nervous System (CNS) will significantly increase. Moreover, Blood-Brain Barrier (BBB) is a key factor in hampering a number of effective drugs to reach the CNS. This review is therefore focusing on possible interventions of nanomedicine-based approaches in selected diseases affecting the CNS. A wide overview of the most outstanding results on preclinical evaluations of the potential of nanomedicine in brain diseases (i.e. Brain Tumor, Alzheimer, Parkinson, Epilepsy and others) is given, with highlights on the data with relevant interest and real possibility in translation from bench-to-bed side. Moreover, a critical evaluation on the rationale in planning nanosystems to target specific brain pathologies is described, opening the pave to a more structured and pathology-tailored design of nanocarriers.

Keywords: Nanoparticles, Liposomes, Nanomedicine, Central Nervous System, Brain Diseases, Blood-Brain Barrier

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List of Abbreviations:

AD: Alzheimer Disease

BBB: Blood Brain Barrier

CNS: Central Nervous System

DOX: Doxorubicin

LPs: Liposomes

LSDs: Lysosomal Storage Disorders

MS: Multiple Sclerosis

NPs: Nanoparticles

PBCA: Poly-butylcyanoacrylate

PD: Parkinson's Disease

PEG: Poly-ethylene glycol

PLA: Poly-Lactide

PLGA: Poly-lactide-co-glycolide

PS-80: Polysorbate 80

Tf: Transferrin

1.INTRODUCTION

The research of efficacious non-invasive therapies for the treatment of neurodegenerative diseases is one of the most important topics faced in the last years by the pharmaceutical technology. The interest for this topic was triggered by the prevision that the number of people affected by disorders of the Central Nervous System (CNS) at the end of the 20th century[1] should significantly increase owing to enhance of the live expectancy.

As the Blood-Brain Barrier (BBB) is the barrier designed to protect the CNS from microbial contamination and exogenous agents, it hampers a number of effective drugs to reach the CNS. The BBB consists of walls formed by capillaries that isolate the brain compartment from the bloodstream. The key features of BBB as the permeability change with respect to macromolecules, or even nanosystems, if in healthy or diseased state.

In order to promote a drug delivery and targeting to the CNS, nanotechnology and nanomedicine are surely representing one of the most efficacious approaches, growing day-by-day in terms of published papers and clinical trials. This evidence is corroborated by simply indexing "*brain*" and "*nanoparticles*" in PubMed: from 1996 up to today this

research totalizes more than 2600 papers. In particular, before 2004, the papers dealing with "*nano&brain*" were quite low (almost 200 units from 1996 to 2004), while only in the last 3 years (2012-2014) more than 1200 research works were dealing with this topic. What is really surprising is that this high number of "*nano&brain"* papers dramatically falls down when the literature research is refined by combining "*nano&brain"* with *"vivo"* (down to 800 papers totally) and again falls down to 100 when refining with "*efficacy*".

This evaluation, evidently superficial, could help in better understanding the rationale of this review, which deals with the evaluation of *in vivo* outputs from nanotechnology application in diseased models mainly.

Regarding clinical trials, few references are available and mainly related to intrathecal injection of nanomedicines, but mostly pro-drugs for brain tumors. Thus, no nanomedicine (NPs or LPs) are now in clinical evaluation.

Several reviews dealt with proof-of-concept experiments in healthy brains, with interesting evidences of BBB crossing and CNS targeting[2-5]. Most of experiments were conducted on "healthy" animals and therefore on "healthy brains", without any pathological hallmarks. This fact inevitably means that, in these studies, before administration of any kind of nanocarriers, the BBB state is preserved, with complete maintaince of its integrity and low permeability rates.

In the following sections, dealing with the application of nanomedicines to the treatment of neurological/neurodegenerative disorders, the state of BBB and the brains are "diseased", with evidences of remarkable changes in BBB permeability and integrity.

These pathologies could vary from brain tumors, neurodegenerative diseases (Parkinson's and Alzheimer's), epilepsy, infectious brain diseases, strokes and many others; the BBB state would be strongly different as shown in table 1.

Pathology	BBB integrity	BBB permeability	BBB influx pathways
Brain Tumor	↓↓↓ *	↑↑↑*	≠
AD	≠	↑↑	↑*
PD	≠	≠	≠
MS	↓↓	↑	≠
LSDs	≠	↑	≠
Stroke	↓↓↓↓	↑↑↑↑	≠
Neuro-infections	↓*	↑*	↑*

Table 1: Summary of BBB features in neurological and neurodegenerative disorders. AD (Alzheimer's Disease); PD (Parkinson's Disease); MS (Multiple Sclerosis); LSDs (Lysosomal Storage Disorders);* (depending on the grade of the pathology).

1.1 Glioma

Glioma is unfortunately the most frequent primary CNS tumors in humans: the various types of gliomas can be identified by histological features reflecting cellular differentiation lineages: astrocytomas, oligodendrogliomas and mixed oligoastrocytomas[6]. Glioma is highly infiltrative, leading to the complete disruption of normal tissue architecture and displays angiogenic features and high degree of vascular proliferation along with endothelial cell hyperplasia[7]. Treatment of gliomas includes surgery, radiotherapy and chemotherapy, however the prognosis remains poor, with a median survival of 12-15 months[8]. Clearly, there is a desperate need for more effective therapies for patients with glioma.

Investigations revealed that BBB and blood-tumor barrier (BTB), the endothelium of new vessels, is remarkably different from healthy BBB. These new angiogenetic microvessels show an increased vascular permeability, derived from deregulation of junctional proteins. Besides, tight junction opening is functionally the most important abnormality, more pronounced with increasing malignancy. Thus, compared with normal healthy brain, gliomas fail to express or express a non-functional form of occludin. Moreover, fenestrations and an increased number and size of pinocytic vacuoles were reported[9-11]. Nonetheless, the altered permeability of BBB remains a local event, evident in the core of tumor, but totally absent at its growing margins and healthy tissue surrounding[12].

In vivo studies are usually performed using xenograft model based on the intracerebral implantation of brain tumor cell lines (usually 9L, C6, 4C8, U87 or 101/8) into immunologically deficient rodents[13]. This specific kind of animal model is wrongly, but unfortunately usually considered as model for proof-of-concept of BBB crossing. This is a big pitfall as BBB does not maintain its integrity, particularly evident at the late-stage of the diseases. On the contrary, at the very early stages, the BBB maintains its barrier function, but loses its low permeability characteristics[14].

This particular situation of BBB, leading to the tumor-accumulation of several kinds of nanocarriers without a real BBB crossing mediated by specific mechanisms or pathways state, strongly impacts on the planning and fate of nanocarriers aiming to treat brain cancer. Thus, more than BBB crossing mechanisms, the majority of studies are taking advantages of Enhance Permeability and Retention (EPR) effect of those tissues. If the nanocarriers are further targeted to the tumor (with specific ligands, proteins or antibodies), the possibility to obtain an enlarged accumulation in the tumor site strongly increases.

