Expert Opinion

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Polymeric nanoparticles for the drug delivery to the central nervous system

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Background: Nanoparticulate polymeric systems (nanoparticles [Np]) have been widely studied for the delivery of drugs to a specific target site. This approach has been recently considered for the therapy of brain diseases. The major problem in accessing the CNS is linked to the presence of the blood–brain barrier. *Objective*: The present review deals with the different strategies that have been developed in order to allow Np drug carriers entry into the CNS parenchyma. Among these, the use of magnetic Np, Np conjugation with ligands for blood–brain barrier receptors, with antibodies, and the use of surfactants have been considered. *Methods*: All the literature available is reviewed in order to highlight the potential of this drug delivery system to be used as a drug carrier for the treatment of CNS pathologies. *Conclusions*: Polymeric Np have been shown to be promising carriers for CNS drug delivery due to their potential both in encapsulating drugs, hence protecting them from excretion and metabolism, and in delivering active agents across the blood–brain barrier without inflicting any damage to the barrier. Different polymers have been used and different strategies have been applied; among these, the use of specific ligands to enhance the specificity of drugs delivered to the CNS has recently been considered. At present, clinical trials are being conducted appeared for the use of these drug carriers but none related to the treatment of CNS diseases.

Keywords: blood–brain barrier, brain targeting, central nervous system, delivery strategies, polymeric nanoparticles

Expert Opin. Drug Deliv. (2008) 5(2):155-174

1. Introduction

Over the last 30 years, a large number of studies have been performed in order to find therapies for the so-called 'difficult-to-treat' brain pathologies, such as brain cancer, HIV-dementia due to neuroAIDS, strokes, ischemia, degenerative disorders (Parkinson's and Alzheimer's diseases), which represent 35% of the burden of the total diseases. The increasing worry about these pathologies is directly connected to the estimate that 1.5 billion people are suffering from CNS disorders [1] and to the forecast that 50% of the total worldwide population will show Alzheimer's symptoms by the end of the 21st century [2].

Thus, this field of research represents one of the most stimulating challenges for the scientific world, as a result of the limited number of therapeutics capable of reaching the most 'secret and sacred' system of the body, the CNS.

In fact, 98% of drugs are not able to cross the blood–brain barrier (BBB) owing to their molecular or chemico-physical properties [3]. The most important obstacle to the delivery of drugs to the CNS is the barriers that drugs have to cross in order to make contact with the target site. Anatomically, the CNS is

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restricted by the BBB and the blood-cerebrospinal fluid barrier. The unique structure of this epithelium is based on the presence of the tight junctions (TJ) that connect the cerebral endothelial and epithelial cells of the choroids plexus. Moreover, glial cells such as astrocytes surround 85% of the surface of the capillaries. They are responsible for making the endothelial cells 'tighten up', so producing an electrical resistance (1000 Ωcm^2), which is much higher than that of the other systemic endothelia [4].

In the past, the BBB was thought to be a static membrane, allowing the passage of substances according to their hydrophobicity/lipophilicity balance and molecular weight (MW).

Nowadays, the BBB is thought to be a dynamic interface through which the substances enter using different mechanisms (i.e., a passive transport [5], active transport, receptor-mediated transport or more complex systems such as endocytosis or transcytosis [6]). All of these mechanisms are called influx systems, allowing the substances, fundamental for the survival of the cerebral cells, to enter the brain from the vascular circulation.

On the contrary, the efflux systems, mainly represented by the P-glycoprotein (P-gp) and the multi-drug resistance protein (MPR), mediate the inverse movement, forcing the substances to escape from the cerebral parenchyma and to re-enter into the peripheral circulation. These efflux mechanisms are responsible for the inefficacy of several drug-based therapies because a large number of drugs (antibiotics, antiviral agents, antiretroviral etc.) are the substrates of these proteins [7].

The permeability of the BBB is significantly influenced by the neurological disorders [8]. As an example, in the neurodegenerative diseases, such as Alzheimer's or Parkinson's diseases, the integrity of the BBB is almost preserved, while in the cerebro-vascular diseases, such as ischemia and the inflammatory diseases, the integrity could be lost [9]. Finally, brain tumors imply several BBB abnormalities, such as hyperplasia of the endothelial cells, the opening of the TJ and an increase in the fenestrations, and the presence of pinocitic vacuoles [10-13].

