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Brain-targeted nanoparticles to overcome the blood-brain barrier

Indrit Seko[1], Adem Şahin[2], Hayrettin Tonbul[3], Yılmaz Çapan[1*]

[1]Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
[2]Department of Pharmaceutical Technology, Faculty of Pharmacy, Selçuk University, Konya, Turkey
[3]Department of Pharmaceutical Technology, Faculty of Pharmacy, İnönü University, Malatya, Turkey

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*Corresponding author:
ycapan@hacettepe.edu.tr

ABSTRACT

The blood-brain barrier is one of the most complicated barrier to pass for therapeutic drugs. Because of the structure of the blood-brain barrier, only a few small molecules with appropriate lipophilicity, molecular weight, and charge can penetrate through the blood-brain barrier and pass in the central nervous system. Because of this unique property, blood-brain barrier is still a major problem for the treatment of central nervous system diseases. In the last decades, many strategies to overcome this barrier have been investigated. Compared to other drug delivery strategies, due to the reduced side effects and no requirement for surgical operations, brain targeted nanoparticle is one the most promising and popular strategy used do deliver drugs to the brain. Many *in vitro* and *in vivo* preclinical studies have been conducted to determine optimum brain targeted nanoparticles. These studies were reported that characteristics of nanoparticles such as particle size, zeta potential, and targeting ligand are critical to achieving the goals. In this review, first of all, the structure of the blood-brain barrier and possible causes of blood-brain barrier disruption were summarized. Later, previous strategies of brain targeted drug delivery and characteristic prosperities for optimized brain-targeted nanoparticles were evaluated. Moreover, different strategies, such as focus ultrasound, which can increase the effectiveness of nanoparticular system applications, are mentioned.

1. INTRODUCTION

About 100 years ago, after the intravenous injection of a proper dye, it was observed that most of the organs other than the brain were dyed. It was understood that this situation is due to a specific structure of vessels between the brain and blood, which is called the blood-brain barrier (BBB) [1]. The BBB is also one of the most complicated barrier to pass for the therapeutic drugs, and because of the structure of the BBB, only a few small molecules with appropriate lipophilicity, molecular weight, and charge can penetrate through the BBB and pass in the central nervous system (CNS). Reports have shown that as much as 98% of small molecules and nearly all large molecules (molecular weight >1kD, i.e., recombinant DNA or gene-based medicines) cannot penetrate through the BBB [2]. On the other hand, the prevalence of CNS's diseases increases with the aging population [3-5]. For the treatment of these diseases, many new compounds were developed, but most of them did not reach the market. After the evaluation to find the causes of these failures, the poor brain penetration of drugs was identified as one of the critical factors [6]. In the last decades, many strategies have been investigated to overcome this barrier [7]. When compared to other drug delivery methods, brain targeted nanoparticle is one of the most popular and critical drug delivery strategies thanks to reduced systemic side effects and no requirement of surgical operation [8]. The physicochemical, pharmacodynamics, and pharmacokinetic properties of the therapeutics could be improved by developing nanoparticular delivery systems [9].

Additionally, passing the biological barriers such as the BBB could be achieved by targeted nanoparticles [10]. These nanoparticles could be prepared with different natural or synthetic materials, and also characteristics of these nanoparticles are critical to achieving the goals. In this review, previous strategies of brain-targeted drug delivery and characteristic prosperities for optimized brain-targeted nanoparticles were evaluated.

2. STRUCTURE OF THE BLOOD-BRAIN BARRIER

The BBB is a selective barrier consisting of endothelial cells from cerebral capillaries, astrocytes, and pericytes (**Figure 1**) [11]. The BBB main function is to separate the brain neural environment from the blood circulation in the brain unlike the peripheral capillaries that allow the relatively free exchange of substances between blood and tissues; the BBB has the least permeable capillaries in the entire body

due to physical barriers (tight junctions) [12]. A single layer of endothelial cells forming the brain capillaries makes up the BBB, which functions as a barrier to create the proper environment for synapsis and neural function [13]. Damaging of BBB's proper function is related to Alzheimer's disease, multiple sclerosis, and Parkinson's disease onset and progression [14,15]. Due to the complexity of the BBB, our knowledge on the issue is limited. The endothelial cells making up the vessel wall form the BBB, which displays biological properties different from other cells. These unique biological properties separated them from peripheral endothelial cells. These properties include;

- A physical barrier created by tight junctions (TJs) between adjacent cells forming the BBB preventing the free transport of molecules to the brain
- Specific transporters are expressed to regulate the influx and efflux of substrates
- Transcellular transport through the cell wall is limited by low transcytosis rate
- The entry of the immune system cells is limited by low expression of leukocyte adhesion molecules in CNS endothelial cells (Glycocalyx is responsible for preventing the immunity system cell penetration into CNS) [16,17].

However, barrier features are not attributed only to endothelial cells. CNS blood vessels are neurons that are separated by pericytes and astrocytes, which serve as an interface. These whole structure formed is called neurovascular unit [18,19].

The cells comprising of neurovascular units have different functions related to BBB.

- Astrocytes
  - BBB integration
  - TJs expression, brain transporters and enzymatic systems associated with BBB regulation [20]
  - Tissue plasminogen activator (TPA) and the anticoagulant thrombomodulin regulation [21]
- Pericytes
  - Regulate by releasing growth factors for vessel formation (angiogenesis) and vessel maturation [22]
  - Crucial for barrier formation, however during adulthood barrier maintenance they can be dispensable [23]
  - Active in the clearance of amyloid aggregates which play an active role in Alzheimer's disease [24]

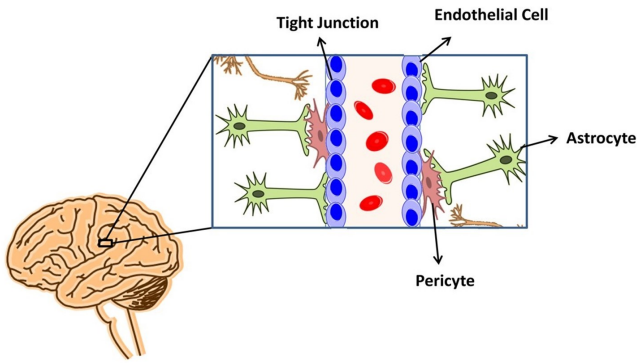

Figure 1. *Schematic representation the blood-brain barrier*

Mutations affecting cells which have different functions in BBB formation and maintenance lead to BBB disruption, which is the cause of many neurological diseases [14]. These mutations provide proof that BBB disruption and other vascular defects in humans contribute to the start and progression of neurological deficits.