FIGURE 1

Non-targeted nanomedicine-based approach was firstly utilized aiming to improve chemo-therapeutic index of doxorubicin (DOX), one of the most active molecule against malignant glioma, unfortunately limited by poor BBB penetration. Interestingly, results appeared in disagreement:

DOX encapsulated in sterically stabilized LPs lead to a significant increase in survival of animals after treatment with some differences in the extents[15-17], not only due to the delivery systems, but mainly to the intrinsic variability in the properties of the tumor and to the different sensitivity to the same drug. Similarly, a "passive targeting" approach was used for paclitaxel[18] or gene material stabilization into LPs[19-20], reporting positive results for tumor prognosis.

Active targeting (whether tumor or BBB aims) was investigated with promising results. The surfactant polysorbate 80 (PS-80) was intensively exploited to cross BBB; notably, even if BBB crossing was not needed in the case of brain tumor over III grade, an anticancer drug loaded PS-80-coated NPs lead to a significant survival improvement in comparison to both free drug and loaded NPs without PS-80[21-25].

Pegylated-cationic serum albumin NPs, binding to negative glycoprotein on endothelial capillary for BBB crossing, were used to deliver a plasmid (pORF-hTRAIL), inducing apoptosis in tumor cell with regimen-treatment-dependent survival improvement and decreased tumor growth[26].

Also liposomes (LPs) were functionalized with tumor-targeting ligands (IL-13-PEG) and used for DOX delivery: as compared to non-target systems, DOX encapsulated into IL-13 pegylated LPs produced higher accumulation in brain tumor, associated with a survival time improvement[27]. Another ligand (transferrin) was conjugated to LPs, which were loaded with the enriched isotope of Boron (B^{10}). These LPs were able to achieve a tumor specific boron delivery system for boron neutron capture therapy, showing an increased and prolonged accumulation in glioma and providing for an increased survival of diseased animals[28].

Recent interesting reviews on innovative approaches based on nanocarriers[29-30] highlighted the role of nanomedicine for brain cancer treatment and imaging, opening the pave to the most innovative frontiers in nano-oncology, namely nano-theranostic for brain cancers. In particular, a step forward in tumor targeting technology was conducted by introducing a second targetor agent, in order to produce double-targeted systems in which the surface of LPs is modified to recognize both receptors for BBB crossing and receptors for tumor cells targeting. In this contest, with confocal microscopy analysis[31] Trojan-horse LPs were investigated for *in vivo* silencing of EGFR by the shRNA clone 967 encapsulated into. Considering that EGFR has a pro-angiogenic function in cancer, the suppression of the EGFR in glioma-bearing mice with the anti-EGFR shRNA therapy was correlated both to a significant reduction in tumor vascular density and to an increasing in median survival. Notably, the double surface conjugation strategy was also exploited to improve the accumulation of cytotoxic drugs to brain tumor[32]. As another example, LPs conjugated with WGA and Tamoxifen and encapsulating Topotecan, lead to an increase in survival of glioma-bearing rats 3-4 weeks after i.v. administration[32]. Double targeted LPs (Mannose and Tf) were also used to deliver Daunorubicin to glioma-bearing rats, showing a significant decrease in tumor growth and a consequent increase in animal survival[33].

1.2. Alzheimer's disease

Alzheimer's disease (AD), the most common form of dementia, is a chronic and progressive neurodegenerative disorder that begins with cognitive and memory impairments, accompanied with behavioral disturbances such as aggression, depression, hallucination, delusion, anger and agitation and eventually progresses to dementia, physical impairment and death[34-36]. The definite diagnosis of AD is made upon histological verification, established by biopsy or autopsy, of two main hallmarks: extracellular amyloid plaques and intracellular neurofibrillary tangles, which enclose hyperphosphorylated tau protein Furthermore, AD neuropathology, based on the *amyloid hypothesis* constituting of extracellular amyloid plaques and intracellular neurofibrillary tangles accumulations[34,37], is characterized by a progressive loss of synapses and neurons in a region- and cell-type-specific manner[38]. Microglia and astrocytes are activated by β-amyloid protein and related oligopeptides, leading to a cascade of events producing toxic molecules, neuronal damage, synaptic dysfunction and alteration of the permeability of the BBB[14]. *In vitro* and *in vivo* evidences highlighted the hypothesis on the involvement of BBB and cerebro-vasculature in AD[39-43] leading to pathophysiological alterations of BBB[40-43]. Thus, leakiness, anatomical modifications in BBB architecture and alterations of BBB influx/efflux pathways as well as deficient glucose transport and an altered excitatory amino acid transporters, with an unbalance of glucose, glutamate and other neurotoxic substances regulation in and out the brain strongly affecting the onset or progression of the pathology[44].

The inflammatory state is another common characteristic of the BBB in AD: this condition is featured by: i) neuroinflammation and relaps of neurotoxins by brain endothelium; ii) oxidative damage; iii) decreased cerebral blood flow with consequent hypoxia and inefficacious transport of nutrients to the brain[45-46]. Even if changed in its dynamics, BBB integrity in AD could be considered as "maintained", therefore orienting the planning of nanocarrier engineering, since a strategy for BBB crossing is required along with a targeting moiety for AD hallmarks targets.

FIGURE 2

Polymeric NPs made of different materials (PBCA, PLGA and chitosan) were employed in *in vivo* with different loaded molecules and different targeting strategies. One of the most used approaches is based on surfactant coating of NPs. In particular, the role of surfactants (PS-80 as well as polaxamers or PEG) as possible trigger-factor for BBB permeability increase was deeply debated by several authors[22-23], hypothesizing the role of apolipoprotein A-I (ApoA-I) absorption onto surfactant-coated NPs leading to a possible interaction with BBB receptors and triggering receptor-mediated transcytosis of NPs. As examples, nerve growth factor (NGF) loaded in PBCA NPs covered with PS-80 (for BBB crossing) was able to reduce amnestic effect, if compared to NGF-free solution treatment[47]. Similarly, PBCA NPs were coated with PS-80 and or with PEG5000 and loaded with tacrine (reversible inhibitor of acetylcholinesterase): both kind of surfactant-coated NPs

were able to improve cognitive function[48], with a greater effect obtained with drug loaded PBCA NPs covered with PEG5000 (with respect to PS-80 coated NPs and free solution) probably 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.

Another example of surface modification of NPs for BBB crossing and application in AD is constituted by the use of ApoE3 and the exploitation of its interaction with LDL receptor. In this view, curcuminoids were loaded into NPs modified with ApoE3 and their anti-inflammatory and antioxidant activities[49] along with their potential in preventing Ab aggregation and toxicity on neurons[50-51] were assessed *in vitro*[52-53].

All the examples cited before are regarding only surface modification for BBB crossing: none of them considering the amyloid targeting. The unique example of a double targeting strategy (BBB crossing plus Ab targeting) is referred to chitosan NPs aiming to develop immunotherapy against Ab1-42. Chitosan NPs, functionalized with a polyamine modified F(ab') portion of IgG4.1, an anti-amyloid antibody [pF(ab')24.1], were able, in *in vitro* model, to cross the BBB and, after administration in animal models, to target the brain amyloid plaques[54-55].