Thus, in order to carry out an effective therapy, it is important to underline two aspects. First, for those diseases producing no changes in the BBB, the major obstacle is represented by BBB characteristics. In the case of pathologies compromising the BBB integrity, crossing the BBB is obviously facilitated; however, efficient delivery to the drug target, for example, the tumor cells, is also necessary [14].

There are two possible approaches [15] to solving the problem of the inability of some drugs to cross the BBB. The first is based on invasive techniques of neurosurgery (intracerebral infusions or implants [16,17]) that deliver the drug directly to the target site. This approach has the advantages of obtaining a definite result in terms of efficacy as the drug is directly placed into the brain. On the other hand, this method incurs neurosurgical costs and increases the risk of infections, in addition to a low patient compliance. Alongside this approach, a temporary chemical or physical disruption of the BBB, produced by some biochemical and immunological changes or by an osmotic shift [18,19], could be used to allow the drug to cross the BBB. The temporary disruption results in several drawbacks such as physiological stress or a transient increase in intracranial pressure; furthermore, an increase in BBB permeability, although only for short periods of time, makes the brain vulnerable to infections and damage from toxins.

Hence, to improve drug delivery to the brain, non-invasive techniques have been investigated. Recent studies demonstrate the efficacy of the medicinal chemistry approach, based on the modification of the physicochemical properties of drugs, [20,21] and the biological approach, based on the conjugation of molecules with antibodies or ligands targeting the BBB [22,23].

The technological approach is a non-invasive method of drug delivery to the CNS. It is based on the use of nanosystems (colloidal carriers), which could be lipid-based (liposomes or solid lipid nanoparticles [Np]) or polymer-based (Np).

The use of colloidal systems in the field of drug delivery is not new [24,25], while the concept of an ideal carrier able to target the drugs to specific regions, organs or cells is certainly more recent [26-30] and is connected to the growth of pharmaceutical technology. The design of nanosystems able to achieve satisfactory drug targeting must include some fundamental properties not yet fully optimized, such as their drug loading capacity and their *in vivo* fate, the interaction of these systems with the body and its mechanisms of defense (i.e., the immunogenic properties of these carriers and the possible defensive reaction of the body's immune system). At the same time, the researchers have to cope with the overall cost, the scale-up production, the stability and finally the acute and the chronic toxicity.

These nanocarriers are quickly removed from the bloodstream depending on their particle size, charge and surface properties [31,32]. This process is mediated by the reticuloendothelial system (RES), a collective group of mononuclear cells originating from bone marrow. After the interaction of the Np with the elements of the complement system, a very fast uptake by the RES is produced.

2. Polymeric nanoparticles

Polymeric Np are nanosized carriers (1 – 1000 nm), made of natural or synthetic polymers, in which the drug can be loaded in the solid state or in solution, or adsorbed or chemically linked to the surface. Nowadays, the use of polymeric Np is one of the most promising approaches for CNS drug delivery [8,33-40].

These Np possess some advantages with respect to single molecules of drugs or prodrugs, such as their high drug-loading capacity. It is, therefore, possible to deliver a

great number of drug molecules into cells for each Np [33]. In addition, Np provide protection to the embedded drugs against chemical or enzymatic degradation thus increasing the chances of the active molecule reaching the target site. Furthermore, it is possible to modify the Np surface properties in order to facilitate drug delivery, for example, escape from the RES system.

This review will focus on the possible strategies of Np modifications in order to improve drug delivery across the BBB.

Different needs must be taken into consideration when developing Np. They could be synthesized from a natural or synthetic starting polymer. This has to be biodegradable and biocompatible, non-toxic, non-thrombogenic and non-immunogenic, and finally inexpensive.

At present, only few polymers guarantee the safety of the polymer-based nanocarriers. One of the most commonly used polymers for the preparation of Np for CNS drug delivery, polyalkyilcyanoacrylate (PACA) [41], is at present not approved by the FDA for intravenous administration. However, some of these polymers have been found to be non-toxic [42-47].