This physiological barrier of BBB is coordinated by a series of physical, transport, and metabolic properties possessed by the endothelial cells that form the walls of the blood vessels. In peripheral vessels, molecules can pass through endothelial cells by the transcellular route, but in the BBB endothelial cells, paracellular transport is more common [11]. Specific transporters to carry specific compounds are located in luminal and abluminal sides of the endothelial cells forming a no transport barrier, facilitating or permitting the entry of necessary nutritious compounds and effluxing of harmful compounds generally large hydrophilic peptides and proteins cannot pass through the BBB, the only way to reach the CNS is using specific transportation receptor-mediated transcytosis or less specific way adsorptive mediated transcytosis (**Figure 2**) [25].

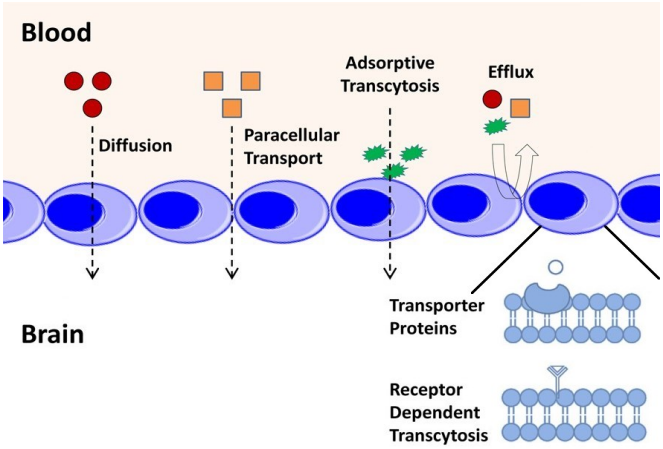

Figure 2. *Schematic illustration of transport mechanisms across the blood-brain barrier*

2.1. Diseases and Mutations Leading to BBB Disruption

2.1.1. Mutations

SLC2A1

Mutations in endothelial cell glucose transporter GLUT1 encoding SLC2A1 genes may lead to microcephaly, seizures, and development delay [26]. In a study conducted in GLUT1 mutated mice, it was reported that BBB disruption occurred within three weeks due to glucose uptake reduction and TJs loss, which led to impaired brain perfusion, vascular regression, the onset of neurodegenerative changes and microcephaly [27].

MFSD2A

Mutations in encoding MFSD2A genes which is responsible for endothelial cell omega-3 transporter (Docosahexaenoic acid transporter) and caveolae-mediated transcytosis regulator across BBB, may lead to microcephaly, neuron loss and mental disability [26,28-30]. MFSD2A gene also suppresses the caveolae-mediated transcytosis; hence mutation of the gene increases the transport through BBB, promoting disruption of endothelial

barrier [31]. Decreased or diminished MFSD2A gene expression in endothelial cells in tumors leads to BBB disruption and reduced omega-3 (DHA) transport. By changing of DHA transport, this specific gene suppression promotes cancer metastasis and creates a suitable environment for cancer development. Therefore, restoration of DHA transport and metabolism functions to normal may be suggested as a method to reduce metastasis and cell growth in brain cancers.

OCLN

OCLN gene is responsible for encoding occludin protein, which is essential for the right function of endothelial TJs. OCLN mutations promote uncontrolled passage of blood elements to the brain leading to severe microcephaly, seizure onset, and development delay. Additionally, occludin gene silencing promoted cancer and metastasis [27].

Various gene mutations related to BBB development and maintenance may promote higher risk of Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington Disease (HD), and Amyotrophic Lateral Sclerosis (ALS).

2.1.2. Neurodegenerative diseases

Alzheimer's Disease

One of the major risk factors for AD is apolipoprotein E4 (APOE4) [32-34]. APOE4 carriers possess a high risk of BBB disruption and vascular pathology [35]. When compared to APOE4 non-carriers, APOE4 carriers can be exposed to BBB disruption, neurovascular unit dysfunction including pericyte degeneration, decreased glucose uptake and damaged cerebrovascular activity [36-41].

BBB disruption may also appear as the cause of amyloid precursor protein (APP) mutations. All these results are supported by human and transgenic animal model studies [42-45].

Some study results reported that AD pathologies appear after the BBB disruption occurs. Besides, tau transgenic animal models show that BBB disruption, leukocyte, red blood cell, and IgG infiltration before any sign of tau pathology verifying the upper statement [46].

Parkinson's Disease

After AD, PD is the second most common neurodegenerative disease. Dopaminergic neuron degradation in substantia nigra and filamentous and oligomeric accumulation leads to motor impairment [47].

MDR1 genes encoding ABCB1 (P-Glycoprotein) are believed to be closely related to PD. Reduced expression of MDR1 in BBB endothelial cells, is associated with the progression of PD [48].

Huntington's Disease

BBB disruption is present in and associated with Huntington Disease (HD). In a study performed in post-mortem HD bearing human brain and R6/2 mice, it was reported that there is a reduction of TJs protein expression (occludin and claudin-5) and increased transcytosis which leads to BBB disruption. These results confirm that vascular pathology and BBB dysfunction plays a role in HD onset and progression [49].