Similarly, despite many examples are reported in literature regarding LPs use for AD treatments, only few papers reported the application of a double targeting technology. In particular, antibodies were employed as ligands for LPs surface to target both to the BBB receptors and to AD target. In fact, LPs modified with Aβ-mAb and OX26 mAb (against TfR) highlighted a very high affinity for Aβ peptides[56] and an increased interaction with human immortalized brain capillary cells (hCMEC/D3)[57]. The preliminary *in vitro* results evidenced serum stability and the ability of cellular uptake, suggesting that dd-LIP can be used as AD targeted therapeutic systems, even if, as exposed before, the affinity of OX-26 for the receptor could affect their trafficking[57] .

1.3. Parkinson's Disease

Parkinson's disease (PD) is a multicentric neurodegenerative disease, which affects 1% of people over age 60[34]. Clinical features of PD include tremor at rest, bradykinesia, rigidity and flexed posture[58] *(Hughes et al., 1993)*. Pathologically, the key deficit in PD is almost connected to the loss of dopaminergic neurons in the *substantia nigra*, with consequent reduction of dopamine level[59]. Cell loss and Lewy body formation (abnormal intraneuronal aggregates of protein, predominantly a-synuclein) occur in the locus coeruleus, dorsal motor nucleus, and substantia innominata[60]. 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. These symptoms include cognitive decline, apathy, depression, anxiety disorders, hallucinations, gait and balance disturbances, sleep disorders, sexual dysfunction, bowel problems, drenching sweats and pain[61].

Dramatically, no permanent rescue and cure for PD is actually present in therapy; this situation is dramatically getting worse since there is a complete un-knowledge of agents able to stop and to reverse the progression of PD. Therefore, the current treatments of PD are only aiming to decrease the impact of symptoms[62-63]. Although symptomatic therapy of PD is effective (Levo-Dopa), novel therapeutic strategies are required. In this view, advanced delivery methods could be reasonably considered as primary goals as the active substances must enter the CNS to exploit their effects.

Animal PD model is obtained through injection of neurotoxins: the most used toxin is 6-hydroxydopamine (6-OHDA stereotaxical injected)[64], but also 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP i.p. injected) is used[65]: both agents destroy neurons by generating active oxygen species such as superoxide radicals.

Remarkably, at present, regarding this pathology no significant evidences of BBB permeability alteration in patients and in animal models are fully described and detailed.

FIGURE 3

Thus, nanotechnology-based strategies were mainly based nanocarriers, which, independently from the type of drugs loaded, need to be modified on their surface to trigger BBB crossing. In this view, PS-80 coating was again employed for BBB crossing of PBCA NPs loaded with nerve growth factor (NGF)[47]. After administration to MPTP mice, some significant improvements in PD symptoms were assessed already after 90 minute and maintained for 21 day.

By using a different animal model (6-OHDA stereotaxical injected rats) LPs were loaded with glial-derived neurotrophic factor (GDNF) plasmid and targeted to the brain with OX-26 (mAb against the mouse TfR). After a single administration to 6-OHDA rats, a general improvement of PD symptoms, a decrease in rotational behavior and a great increase of Tyrosine Hydroxylase (TH) activity were achieved[66]. These remarkable results were improved with multiple pulses injection of the LPs[67]. In other works[68-69], the same kind of targeted LPs were loaded with 877 cDNA (a complementary DNA of TH gene) and after administration at different doses, an improvement in PD symptoms was allowed until 3 day post injection, over 6-9 days. The same targeted LPs were loaded with TH plasmid, allowing a prolongation of the neuroprotective effect limited to 3 days after the injection[70].

Haloperidol and chlorpromazine were also used to induce extra pyramidal effects in rat or mice. In particular, LPs formulated with two surfactants (span 80 or tween 80) and loaded with dopamine were injected in rats treated with haloperidol. Both formulations lead to an increase in muscular coordination activity, tested with the rotarod test, along with in locomotor activity[71].

1.4 Multiple Sclerosis

Multiple Sclerosis (MS) is a disorder of the CNS with inflammatory and neurodegenerative components, featured by a variable clinical pattern, ultimately leading to a progressive neurological dysfunction[72]. MS pathological hallmarks consist of demyelination and cellular infiltration of T cells and macrophages. Up to today, no current resolutive therapy exists for MS: immunotherapy and repeated injections of high dose glucocorticosteroids (GS) or potent anti-inflammatory drugs represent the most common treatment protocols.

In MS, BBB integrity is progressively lost with the increase of disease severity due to a partial loss and delocalization of brain endothelilal junction proteins (occluding and VE-cadherin mainly)[73-74] . The experimental autoimmune encephalomyelitis (EAE) is the most applied *in vivo* experimental model of MS, featured by high tissue inflammation and partial disruption of the BBB[75-77].

FIGURE 4

Thus, in this particular case of CNS-disease, the engineering of the surface nanocarriers is not needed, or at least it does not represent the first goal. The strategy to be applied for innovation in MS therapy could be based on the planning of nanosystems able to extravasate and to circulate in the bloodstream for a long period of time.

As examples, long-circulating pegylated LPs loaded with prednisolone, without any targeting ligands for BBB crossing, were able to efficiently deliver the glucocorticalsteroids[78-79] up to a high concentrations in the inflamed cerebral area of autoimmune EAE rats model as compared with an equivalent dose given as free drug. This approach leads to a reduction of inflammation and to a significant improvement in the disease course of EAE. Notwithstanding the authors hypothesized a selective targeting, it is almost clear that the altered permeability of BBB remarkably enhanced the penetration and the localization of LPs in inflamed tissue.

Similar outputs were achieved with long-circulating pegylated LPs[80] delivering a potent antioxidant (tempamine) up to brain concentrations able to significantly attenuate clinical symptoms of EAE in the mouse model[81]. The authors hypothesized that the size and the long-circulating properties of these LPs could favor their extravasation into permeable inflammation sites.

1.5. Epilepsy

Epilepsy is one of the oldest known conditions to mankind and still the most common neurological condition affecting individuals of all ages; 50 million individuals worldwide have a diagnosis of epilepsy[82-84]. It is defined as a condition featured by recurrent (two or more) epileptic seizures, unrelated to any immediate identified cause[85].

Considering BBB state, epileptic seizures are known to alter BBB properties and to increase its permeability[86-88]. This condition remains overtime and may contribute to the progression of epilepsy and development of novel seizures due to an increased excitability in the epileptogenic foci[88]. Despite this change in permeability, a clear disruption of BBB is not reported for epileptic brains in contrast with what reported for gliomas and strokes. Thus, the planning of nanocarriers for possible novel approaches in epilepsy treatments could vary from "passive" targeting to "active" targeting for BBB crossing, depending on the grade of the pathology.

FIGURE 5

As an example of "passive" targeting, LPs without any surface engineering and loaded with valproic acid and g-butyrolactone-g-carbonyl-L-histidyl-L-propynamide citrate produced significant anti-convulsive effects after i.v. administration in amygdaloid-kindled rats[89-90]. Since positively charged LPs lead to prolonged anti-convulsive effects, a combination of higher protection of the drug from the enzymatic degradation and adsorbtive-mediated endocytosis for BBB crossing were hypothesized as responsible for the anticonvulsivant effect, even if a real proof of both mechanisms was not definitely given.