On the other hand, PACA Np have been considered for the treatment of resistant tumors [48]. Phase I studies with PACA Np loaded with the antitumoral drug doxorubicin [49] have shown good tolerance. To date, these Np have reached Phase II clinical trials for the treatment of resistant cancer [48]. They have also been tested as doxorubicin drug carriers in a Phase I intra-arterial administration to treat patients with advanced hepatocellular carcinoma with promising results [50].

Polylactide-co-glycolide (PLGA) or polylactide (PLA) polymers are FDA-approved and, therefore, are two of the most promising polymers for the preparation of Np, but clinical trials testing the use of these Np as drug carriers are still lacking. Degradation of PLA or PLGA into oligomers and monomers of lactic and glycolic acids (substrates of the Krebs' cycle) occurs by an autocatalytic cleavage of the ester bonds through spontaneous hydrolysis [51]. Depending on their MW and their conjugation with other polymers (such as PEG) these biodegradable polymers show different times of elimination from the body [51,52].

However, it has been shown that Np made of PLA or PLGA undergo sudden removal when injected into the peripheral circulation, owing to RES activity (PLGA or PLA Np accumulate in the liver and the spleen) [52-54], resulting in a blood half-life of ~ 2 – 3 min [55-57]. The activation of this defensive system is strictly connected with the surface characteristics of the Np (charge, functional groups etc.), the system geometry and size. Considering the surface properties, Np with a hydrophobic surface and negative charges promote protein adsorption and activate the complement system [32]. On the contrary, Np < 100 nm in size have a lower possibility of being uptaken by macrophages or recognised by opsonins. On the other hand, a small diameter of the Np corresponds to a large relative surface area, which could promote their aggregation.

The final size of the Np is an important parameter that partially determines their biological fate when administered [32]. Particles < 10 nm are rapidly removed after an extensive extravasation and renal clearance, whereas Np of > 200 nm are rapidly filtrated by the spleen and removed by the reticuloendothelial cells. In addition, carriers with diameters > 5 μm will induce capillary blockade. If the particles are physically retained in the target tissue capillaries, drug diffusion through the capillary wall (a step limited by the MW of the drug) will induce the therapeutic action. If this action requires extravasation, sizes between 0.5 and 5 μm may also be suitable for that process. The permeability of these vessels is increased in the presence of solid tumors and, therefore, Np can extravasate across the endothelium barrier.

Moreover, the drug loading efficiency of Np has to be determined carefully [58].

Np can be engineered with specific ligands or, more generally, substances able to increase their ability to cross the BBB by means of specific mechanisms, such as absorptive-mediated transcytosis or receptor-mediated endocytosis. Since specific receptors have been identified on the brain capillaries for transferring (Tf), insulin (INS) and INS-like growth factor [59], attempts have been made to link these ligands on the Np surface in order to realize BBB targeting.

3. Strategies for crossing the BBB using modified Np

Different approaches have been developed in order to target Np to the CNS (**Figures 1** and **2**):

- Magnetic-Np approach is based on magnetic fields that drive the nanocarriers to the target site;
- Nanogel approach is based on the use of a colloidal carrier made of a nanoscale polymeric network of crosslinked ionic polyethyleneimine (PEI) and non-ionic PEG chains;
- Emulsifying wax and Brij 72 approach is based on the use of emulsifying wax and Brij 72 as oil phase and Brij 78 and T-80 as surfactant for the preparation of warm oil-in-water microemulsion templates;
- Surface charge-based approach takes advantage of the mainly positive surface charge of the Np to induce an adsorptive-mediated transcytosis;
- Surfactant-based approach is based on the use of surfactants (T-80 etc.), as a coating or linked to the Np and acting as a targetor molecule for the BBB;
- PEG approach is based on the long-circulating characteristics of Np coated or linked with PEGs;
- Ligand-based approach is related to conjugation with specific ligands (i.e., antibodies, proteins, peptides etc.) able to increase or promote movement across the BBB crossing.