Amyotrophic lateral sclerosis

ALS is a fatal neurodegenerative disease affecting human motor systems. The main cause of the disease is not fully understood yet, and the progress in treatment has been very slow [50]. According to a study performed in ALS transgenic mice, BBB disruption, and endothelial cell damage before any symptoms of weakness and motor injury, indicating that BBB dysfunctions affect the ALS progression. Decreased expression of TJs proteins occurred after the onset of ALS symptoms [51].

Stroke

Ischemic and hemorrhagic stroke are closely related to BBB disruption and have a worse prognosis [52]. As a result of increased paracellular and transcellular permeability and BBB endothelial cell disruption, the blood components cross into the brain. Water and ion balance affect the brain's neural environment, and leukocyte infiltration leads to inflammation, which increases the damages to the brain tissue [53,54]. Overall, BBB disruption is one of the main causes of ischemic strokes and drastically increases the risk of a brain hemorrhage, which is a deadly condition.

Epilepsy

IgG leakage and TJs loss characterized BBB disruption was reported in temporal lobe epilepsy humans and transgenic rodents. The BBB disruption was located in the affected area of the seizures indicating BBB plays an important role in epilepsy. The increased permeability of the BBB was associated with the frequency of epileptic seizures, which means in chronic periods, BBB impairment is also chronic, leading to other complications as well [55].

2.1.3. Brain tumors

Although the neurovascular unit regulates the environment for the optimal neuronal activity, it also inhibits the delivery of the therapeutic agents through BBB into CNS for the effective treatment of brain tumors. As tumor progresses, the BBB is disrupted and named the blood-tumor barrier (BTB). When compared to BBB, BTB is more heterogeneous, has increased permeability to small and large therapeutic agents, and allows accumulation of agents in tumor regions [56-58]. Due to the condensed space inside the brain, the tumor mass can also disrupt the normal blood flow by compression of vessels in areas nearby [59]. The blood vessels in the tumor core are more permeable (leakier) compared to vessels in the periphery of the tumor, which has an intact BBB [60], which leads to a heterogeneous vasculature. The leakiness of BTB is detected by the therapeutics in the tumor area and circulating tumor cells and DNA of glioma cells in the blood. T-cells and monocytes immune cells can have been located in brain tumor areas, and the TJ protein decrease in endothelial cells indicates the leakiness of the BTB.

Together with tumor expansion, increased angiogenesis induces the formation of new vessels with the increased need for tumor nutrition. The vascular endothelial growth factor (VEGF) is deregulated during tumor expansion to create leaky and immature vasculature and a hypoxic and acidic environment that promotes tumor progression [61-63]. Anti VEGF therapies decrease the permeability and formation of

new vessels; however, it also decreases the permeability of therapeutic agents by restoring the normal function of BBB [64,65]. The anti-VEGF therapies must maintain a balance between the BBB restoration, cancer progression through hypoxia, and decreased agent delivery through BBB [66,67].

Different types of brain cancer display different permeability and BBB properties. For example, there are four subtypes of medulloblastoma displaying different permeability properties. The best treatment prognosis with antineoplastic drugs is received in the WNT medulloblastoma subtype, showing higher fenestration of vasculature, indicating more drug accumulation in the tumor area [68]. In glioma model animal studies, the tumor permeability of drug-loaded liposomes and targeted therapies is higher in BTB regardless compared to BBB as expected [69,70]. The BTB in Glioblastoma features disruptive properties by TJs reduction and glioma stem cell derived pericyte cells, which decrease the integrity of the vasculature [71,72]. Therefore, targeting stem cell pericyte cells can increase the therapeutic agent delivery to glioma improving prognosis [73,74]. In order for cancer cells to enter the brain and cause secondary brain metastasis, they must first cross through BBB. Studies show that metastatic cells can cross BBB by disrupt claudin TJ [75]. Once the metastatic cell is the brain capillaries, the cell expresses proteases and ligand to facilitate the infiltration of other metastatic cells across BBB and create an appropriate microenvironment for cell growth. The BBB properties and functionality varies for different types of breast cancer brain metastasis. In HER-2 positive breast cancer brain metastasis, and increased expression of GLUT-1 and BCRP efflux pumps can be seen. When considering the treatment of brain cancer with therapeutic agents, all these properties should be considered for an effective treatment.

3. GENERAL STRATEGIES TO OVERCOME THE BLOOD-BRAIN BARRIER

There are three different approaches to deliver the therapeutic agents to the brain by penetrating through the BBB; invasive, pharmacological, and physiological [76]. Firstly, in invasive approach, all the technics used to deliver therapeutics to the brain are physically based. Invasive approach by mechanically penetrating the BBB delivers the drug by intra-cerebroventricular (ICV), convection-enhanced delivery (CED), or disruption of the BBB [77,78]. The disadvantage of ICV infusion is the low drug diffusion of brain parenchyma. If the target is not located near the ventricles, then this method is not an effective one [79]. CED, in general, is the insertion of a small stereotactically guided catheter into the brain parenchyma. The drug is pumped through this catheter and penetrates to the interstitial fluid.

The limitation of this method is that in some parts of the brain, for drugs applied through infusion is hard to have a high drug concentration. Placement of the catheter is a major factor in the achieved drug amount to the targeted site [80]. Another method for delivering drugs to the brain is the disruption of the BBB. This method can be applied in different ways; disruption by osmotic pressure, MRI-guided focused ultrasound the BBB, application of bradykinin-analog [81-83]. All these methods are expensive, require hospitalization, and are non-friendly patients. In addition to this, disruption of the BBB allows harmful blood components to enter the brain and may even cause permanent damages. Secondly, in the pharmacological approach, passive transportation through the BBB depends on molecule properties like molecule charge (low hydrogen bonding), molecular weight (<500 D), and lipophilicity (for a better transport lipophilicity should increase) [84]. Using these properties, some molecules can be chemically modified to pass through the BBB by adding more lipophilic substances and increasing lipophilicity. Sometimes, chemical modification of the molecule causes pharmacological activity loss [85]. The newly formed compound by molecule modifications may have become a substrate for P-glycoprotein and ending effluxed from the brain [86]. Lastly, although there are many transport ways to penetrate the BBB, in the physiological approach, brain drug delivery is based on uptake by specific receptors for specific ligands such as low-density lipoprotein (LDL) and transferrin (Tf) [87,88]. The best way to deliver neuroactive drugs from blood capillaries into the brain is by means of specific transporters and receptors. The molecular structure of drugs can be modified, or specific ligands can be conjugated to the molecule so that the molecule is recognized by specific receptors or transporters (**Figure 2**).