The same technology was applied for the evaluation of the effect of phenyntoin in rat model of epilepsy. Interestingly, the suppression of central amygdalic discharges was greatly achieved by multiple pulsed administrations of loaded LPs, leading to a higher blood concentration of LPs. In fact, in epilepsy, the blood flow is reliably increased in the epileptogenic focus as well as the permeability of BBB increases over the disease progression. Both these conditions could lead to locally augmented concentrations of LPs, especially in brain area[91] .

Considering the "active" targeting, PS-80 coated PBCA NPs were used for BBB crossing and to allow brain delivery of a novel non-competitive NMDA receptor antagonist MRZ 2/576. This drug was potent but rather short-acting anticonvulsant, rapidly discharged from the central nervous system by transport processes that are sensitive to probenecid. After i.v. administration in mouse model of convulsion, these NPs reasonably operated as drug delivery systems able to protect and to prolong the release of drugs leading to an improvement in the length of the anticonvulsive effect (up to 210 min)[92]. 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, even if, remarkably, no evidence of BBB state (healthy or diseased) was given with regards to this animal model.

1.6. Lysosomal Storage Disorders

The lysosomal storage diseases (LSDs) consist of heterogeneous group of disorders which affect 1/7,000 live-born infants, the majority of which develop CNS disease. Each LSD (more than 40 types) results from a deficiency of a single lysosomal enzyme, pivotal for degrading

macromolecules that must be turned over in lysosomes[93]. The neurological compromission is generally refractory to treatment, usually based on enzyme replacement therapy (ERT). The failure of treatments is due to the inability of enzymes in crossing the BBB[94]. Moreover, no clear data are present in literature concerning the status of BBB in LSDs. The only available evidences are limited to a restricted number of LSDs in which BBB state is almost conserved in its integrity with few features compromised (i.e. increase in macropynocitotic and endocytotic processes)[95].

Thus, if an increased in BBB endocytosis and macropynocitosis is confirmed also for the other types of LSDs, it appears almost clear that nanotechnological interventions could represent the most promising approach, since the above described pathways for BBB crossing (namely endo- or macropinocytosis) are nicely shared with those used by nanocarriers to overcome BBB.

FIGURE 6

In this contest, LPs loaded with β-glucuronidase (GUSB) expression plasmid (p-CMV GUSB)[96] and superficially engineered with the rat 8D3 MAb to the mouse transferrin receptor (TfR) to achieve BBB crossing were tested in mice model for type VII mucopolysaccharidosis (MPS-VII), deficient for GUSB lysosomal enzyme. The results demonstrated that therapeutic brain levels of GUSB enzyme were improved after i.v. administration of the TfR MAb-targeted LPs encapsulating p-CMV GUSB plasmide. Notably, 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[97].

1.7. Stroke

After heart disease and cancer, stroke is the third largest killer, second most common cause of neurologic disability after AD[98], with over five million deaths/year and over nine million stroke survivors. The etiology of stroke is brain vascular occlusions (thrombotic stroke) or rupture (hemorrhagic stroke) and its neuro-pathological hallmarks consist of necrotic infarcts of variable size with inflammatory gliosis.

In the case of stroke, BBB integrity is almost lost[99-101]: the hypoxic/ischemic condition leads to a wide disruption of tight junctions and increased BBB permeability, probably mediated by cytokines, VEGF, and NO. Moreover, elevated levels of proinflammatory cytokines, IL-1β, and TNF-α have been demonstrated in animal brains after focal and global ischemia[102] and in cerebrospinal fluid of stroke patients[103]. The same BBB leakiness is shared by animal models of stroke (focal cerebral ischemia) produced by the method described by Chen and colleagues[104] based on an extensive, but reproducible infarct.

As in other previously described neuro-pathologies, since the BBB is not functioning as protective barrier, nanocarriers do not need any BBB crossing strategy.

FIGURE 7

Consequently, "passive" targeting is mostly applied considering that nanocarrier goal principally lies in protecting and modulating the delivery of un-stable drug during the ischemic insult or at least to prevent the neuronal damages after ischemic attack. The success of this strategy is correlated to the facilitated diffusion of nanocarriers through the compromised and broken BBB. As one of the first application of nanocarriers to manage stroke, superoxide dismutase (SOD) loaded into LPs, achieved an ameliorated condition[105] of model animals of stroke, manifesting the reduction of brain superoxide radicals levels. These outputs were due to both the protection of SOD exploited by LPs along with their ability to operate a prolonged delivery of drug. Using the same animal model, LPs were able to mediate an increase in SOD activities into the brain, not only in the infarct, but also in the non-infarcted subcortical areas. To give explanation of this interesting side-result, the authors hypothesized that SOD loaded LPs after crossing of leaky BBB in the ischemic zone were up-taken by adjacent neurons and microglia, thus extending the area of action. Otherwise, SOD loaded LPs could exert their action on extracellular superoxide on the luminal side of capillaries, without penetrating the BBB[106] .

The same active substance (SOD) was encapsulated into PLA NPs, able to stabilize SOD from degradation, to provide a localized modulated brain delivery and letting to a prolonged neuronal protection against the mediators of reperfusion injury, namely reactive oxygen species[107].

Above SOD, citicoline was loaded into LPs, letting to increased survival rate in ischemic-reperfused rats and a reduction of lipid peroxidation, compared to the free drug[108]. Similarly, LPs were used to protect the drug (calpain-inhibitor), to maintain high drug concentration both in blood and in brain and to finally promote rescue form ischemic neuronal damage after administration in gerbils stroke models[109].

Antioxidant quercitin encapsulated in LPs[110-111], administered i.v. to rats (pre-treated with arsenic), was able to attenuate oxidative damage induced by ischemia reperfusion injury. Recently, the same research group proposed the use of PLGA NPs as quercitin carriers in combating arsenic induced oxidative damage in rat brain. After oral administration of quiercitin loaded NPs, arsenic cerebral oxidative damage was prevented[112]. Even if the authors hypothesized the ability of these NPs to cross the BBB via particle uptake mechanism based on an endocytotic pathway by the brain capillary endothelial cells, no data is available concerning the maintenance of BBB integrity in arsenic-animal model. Therefore, it is impossible to draw an absolute conclusion on the real efficacy and BBB crossing pathway by these NPs. More realistically, the increased efficacy of these formulations correlated to their ability to stabilize the drug and to prolong drug brain delivery, since, in these pathological conditions, BBB is inactive and broken.

Another case of use of PLGA NPs as drug protecting carriers was recently discovered: the effect of cerebrolysin (CBL)

loaded into PLGA NPs on neuroprotection and neurorepair after rat model of concussive head injury was assessed in comparison with free drug. Changes in blood-brain barrier and brain edema formation, measured as parameters of neuroprotection in CHI clearly showed that delivery of CBL by NPs has superior neuroprotective effects following CHI as compared to normal CBL[113].

Finally, after administration in focal cerebral ischemia model rats, the novel glycine-b site NMDA (N-methyl-D-aspartate) receptor antagonist MRZ 2/576 was formulated in PBCA NPs[114] providing for neuroprotection and modulation of the delivery of the drug to the CNS.