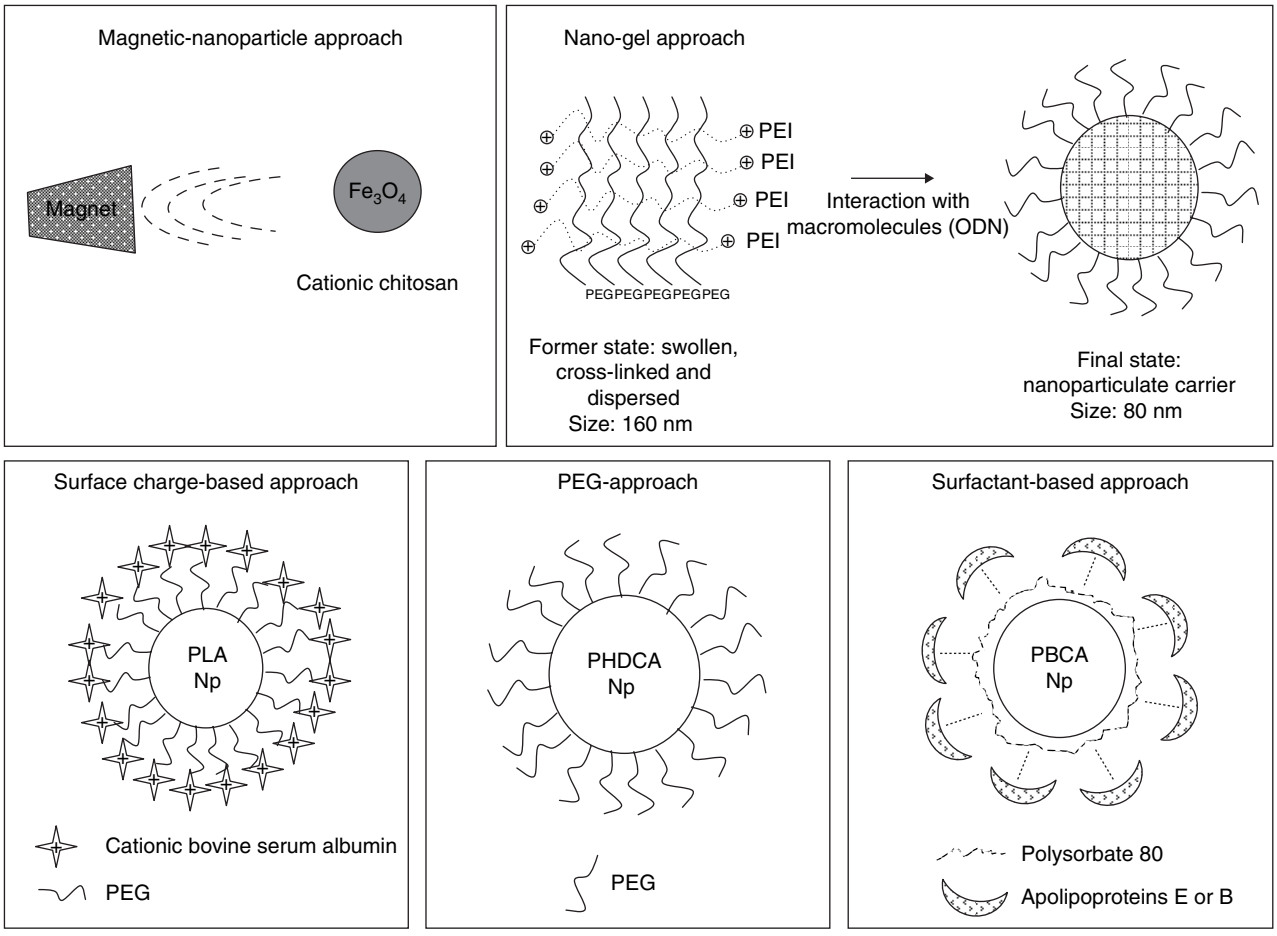

Figure 1. Schematic representation of possible approaches in Np-mediated brain drug delivery.
Np: Nanoparticle; ODN: Oligonucleotide; PBCA: Poly(butylcyanoacrilate); PEG: Polyethylene glycol; PEI: Poly(ethylene imine); PHDCA: poly(cyanoacrylate-co-hexadecylcyanoacrylate); PLA: Polylactide.