4. OPTIMIZATION OF NANOPARTICLES FOR BRAIN DRUG DELIVERY

4.1. Particle Size

Today the dynamic light scattering (DLS) and nanoparticle tracking and analysis (NTA) are the most appropriate, most commonly used, and the fastest way to determine the size of nanoparticles. One of the most important characteristics of NPs is particle size and size distribution [89]. The particle size determines the biological fate, *in vivo* distribution, targeting abilities, and the toxicity of a drug delivery system [90,91]. Additionally, they also affect nanoparticles stability, drug loading, and drug releasing [92-94]. The advantages of nanoparticles over microparticles are demonstrated in many studies. Nanoparticles, according to other larger particles have a higher cell uptake of the therapeutics and can target a wider range of intracellular and cellular components because of their mobility and smaller size.

In a study performed in Caco-2 cells, 100 nm nanoparticles had 2.5 times and 6 times greater uptake rate than respectively 1 μm microparticles, and 10 μm microparticles [95]. These results indicate that particle's biodistribution can be partially arranged by controlling particle size. Drug releasing is also affected by particle size. Smaller particle size means larger area/volume ration, so most of the drugs are attached to these nanoparticles are present at the surface or close to the surface, and as a result, there is a faster drug release. Since larger particles have bigger cores more drug can be loaded, but this situation causes slower drug release due to longer distance from the core to surface. Therefore, by controlling the particle size, we can affect the drug release rate in both ways. The aggregation risk is higher for smaller particles. During redispersion, transportation and storage polymer degradation are affected by particle size too. For example, the PLGA nanoparticles' degradation rate increases when its particle size increases [96,97].

Particle size is a crucial parameter not only for the reasons listed above, but also is responsible for different amounts of drug delivery across the BBB. In a study, using an *in vitro* model of the BBB, three different sized silica nanoparticles (30, 100, and 400 nm) were compared in terms of the permeability amount through the BBB. The results of this study show that nanoparticles between 30 nm and 100 nm can pass the BBB more efficiently [98]. Although the nanoparticles' material is relevant in the BBB crossing, another study performed with different gold NPs showed similar results that 70 nm is the optimal particle size [99]. After crossing the BBB, the extracellular space (ECS) is another obstacle for the drug to be delivered to the target site, which may be relatively far from the area of drug. The diffusability of the nanoparticles should be high, and it is highly dependent on the particle size. In a study done at Johns Hopkins University, the simulation of the ECS showed that in order for the drug to penetrate through ECS it should have a particle size at least smaller than 114 nm [100]. Even though, most of the nanotechnology drugs that are market available have a nanoparticles size above 100 nm, for the BBB crossing and acceptable ECS penetration it is recommended to have a NP size smaller than 100 nm (**Table 1**).

Table 1. *Key points of brain drug delivery with nanoparticles*

Parameter	Optimum Value
Particle size	<100 nm
Zeta Potential	Lipophilic moieties and electrically near neutral charge [101]
PEGylation	A low PEG density below %10 with mush-room configuration

4.2. Surface Properties of Nanoparticles

Surface modification determines the interaction between nanoparticles and the environment, whether it is plasma protein (antibodies), cell surface (cell membrane), or another nanoparticle. Drug loading into conventional carriers shows different biodistribution profiles from the drug itself because it is targeted by the mononuclear phagocyte system (MPS) like spleen, liver, and bone marrow. After being intravenously administrated, nanoparticles are identified by the phagocytic cells of the immune system and are eliminated from the blood circulation [102]. Except the particle size, the amount of proteins (i.e., opsonins) binding to the surface of the nanoparticles is determined by the hydrophobicity of the surface [103]. Hence, the *in vivo* fate of nanoparticles is determined by the surface hydrophobicity. Biological processes like interaction with biological membranes, protein adsorption, immune response, cellular uptake, and haemolytic activity are directly affected by the hydrophobicity of nanocarriers. This parameter directly affects the distribution, stability, and immune reaction to the nanocarriers; hence it should be characterized and controlled. Therefore, if the surface of nanoparticles is not modified, it is opsonized and cleared from blood circulation by phagocytic cells.

In a study performed by Gessner et al., nanocarriers with different degrees of hydrophobicities were investigated in terms of plasma protein adsorption quantitatively. The results showed that the higher is the hydrophobicity of the nanocarrier, the more plasma proteins are adsorbed that lead to reduced blood circulation time and hindered targeting besides RES organs [104].

In a study done by Shima et al., different degrees of amphiphilic poly(γ-glutamic acid) nanoparticle hydrophobicities were investigated in terms of immune response once introduced into the blood in mice and *in vitro*. The immune response was evaluated for each increasing hydrophobic degree of NP. It was reported that immune response to nanocarriers could be controlled to a large certain extend by optimizing the surface hydrophobicity [105].

In a study reported by Zhu et al. at the University of Massachusetts, how the nanocarrier hydrophobicity and protein adsorption influence the cellular uptake. 14 different gold NP with various hydrophobicities were synthesized and investigated for uptake in the HeLa cell line. The gold NP with the most hydrophobic surface showed the higher degree of protein adsorption leading to lower cell uptake. The opposite is true for the gold NP with the lowest hydrophobic surface showing higher cellular uptake [106].

In another study performed by Saha et al., various degrees of hydrophobic gold NP were synthesized and evaluated in terms of haematolytic activity. It was observed that higher hydrophobic surface gold NP had higher haematolytic effect, and the adsorbed protein corona decreases the haemolysis of red blood cells. It was concluded that both hydrophilic and hydrophobic had no haematolytic effect after 30 minutes in the presence of plasma. However, higher hydrophobic gold NP maintained the haematolytic activity due to aggregation despite protein adsorption in the plasma environment for at least 24 hours [107].