1.8. Infectious disease

Brain inflammatory diseases such as meningitis and encephalitis 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) or viruses[115-117]. Despite the general efficacy of antibiotic treatment, high mortality and morbidity are frequently recovered due to the difficulty of drugs to cross the BBB and access the brain.

In this category of neuro-pathologies, CNS is often featured by a general state of inflammation and the BBB permeability changed depending on the period of the infection with evidences of some "enhanced" specific pathways exploited by pathogens for BBB crossing and to invade the CNS. These pathways, used by pathogens to enter the brain, the CNS, could also be exploited by nanomedicines to the same goal. Thus, nanocarriers aiming to treat neuro-infections could be properly designed in order to cross the BBB maybe taking advantage of some "enhanced" brain-entry pathways and to finally eradicate the pathogens from CNS.

FIGURE 8

The most consolidated approach for brain infection is based on the use Ambisome ®, a liposomal formulation of amphotericine B in which the drug is strongly associated with the bilayer structure of unilamellar and un-targeted LPs.

Several works, using different animal models, provided evidences of AmBisome effectiveness against brain infections. Treatment of un-infected and Candida albicans-infected rabbits[118] with AmBisome and the other commercially formulation of amphotericine B (Amphotec ®), highlighted the superiority of AmBisome treatment able to completely clear Candida albicans from the brain of infected animals. Similar results in terms of efficiency of AmBisome therapy (20-30 mg/Kg) were achieved with a mouse model of meningitis caused by Cryptococcus neoformans[119], letting to disappearance of Cryptococcus in the brain. This dosage was reduced (10 mg/Kg), but still maintaining efficacy and more importantly directly correlating the brain penetration with the progression of cryptococcal meningitis[120]. AmBisome was also tested to treat different brain infections, as coccidioidal meningitis (in rabbits)[121]. More recently, the authors studied the efficacy of AmBisome in comparison to micafungin (MICA), caspofungin (CAS), amphotericin B, voriconazole (VCZ). After administration in immune-suppressed mice, intracerebrally infected with Aspergillus fumigates, the brain infection was significantly reduced by Ambisome treatment if compared with fluconazole and amphotericine B treatments. Moreover, combination regimens were tested, highlighting that only AmBisome and VCZ, at suboptimal doses, improved the outcome in CNS aspergillosis[122]. Thus, since AmBisome technology is based on LPs without any BBB-crossing ligands, its ability in treating brain infections is surely related to alterations of the permeability of BBB, which is strongly affected by pathogen-driven brain inflammation.

Recently, polymeric NPs were investigated as an alternative treatment in brain infection diseases. A very elegant example of this strategy consists of the creation of auto-assembling NPs made of amphiphilic cholesterol conjugated with a cationic peptide containing TAT sequence. NPs are simultaneously vehicles and drugs, as they possessed a broad spectrum of strong antimicrobial activities, much stronger than the hydrophilic peptide, incapable of forming NPs. These NPs were found to be as efficient as vancomycin in treating the meningitis in rabbits[123] and against brain infections by Cryptococcus neoformans in rabbit model. NPs were able to suppress the yeast growth in the brain tissues with similar efficiency as amphotericin B did[124]. Interestingly, as NPs are decorated onto their surface by a cationic peptide, BBB crossing was supposed to take place through adsorptive-endocytosis.

PLA-b-PEG NPs loaded with amphotericin and coated with polysorbate 80 for BBB crossing[25, 125] were exploited for the treatment of cryptococcal meningitis-in mice model. 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[126].

2. CONCLUSION and FUTURE DIRECTION

The planning and the design of nanosystems aiming to be tools for the treatment of neurological disorders is a critical point which strongly impacts on the effectiveness of the application of nanomedicine for brain diseases treatments.

Beside the aspects of innovation in biopolymers and beside some relevant issues connected to pharmaceutical nanotechnology (i.e. optimization of formulation protocols of selected drugs, coupling strategy for surface modifiers, etcc), the most urgent need is connected to the clear individuation of those features of nanomedicines which are essential for the selected pathology. If in the past, the only topic (and the main goal) was only to create nanovectors able to cross the BBB, nowadays it is evident how the strategy for "targeting" selected pathology must come out from the fusion of different inputs by nanotechnology, pathophysiology and pharmacology, which obviously differing from pathology to pathology.

As shown, depending on the pathology, BBB crossing could be not essential (stroke or gliomas), in other cases some BBB features are changed (i.e. permeability in AD or MS) and in other situations some changes in BBB dynamics could be exploited by nanomedicinesto increase the chances of success (LSDs and Infectious Diseases).

Thus, in the next future, these evidences inevitably will change the rationale of the planning of the most suitable *nanomedicine*, leading inevitably to "*pathology-tailored personalized nanocures*".

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Figures and figure captions:

Figure 1: Glioma. Blood-Brain Barrier features and nanomedicine approaches. For references to existing strategies, please see chapter 1.1.