3.1 Magnetic-nanoparticle approach

The use of an external magnetic field for drug targeting could be considered as one of the so-called 'physical-targeting' methodologies [60].

The rationale for this approach is based on the eventuality that particles in the bloodstream could be concentrated at a specific target site within the body by using external or internal high-gradient magnetic fields. Once the Np are accumulated at the target site, the drug could be released via enzymatic activity or changes in the physiological conditions (pH, osmolality, temperature) [61].

The effectiveness of the therapy depends on several parameters, such as the field strength, the volumetric and magnetic properties of the particles, the infusion route, the depth of the target site, the strength of the drug–carrier binding and the tumor volume. In general, the Np are made up of a magnetic inner core coated by a compatible polymer, which acts to shield the magnetic particles from the environment, and also enables the surface to be chemically modified. There are several examples of magnetic particles that have been directed to the target sites, particularly tumors, using an external magnetic field [62,63]; the feasibility of these carriers has been demonstrated by several studies, up to Phase I clinical trials, in which a good body tolerance of these carriers was shown along with promising delivery of the particles to the target site [64]. Finally, this approach was also used more recently for the imaging of cerebral areas [65,66], as well as for monitoring brain tumors by using magnetic iron oxide Np.

This technique has also been employed to target cytotoxic drugs to brain tumors. Hassan and colleagues proposed to use of a magnetic field in order to carry magnetic Fe_3O_4 microspheres of cationic chitosan loaded with oxantrazole into the CNS [67]. After intra-arterial administration, a higher brain concentration of the embedded drug was achieved when a magnetic field (6000 G over 30 min) was applied. The increased brain localization could be ascribed both to the magnetic field and to the possible interaction of cationic Np with the anionic BBB. This method was also applied by Puffer and Gallo to direct magnetic