To increase the success of targeting, it is required to lower the opsonization and increase the *in vivo* blood circulation time of drug. All these can be accomplished by coating the surface of nanoparticles with hydrophilic polymer/surfactant or hydrophilic biodegradable copolymer (i.e., polysorbate 80, PEG, poloxamine, polyethylene oxide, and poloxamer) [108-110]. In many studies, it is reported that PEGylation of the surface of the nanoparticles inhibits the opsonization from blood components. If PEG molecules have a brush-like configuration or an intermediate configuration, the complement activation and phagocytosis are reduced, but surfaces coated with PEG mushroom-like configuration favored complement activation and phagocytosis [111]. Zeta potential is used to determine the surface charge of nanoparticles, and it is affected by the content of nanoparticles and the type of medium it is dispersed in [112]. Surface modification is also important to prevent agglomeration of the nanoparticles [113]. If the zeta potential is high enough the nanoparticles, because of their opposite potentials, will repel each to reach dispersed and redispersible solution and prevent agglomeration. As zeta potential is a function of dispersion stability, if zeta potential has a value higher than ±30 mV, the dispersion is physically stable, and the aggregation between particles is inhibited. Aggregation starts at 5 mV and smaller values of zeta potential [114]. *In vitro* study performed in hCMEC/D3 BBB cell model with similar size (ranging from 105 nm to 126 nm) liposomes but different surface zeta potentials, it is concluded that a significant difference in cell uptake is determined between neutral and non-neutral surface zeta potential liposomes whereas no significant cell uptake

difference was found between -6 mV potential and larger zeta potential values [115]. The best candidates for BBB overcome are electrically near neutral and lipophilic molecules [101].

4.2.1. The PEGylation of nanoparticles to overcome the blood-brain barrier

The PEGylation of the nanoparticles surface is very important in the formulation development of nanoparticles. PEG coating protects the nanoparticles from the phagocytes, and it is dependent on PEG molecular weight and density [116]. Surface-grafted hydrophilic polymers coat the NPs as a dense cloud preventing at even low concentrations the interactions with other polymers. To have a low protein adsorption, long chain and high surface density are necessary. However, the density of the surface has a greater effect on steric repulsion than the length of the chains [117]. Methoxy PEG-PLA nanoparticles were developed and compared to uncoated nanoparticles. The labeled 14C PEG-PLA nanoparticles were phagocytosed slower than F68 coated PLA nanoparticles by THP 1 monocytes cultured cells. ME-PEG PLA nanoparticles improved its half-life by 360 minutes comparing to uncoated F68 nanoparticles. Due to particle circulation, a high radioactivity was found in blood vessels and heart. After 6 hours of iv administration of nanoparticles, radioactivity was found in phagocytic organs to indicate the delaying of phagocytosis [118]. In another study, PEG-coated PLGA nanoparticles in combination with focused ultrasound (FUS), which is used to temporarily and locally open the BBB for the PEG-PLGA, without any extra conjugated ligand crossed the BBB [100]. This means that the blood circulating time of the PEGylated PLGA nanoparticles was enough for FUS to induce BBB disruption and NPs to pass to the brain. PEG density and conformation are very crucial characteristics in improving the pharmacokinetics and biodistribution of NPs. In a study performed by Sheng et al., PLA NPs were coated with different concentrations of 5%, 10%, and 20% by weight. After the preparation of PEG-coated and uncoated PLA NPs, the time needed for the macrophage cells to uptake the NPs were measured for each PEG concentration. The optimum PEG coating leading the longest blood time circulation is 10% with 34.3 hours of circulation time [119]. In another study, the effect of PEG density in targeting potential of NPs was investigated. The results show improved NP targeting with a low PEG density below 10% and mushroom configuration, which is compatible with the PEG density range in pharmacokinetics and biodistribution studies [120].

5. PHARMACOKINETICS AND ORGAN DISTRIBUTION

The pharmacokinetics of small molecules, large molecules like protein and drug delivery systems like nanocarriers differ very much from each other.

Pharmacokinetics is also defined as what the body does to the therapeutics; hence it is considered into four subtitles as absorption, distribution, metabolization, and elimination. For small molecules and large molecular weight molecules administered parenterally, all these processes are meaningful; however, most of the nanocarriers do not have or have very limited gastrointestinal absorption. Distribution of the nanocarriers is closely related to its design characteristics such as size, shape, surface hydrophobicity, zeta potential, and targeting moieties. The primary route of elimination is through RES organs like spleen and liver. Nanocarriers are vehicles that intent to provide better efficacy, lower side effects, and better pharmacokinetic properties for the encapsulated therapeutic agent. To achieve these goals, the particle design should consider both the physiological properties of the body and the features of the nanoparticle itself.

The nanocarriers, once they are injected, the distribution and clearance start simultaneously. The blood flow distributes them to organs of RES, targeted tissues, and mostly are cleared these organs as well. All nanocarriers administered in vein firstly pass the lungs, and then they are transported to other tissues and organs through arterial blood flow. The nanocarriers are cleared from the organism in two ways. One of them is the RES or also known as the mononuclear phagocyte system (MPS). The macrophage cells phagocytose the nanocarriers and clear them from the bloodstream in addition to retention in RES organs. The second system of nanocarrier clearance is the liver and kidneys, which function as the main clearance organs. The systems and organs active in nanocarrier are summarized below;

Blood

The plasma proteins bind to form a protein corona around the nanocarrier named as opsonins. The opsonins facilitate the nanocarrier clearance from the bloodstream, making it the first barrier. The reduction of opsonization is one of the strategies to consider during nanocarrier design for a longer blood circulation time.