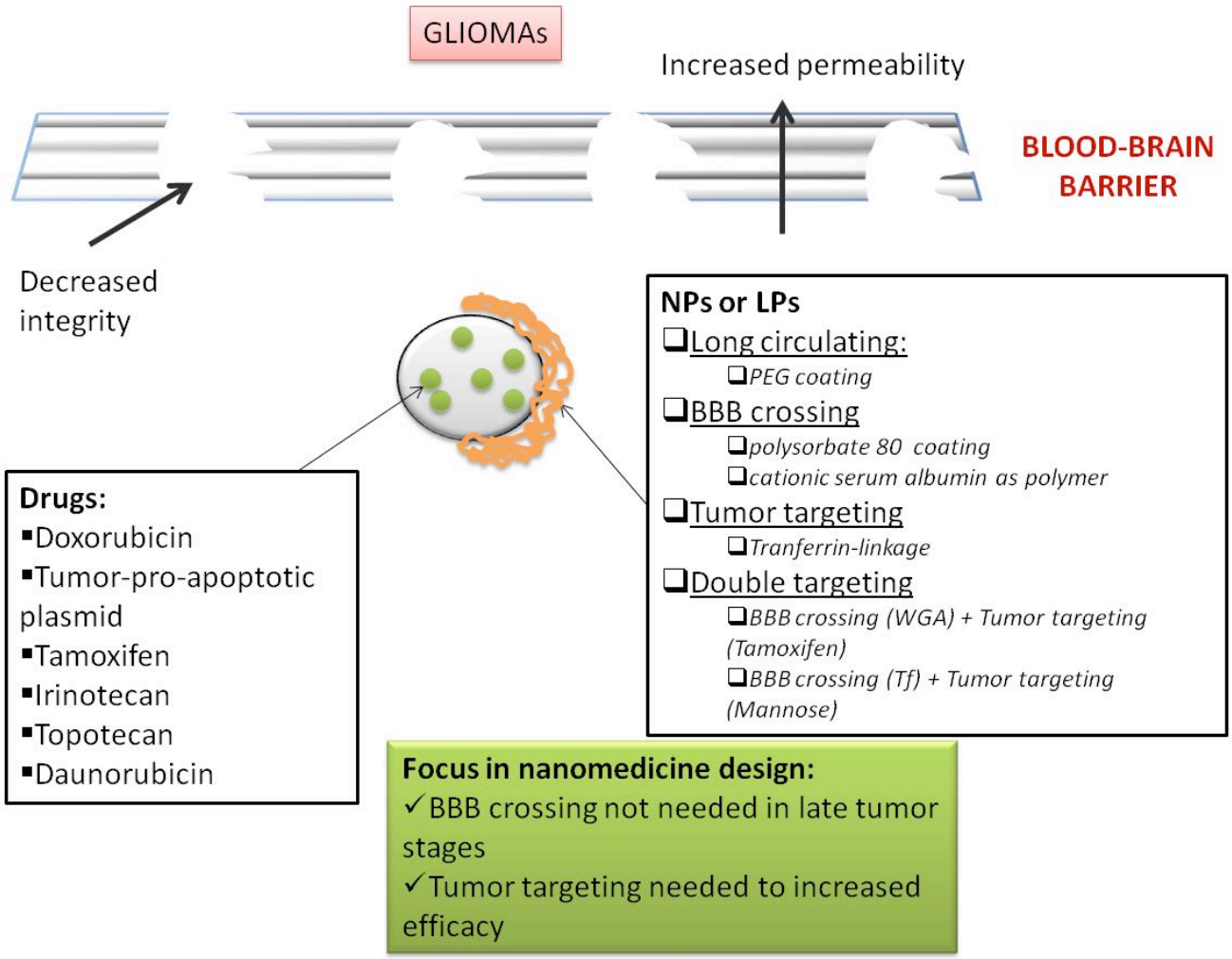

Figure 2: Alzheimer Disease: Blood-Brain Barrier features and nanomedicine approaches. For references to existing strategies, please see chapter 1.2.

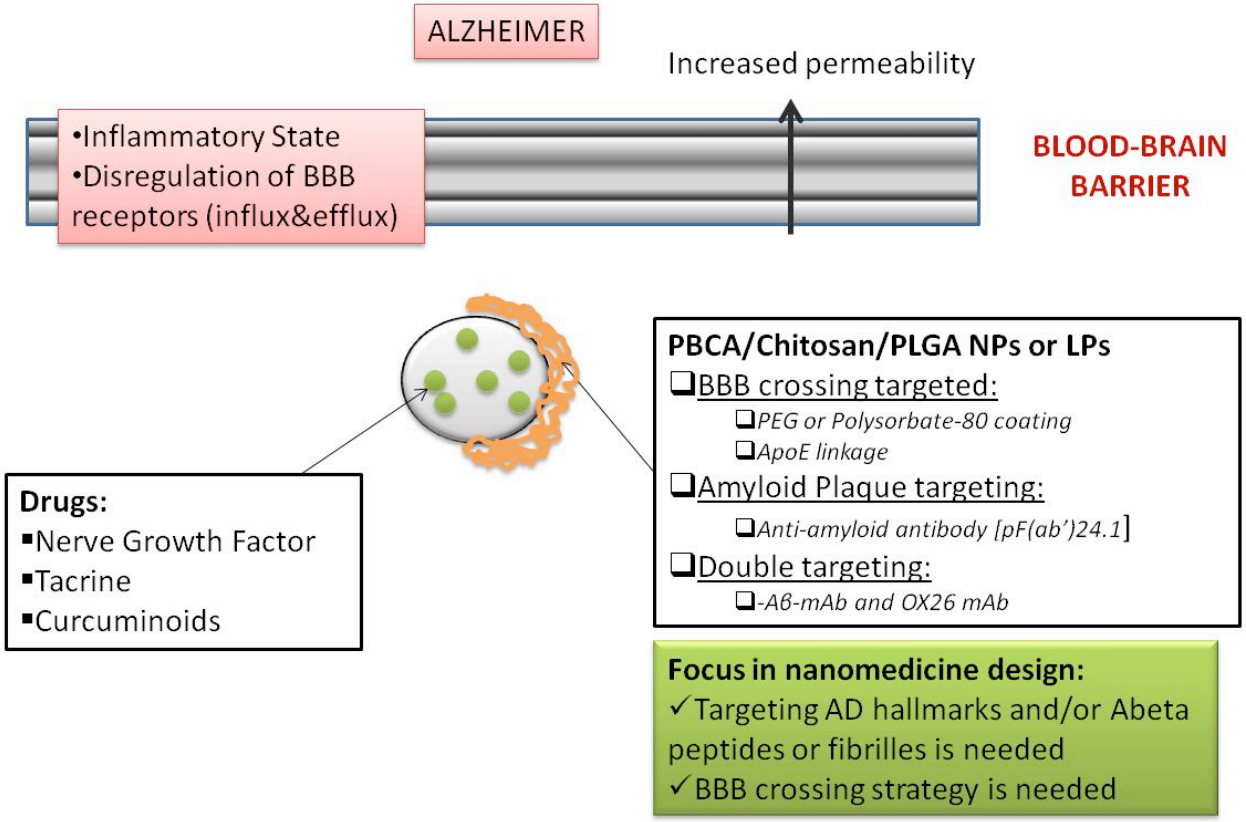

Figure 3: Parkinson's Disease: Blood-Brain Barrier features and nanomedicine approaches. For references to approaches, please see in the chapter 1.3.