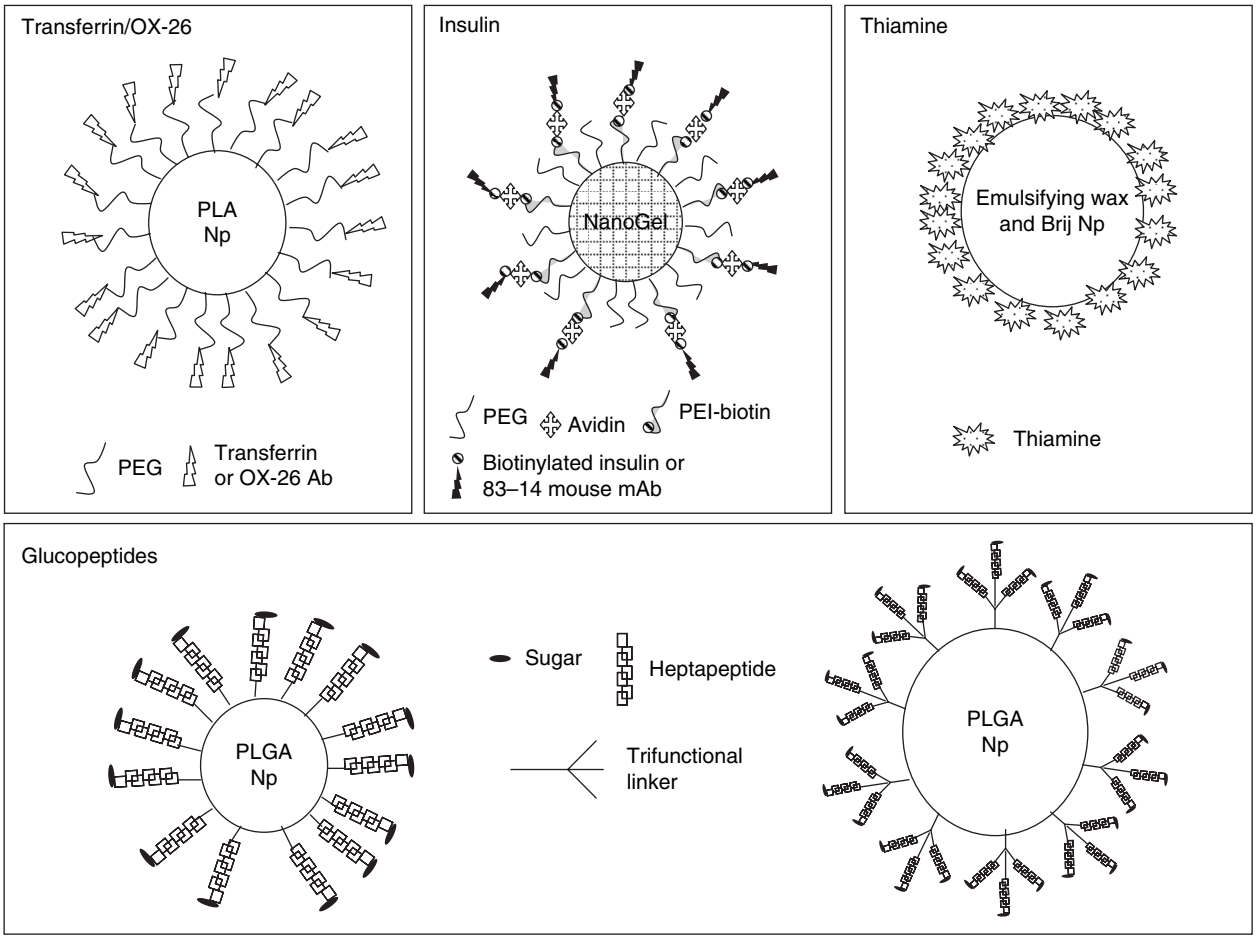

Figure 2. Schematic representation of the ligand-based approach.
Np: Nanoparticle; PEG: Polyethylene glycol; PLA: Polylactide; PLGA: Polylactide-co-glycolide.

microspheres to a brain glioma after an intracarotid injection in animals [68].

In order to understand the relevance of the magnetic field or the Np surface charge, these authors reduced the size of the particles to 10 – 20 nm and eliminated the surface charge by using a neutral polymer [69]. The experiments showed that the neutral magnetic Np, administered to rats with brain tumors (glioma), localized mainly into the brain tumor tissue after the activation of the external magnetic field. If compared with larger magnetic microparticles (1 μm), the neutral magnetic Np showed a higher level of brain tumor distribution, configuring themselves as drug delivery systems for targeting brain tumors.

As a general consideration, notwithstanding the very promising results of the studies and trials on magnetic Np, it is important to underline the problems connected with this approach, such as the possible embolization of the blood vessels as a consequence of Np aggregation, the difficult scale-up from animal models because of the distance between the target site and the magnet, the non-magnetic properties of the drugs once released from the Np and the possible body toxicity connected with the magnetic carriers.

In order to improve the selectivity of these carriers and to optimize the capture and localization of carriers at desired sites in the body, internal magnets can also be positioned in the vicinity of the target by minor surgery. Furthermore, the simultaneous use of an external magnetic field and a magnetic implant to direct the magnetic Np is also a promising strategy [70].

3.2 Nanogel approach

Nanogel possesses a unique dynamic architecture, which, in a former state, is swollen, crosslinked and dispersed in solution. The electrostatic interactions produced by the binding of macromolecular drugs with PEI chains cause the structure to collapse, and therefore decreases the volume and size of the particles. PEG plays a key role in obtaining a stable dispersion with an approximate size of 80 nm.

The potential of nanogels relies on the possibility that they spontaneously adsorb biomacromolecules, in particular

gene material as the cationic charges of the gel interact with the negatively charged oligonucleotides (ODN) thus allowing their delivery [71].

One of the most important advantages of the nanogel-ODN approach is the probability of obtaining a high loading capacity (from 40 to 60% of ODN by weight), which has not yet obtained with other carriers used in gene therapy, along with the protection of ODN against enzymatic degradation.