Spleen

Spleen is a highly perfused organ which store blood, clears the old blood cells, filtrates the foreign particles from the blood, and produces phagocytic cells. Moghimi et al., reported that the safe limit for spherical nanocarriers to avoid spleen filtration is 150 nm, larger particles are highly prone to filtration at interendothelial cell slits of venous sinuses in the spleen, whose width is approximately 200-250 nm [121].

Kidney

Clearance via kidney includes tubular secretion and glomerular filtration. Particles with size less than 5.5 nm and proteins less than 3 kDa depending also from the shape can be filtered through glomerular filtration [122,123]. As the endothelial cell in glomerular filters possesses fenestrations from 50-100 nm, hence nanoparticles smaller than 100 nm in size can be filtered through the kidney [124,125].

Liver

The liver's function is to remove foreign particles such as bacteria, viruses, and nanocarriers from the bloodstream [126]. The fenestrations in endothelial cells similar to the EPR effect allow foreign substances to be trapped in the liver to interact with hepatocytes, Kupffer cells, and BB cells [127]. The Kupffer cell comprises 80-90% of the macrophages in the human body [128]. These cells are responsible for phagocytosis of most nanocarriers and liver accumulations [129,130]. A study by Wisse et al. reported that the fenestrae in humans, which allow the passage of particles from susoidal lumen to the surface of hepatocytes necessary for liver filtration is 107 ± 1.5 nm [131].

Another parameter that influences the nanocarrier distribution and clearance is the shape. Most of the studies cited in the literature regarding nanocarriers are shaped spherical. It is the easiest way to manufacture, and the data available is larger. It is very difficult to summarize that what kind of shape or even charge is the best for any specific tumor, as most of the nanocarriers, regardless of their shape and charge, are accumulated in the liver and spleen [132,133]. Geometric shapes play a crucial role in nanocarriers' pharmacokinetics, such as flow properties, cellular uptake, vascular adhesion, and escape from blood vessels [134]. However, there are some tendencies of specifically shaped nanocarriers toward specific organs. For example, irregularly shaped nanocarriers are accumulated mostly in spleen, and rod-shaped particles are accumulated in the lungs [135,136]. The shape also plays an important role in renal filtration. It was reported that single-walled carbon nanotubes (SWCNT) of 200-300 nm of length undergo glomerular filtration, which is a conflict with the fenestrations around 100 nm [137]. Worm-like shaped nanocarriers display different flow properties, increasing the surface of interaction with the blood component as a single, minimizing the risk of phagocytosis from macrophages as well [138].

Another parameter which is crucial for pharmacokinetics of nanocarriers is the surface modification, which is also explained in detail in the surface properties and zeta size section.

6. TARGETED DRUG DELIVERY

NP carrier system development as targeted drug delivery systems is being revised recently. The targeting strategy can be classified as passive and active targeting. The therapeutic agent or the carrier of the therapeutic agent should be conjugated to specific tissue or cell ligand for active targeting aim. In passive targeting, the therapeutic agent is conjugated to a macromolecule or entrapped in a NP and passively delivered to the target site. Drugs entrapped to NPs or conjugated to macromolecules can target tumors with enhanced permeability and retention (EPR) effect.

NPs can be formulated to penetrate through biologic barriers and deliver drugs. Drugs like antineoplastics, antivirals cannot penetrate through BBB to pass into the brains, which considerably limits their treatment abilities for CNS diseases. Adsorption or covalent binding of a specific ligand or monoclonal antibody (mAb) to the surface of the nanocarrier is used as means of targeted drug delivery system to the brain. The ligand or mAb interact with specific receptors located in endothelium cells of the brain capillaries to penetrate BBB as an endogenous agent. The NP application as a delivery system across BBB is a promising approach.

6.1. Brain Targeted Drug Delivery Using Ligand

Receptor-mediated endocytosis (RME) requires specific ligand to bind to the appropriate receptors located in the luminal side of the endothelium of BBB. Once the ligand binds to the receptors, the receptor-ligand complex is formed, and the endocytosis begins. The newly formed complex internalizes as a vesicle into the endothelial cell. After internalization of complexes four different mechanisms can occur [139];

- Ligands can be degraded by the lysosome, and the disconnected receptors return to the membrane of the cell
- Simultaneously degradation of receptor and ligand by lysosome
- Post internalization receptor and ligand are recycled (retroendocytosis)
- Receptors bound to ligands are transported inside the cell to reach another domain of plasma membrane

RME systems can use endogenous or chimeric ligands to achieve active drug targeting into the brain. For a long time, blood ligands like Tf, insulin (Ins), Ins-like growth factor (IGF1&2), leptin, IgG, folic acid, and modified low-density lipoprotein (LDL ligands as ApoE) were a focus point for brain targeted drug delivery (**Table 2**). These kinds of endogenous ligands are non-immunogenic and biocompatible; on the other hand, the main advantage is the high affinity to tumor and brain cells.

It has been shown that cerebral capillaries have a higher level of Ins receptor expression than peripheral capillaries in animals and humans [140]. The high affinity of Ins to tumor cells makes it a promising target for targeting drug delivery. However, peptidic Ins hormone has a short half-life time, and in high concentrations it may cause hypoglycemia. This side effect can be avoided if the Ins-like growth factor (IGF) was used in place of Ins because it can be administered in high concentrations without causing hypoglycemia. Most of the researchers have used the Ins receptors' properties for brain targeting because of the high density of Ins receptors on the cerebral microvessels and transcytosis triggered through them. Similar to Ins, IGF1 and IGF2 can also pass into the brain by penetrating through BBB, but there are no recent studies using this targeting ligand [141]. Tf is a monomeric glycoprotein that contains one (monoferric) or two (diferric) iron atoms. TfR is overexpressed on the brain capillary endothelium and at the surface of proliferating cells such as brain tumor cells, especially glioblastoma multiform. Besides, in healthy individuals TfR levels are low. TfR can be saturated even in the physiological state because of the high amounts of endogenous Tf found in blood [142]. Folate receptor (FR) is expressed in brain capillaries endothelial cells. Due to FR is overexpressed in several tumors; it is a tumor marker in ovarian carcinoma and brain cancer. Folates like folic acid (FA) can be carried through the membrane by three mechanisms; reduced folate transporter, FR, and FA export pump [143].