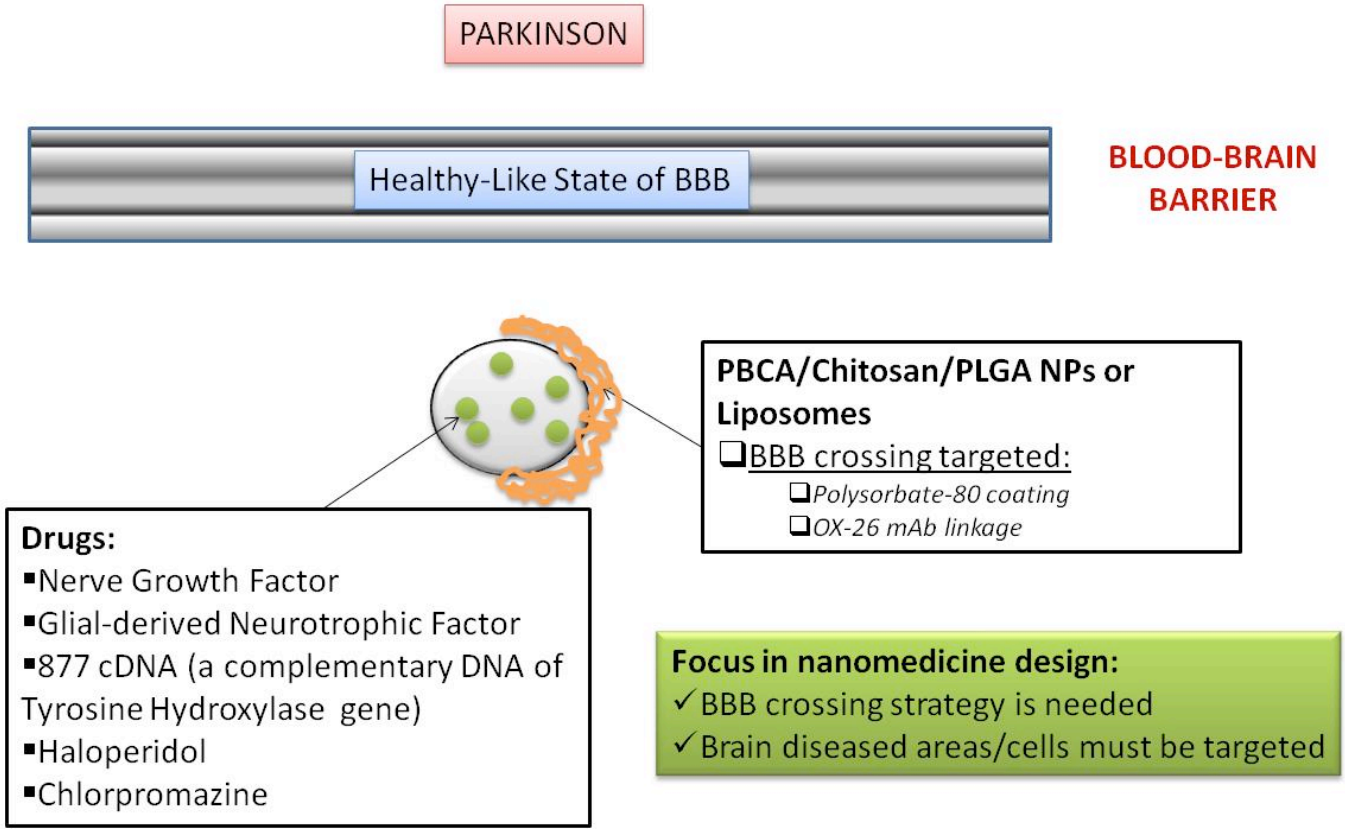

Figure 4: Multiple Sclerosis: Blood-Brain Barrier features and nanomedicine approaches. For references to approaches, please see in the chapter 1.4.

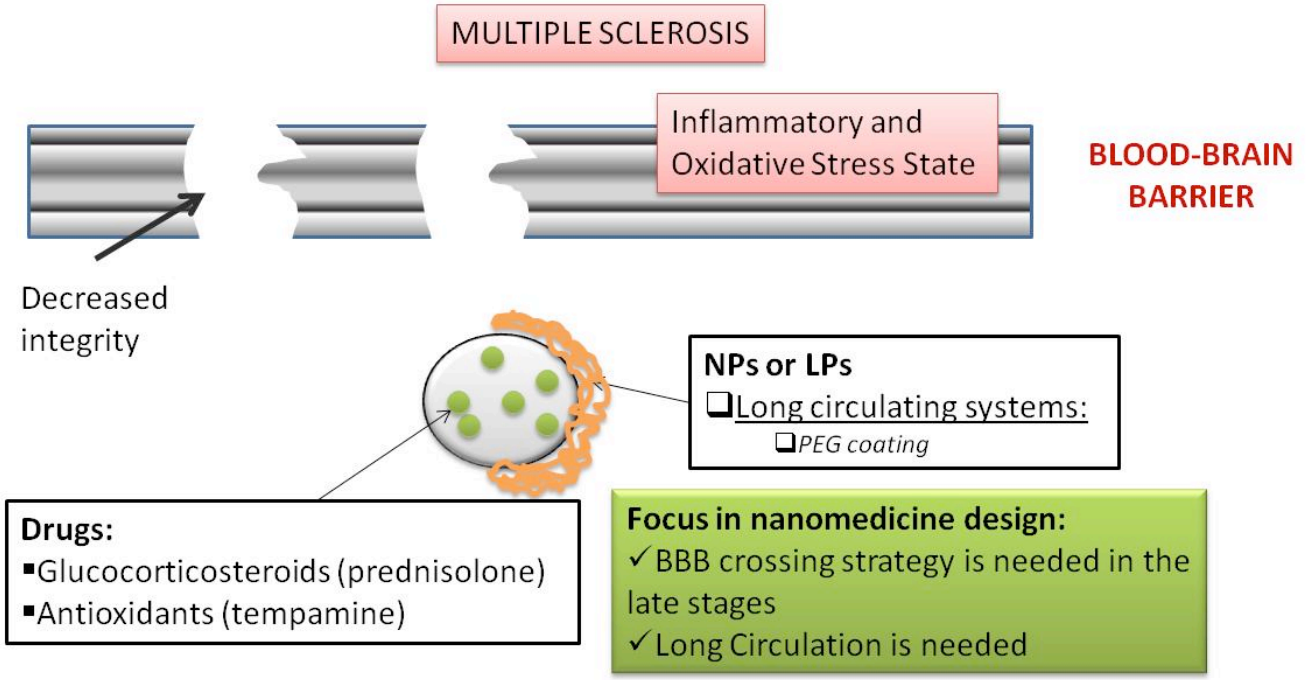

Figure 5: Epilepsy: Blood-Brain Barrier features and nanomedicine approaches. For references to approaches, please see in the chapter 1.5