6.2. Brain targeted drug delivery using a monoclonal antibody

As another approach to brain targeted drug delivery are chimeric ligands like peptidomimetic mAb, which bind to specific receptors found in BBB. As another approach to brain targeted drug delivery are chimeric ligands like peptidomimetic mAb. By binding to different sites other than endogenous ligands, they do not interact or compete with these ligands unless it is administered in high doses. mAb are macromolecules and can penetrate BBB by binding to specific receptors like Tf and Ins to induce transcytosis (**Table 3**). But the main question still remains as how much of the i.v. injected amount can actually penetrate BBB and pass to the brain and what is the result compared with.

Table 2. *Examples in literature of drug delivery systems incorporating ligands as targeting moieties*

Ligand	Nanocarrier	Polymer/ Coating	Drug	Results	Ref
Tf + Folate	Carbon dots	-	Doxorubicin (Dox)	Better Tf receptor mediated transport across BBB and better *in vitro* cell uptake compared to Dox alone	144
	Gold nanoparticles	-	-	Gold NPs bypassed the BBB *in vitro* model and *in vivo* mice model. Tf ligand conjugated NPs had better brain uptake than Tf mAb conjugated NPs.	145
	Core-shell NP	Cationic liposome	Si-RNA	SiRNA (SiEGFR) carrying, Tf (T7), which is a modified ligand, was conjugated to core shell liposome NP. According to PEGylated and not targeted core shell NP, Tf conjugated core shell NP showed a higher accumulation in the tumor site.	146
	Chitosan	TPGS	Docetaxel	C6 Glioma cell MTT assay calculated IC_{50} values of TPGS-Chitosan and especially Tf-TPGS-Chitosan NP compared to commercial Docel™ are significantly lower. *In vivo* AUC values of Tf-TPGS-Chitosan values were increased referenced to Docel™.	147
	PLGA	PEG	Doxorubicin	A modified Tf was conjugated to PEG coated doxorubicin loaded PLGA NP (TPDP), which is incorporated in PLGA scaffold to control the release rate of Dox.	148
	PLGA	-	Etoposide	The efficacy of antiproliferative activity in U87MG cells was as follows in increasing order: Tf+Fa-PLGA NPs > Fa-PLGA NPs > PLGA NPs > free etoposode	149
Folate + Des-octanoyl Ghrelin	Polymersome		Doxorubicin, CY5.5	Dual ligand conjugation to the polymersome increased the crossing through BBB and inhibited the glioma tumor growth	150
	SPIO NPs	Bovine Serum Albumin (BSA)	Fluorescein Isothiocyanate (FITC)	BBB crossing was not shown. Study results performed with folic acid conjugated SPIO NPs showed the combination is biocompatible, did not affect the cell cycle and proliferation. Increased internalization in U251 cell was also observed.	151
	Cationic microbubbles (MB) + FUS	-	DNA	FUS is used to transiently disrupt the BBB to deliver the DNA loaded folat-MB to the brain tumor site enabling targeted local gene therapy. Folate targeted MB had better gene transfer than nonconjugated or DNA gene alone.	152
ApoE + Phosphatidic Acid	SLN	-	-	A cellular uptake study in hCMEC/D3 cells comparing SLN and ApoE-SLN uptake speed and uptake amount. ApoE-SLN showed 1.8 times higher uptake than unconjugated SLN. BBB is thought to be crossed transcellularly.	153
	BSA NPs PBCA NPs	-	Sumatriptan succinate	This is a comparative *in vivo* study in rats investigating migraine efficacy between ApoE-BSA NPs and polysorbate 80-coated PBCA NPs. LDL receptor targeting ApoE-BSA had the highest drug amount delivered in the brain.	154
	Liposome	-	-	Two ligands are conjugated to liposome for therapy of Alzheimer disease. ApoE to facilitate the BBB crossing and phosphatidic acid having a high affinity to amyloid β peptide. Results show that double targeted liposome inhibits the amyloid β aggregation and enhances the starting disaggregation of aggregates. BBB crossing amount increased 5 times according to single ligand conjugated liposome.	155
Glutathione (GSH)	Liposome	PEG	Amyloid targeting Ab fragments	The Ab fragment was labelled with radioisotope to follow the progress and route. GSH-liposome crossed the BBB in Alzheimer transgenic mice models.	156
	Poly(ethyleneimine) (PEI)	-	-	The ability of GSH ligand to cross through *in vitro* endothelial cell BBB model was studied. GSH-PEI NPs showed a promising approach to BBB crossing.	157
	PLGA NPs	PEG	Docetaxel	In this study cytotoxicity tests in RG2 and C6 cells and *in vitro* Transwell cellular BBB model for BBB penetration were performed for GSH-PEG-PLGA NPs. BBB permeation increased for GSH-PEG-PLGA NPs compared to free Dox solution and there is a selectivity between healthy cells and glioma cells for GSH-PEG-PLGA NPs.	158

Table 3. *Examples in literature of drug delivery systems incorporating monoclonal antibodies as targeting moieties*