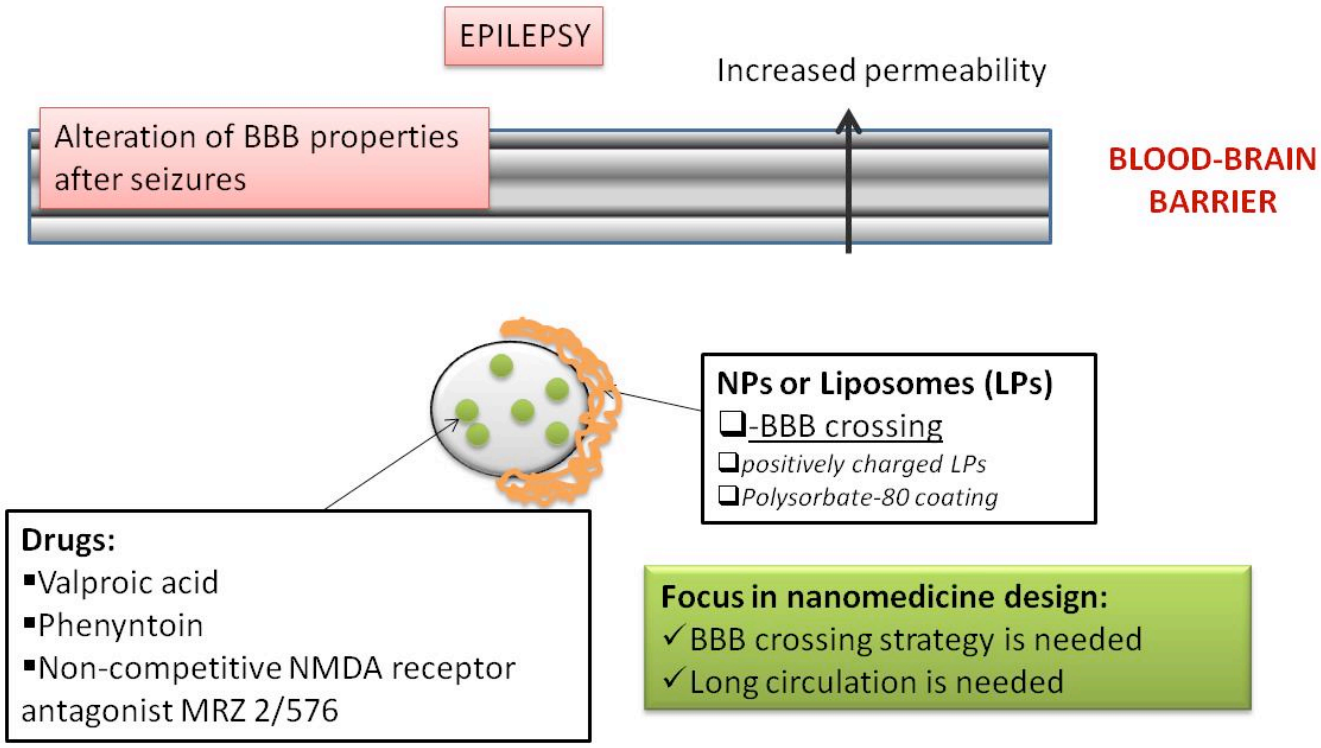

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Figure 6: Lysosomal Storage Disorders: Blood-Brain Barrier features and nanomedicine approaches. For references to approaches, please see in the chapter 1.6

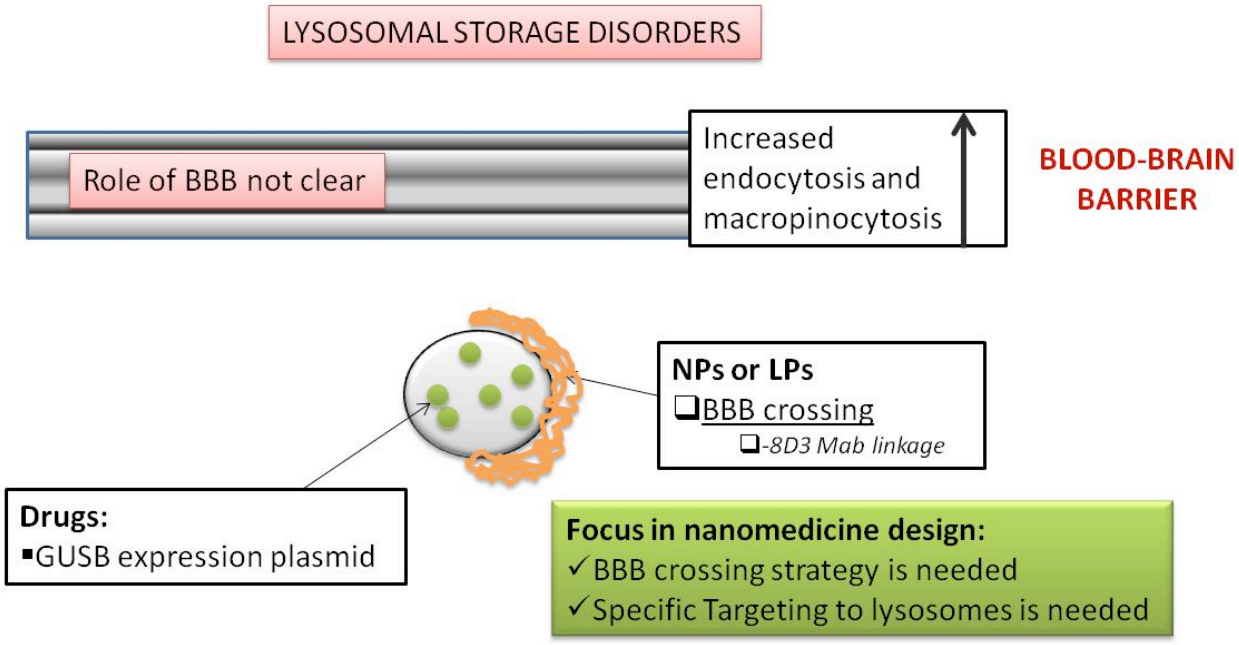

Figure 7: Stroke: Blood-Brain Barrier features and nanomedicine approaches. For references to approaches, please see in the chapter 1.7.

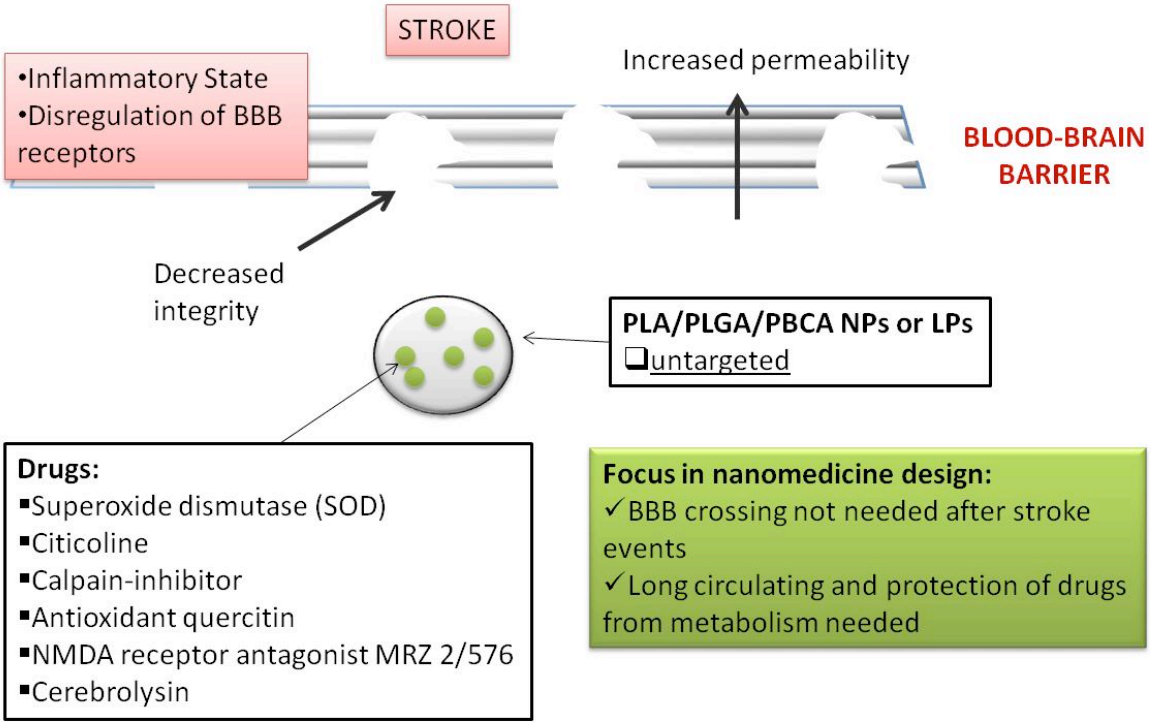

Figure 8: Infectious Disease: Blood-Brain Barrier features and nanomedicine approaches. For references to approaches, please see in the chapter 1.8.