mAb	Nano-carrier	Drug	Results	Ref
Anti-Transferrin (OX26)	PLGA NPs	iAβ5	Anti-Aβ (DE2B4) peptide and OX26 dual conjugated PLGA NPs, were investigated for BBB penetration and toxicity in a porcine brain endothelial cells comprising BBB model. The results show better cellular uptake compared to non-targeted NPs. More studies are needed to show intracellular and intracerebral uptake increase of iAβ5.	159
+ Chlorotoxin (CTX) Ligand	PEGylated liposome	Plasmid DNA	BMVECs/C6 cells co-culture model of BBB confirmed penetration of BBB endothelial cells and decrease of C6 viability. By dual targeting of OX26 and CTX respectively for BBB penetration and tumor targeting resulted in tumor volume decreases in C6 glioma rats.	160
Murine Anti-Tranferrin Receptor (Ri7)	Quantum dots (QD)	-	Endocytosis by receptor mediated transport was investigated and confirmed in bEnd5 and N2A cells. *In vivo* studies in mice supported the *in vitro* results by showing increased internalization of Ri7-DQ in the brain. Several hours after administration of Ri7-DQ, high concentrations were determined in the brain, which means that this approach is promising to deliver drugs in therapeutical concentrations to the brain tissue.	161
Anti-transferrin Receptor (TfR) mAb	Polymalic acid (PMLA) PolycefinTM nanoplatform	Morpholino Antisense Oligonucleotides (AON)	AON is conjugated to PMLA nanoplatform to inhibit gene expression responsible for tumor growth. *In vivo* results showed increased animal survival confirming the internalization of TfmAb-PMLA nanoplatform to the brain. AON induced inhibition of gene responsible for tumor growth.	162
Anti-transferrin Receptor (TfR) mAb + ApoE ligand + Curcumin	LUV liposome	-	TfR-Mab-ApoE-Curcumin-LIPs have 3 different targeting ligands and the study investigates the potential of liposome targeting *in vitro* BBB model and *in vivo* in normal and transgenic mice. Curcumin was found to inhibit Aβ peptide aggregation and penetrate through BBB by active targeting of LDL receptor and transferrin receptor. It should be noted that different doses of liposomes demonstrated different brain targeting capabilities.	163 164
Anti-Human Insulin Receptor Antibody (HIRMAb)	HIRMAb-IDS protein fusion	Iduronate 2-sulfatase (IDS) Protein	HIRMAb-IDS fusion was administered to Rhesus monkeys to observe the pharmacokinetics after IV infusion of different doses. The safety of the protein fusion was investigated and with the exclusion of hypoglycemia from high HIRMAb doses, no major adverse effects were detected. Safety profiles of *in vivo* studies in monkeys confirm the possibility of clinical studies of IgG anti-receptors protein fusions in CNS diseases.	165
	-	-	After SC injection of a range of HIRMAb doses, it was concluded that the lowest dose of HIRMAb is stable and has a long blood circulating time. The lowest dose of HIRMAb, has enough for BBB penetration to deliver IgG protein fusion that can deliver therapeutical relevant dose in the brain.	166
	SLN	Saquinavir (SQV)	Different parameters like palmitic acid weight fraction, the amount of conjugated MAb and poloxamer 407 weight fraction and their effect over SLN characterization were investigated. Cytotoxicity, cell uptake in RAW264.7 cells and BBB penetration in HBMECs/Has BBB model experiments were performed for all changed parameters. 83-14 MAb/SQV-SLNs results show an increased BBB targeting efficacy.	167
Anti-Human Insulin Receptor Antibody (83-14 Mab)	Polymersome (PDMS-b-PMOXA)	-	HIRMAb-Polymersome was investigated in terms of BBB penetration on a human insulin expressing hCMEC/D3 cell BBB model. Endocytosis in endothelial cells and competitive inhibition uptake by inclusion of excess free 83-14 MAb were confirmed.	168

There are many studies performed dedicated to this specific question. The range of BBB penetration was measured as % of drug penetrated according to injected dose over gram (%ID/g) is between 0.2-3.1% for Tf targeted liposomes [169]. As described in the recent articles, the uptake and transport of drugs should be compared to polyclonal IgG to see the difference between targeted and nontargeted drug delivery systems [170,171]. Although IgG transport to the brain is very low, it suggests that other means of transport across BBB are possible other than receptor-mediated transport. The increased transport of drug delivery systems conjugated to TfR-mAb and HIRmAb could be used to deliver therapeutical drug doses to the brain [165,171,172].

7. CONCLUSION

Many large molecules like peptides, proteins, genes, antisense agents, and mAb have the therapeutical potential for CNS disease treatment. Nanotechnology provides clinical advantages for drug delivery like increased drug stability and half-life, decreased side effects, and drug dose. Despite the many research on new macromolecules, drugs, and drug delivery systems, there is still the very low translation of

these studies to clinical trials for CNS diseases, and one of the main reasons is the BBB. Modification of drug delivery systems, transient disruption of BBB, and their combination are the main approaches to overcome this problem such as a tumor or Alzheimer targeted modified NPs delivery in combination with FUS to transiently disrupt BBB.

Even after many years of study in this field, many researchers have failed to acquire sufficient prove and quantitative data supporting the efficient and clinically relative doses of drugs delivered to the brain parenchyma. More mechanistic studies like investigating the intracellular sorting mechanisms after uptake of nanocarriers into the endothelial cells should be performed. Not if but when the brain drug delivery problem is solved, and with the advancement in protein and gene modification, material design, and innovations in fabrication scale-up, the rate of new drug developments will accelerate. In order to better understand the nanocarrier drug delivery system, the influence of the formulation characterization parameters like particle size, shape, zeta potential, and PDI influencing brain drug delivery should be fully understood. The optimum value of particle size is less than 114 nm, and zeta potential value is near neutral. Varies tumor-targeting moieties have been incorporated in nanocarriers, but the most effective and better studied in literature are folate, Tf, ApoE, or their corresponding mAb and HIRmAb. Although one of the most efficient brain targeting ligands like Tf has a relatively low % ID/g drug efficiency future studies should include more pharmacokinetic data regarding brain accumulative drug amounts in order to better evaluate the efficacy of the treatment and dosage.

In recent years, many studies are describing FUS as a tool to boost the transportation of nanocarriers through BBB transiently and with minor or no side effects [173,174]. In the future, if FUS technology can be more reachable and less expensive, it has the potential to cross even larger carriers into the brain.

AUTHOR CONTRIBUTIONS

Concept: IS; Design: IS, AŞ, HT; Supervision: YÇ; Materials: IS, AŞ; Data Collection and/or Processing: IS, AŞ; Analysis and/or Interpretation: IS; Literature Search: IS, AŞ, HT; Writing: IS; Critical Reviews: YÇ.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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