A further development of this system, which is connected with the chemical structure, is the conjugation of specific ligands by means of amidic linkage to the nanogel or by a 'biotin-avidin' coupling chemistry for improved targeting [72]. An increase in BBB transport was noted when the PEI-PEG network was conjugated with Tf or INS (see Section 3.7.2) [73]. *In vivo* experiments with mice, treated with phosphorothioate ODN loaded into nanogel particles, showed a low toxicity in addition to increased brain localization (15-fold with respect to free ODN) [73]. This finding could be related to the decrease of both liver and spleen accumulation in comparison with the free ODN [74].

3.3 Emulsifying wax and Brij 72 approach

Recently, Koziara and colleagues developed a new kind of Np for the BBB crossing, using emulsifying wax, Brij 72 (B-72), Brij 78 (B-78) and T-80 [75-77].

The Np were obtained using warm oil-in-water micro-emulsion templates in which the oil phase was constituted by emulsifying wax and B72, while B-78 and T-80 were used as the surfactants [78]. The *in vivo* studies were mainly performed by means of *in situ* brain perfusion technique [79] in order to obtain kinetics and mechanisms of BBB transport data in an *in vivo* environment. These Np did not produce adverse effects on the BBB parameters [77]. To further confirm the non-toxic effect of Np administration on the integrity of TJ, different assays have been completed (western blot analysis) in order to check the expression of claudin-1 and zona-occludens-1, whose decrease is considered as an index of TJ disruption.

By using ^{3}H-Np, brain distribution parameters have been evaluated over a timeframe of 0 – 60 s, without significant changes. These Np were found to be not associated with brain endothelium and no Np efflux from the BBB took place [80]. The effect of the surface charge of these new Np on the BBB crossing capacity was studied by adding 10% (w/w) *N*-octadecyl choline to obtain cationic Np, while B-78 and sodium dodecyl-sulfate were introduced to prepare anionic Np [81]. The results indicated that a low amount (10 µg/ml) of anionic Np did not affect the integrity of the BBB, whereas doubling the amount of both anionic and cationic Np caused the TJ to open.

The authors also formulated an antitumoral agent (paclitaxel), not capable of crossing the BBB, in the emulsifying wax/B-72 Np, confirming, after both *in vivo* and *in vitro* experiments, the usefulness of these Np for CNS targeting [80].

3.4 Surface charge-based approach

The surface properties of the Np represent a key factor in the drug delivery system for CNS targeting (**Table 1**); owing to this, different studies have been performed in order to investigate the role of the surface charge. Fenart and colleagues studied the penetration of different charged malto-dextrin Np on a bovine brain capillary culture showing that ionically charged Np increased BBB penetration in comparison with the results achieved in the presence of uncoated Np [82]. Nevertheless, the ability of charged (mainly positive) Np to cross the BBB was dramatically decreased by a strong interaction with red blood cells.

Considering the surface-charge based approach, interesting studies have been carried out using PEG-PLA Np conjugated with cationic bovine serum albumin (CBSA) [83]. The rationale behind the use of CBSA as a brain molecular targetor comes from the evidence that neuropeptides conjugated with CBSA are able to cross the BBB. Therefore, CBSA was conjugated with PEGylated Np in which 6-coumarin was loaded as the fluorescent probe. The *in vitro* and *in vivo* experiments showed that the amount of Np in the brain was higher than that obtained by conjugating normal neutral bovine albumin (BSA) to the Np thus possessing a slightly negative surface charge (-10 mV).

A plasmid (pORF-hTRAIL) with therapeutic activity against brain malignant glioma was encapsulated into CBSA Np [84] and then tested both in *in vitro* and *in vivo*. After transfection in C6 glioma cells using the CBSA Np, the presence of the plasmid in the cytoplasm was demonstrated *in vitro* at 30 min while the capacity of Np to transport the plasmid into the nuclei was recorded at 6 and 48 h, inducing apoptosis. The *in vivo* experiments confirmed the *in vitro* results, showing the localization of the plasmid in brain and tumor microvasculature after 30 min. Repeated injections of plasmid-loaded CBSA Np induced *in vivo* apoptosis and decreased the tumor growth, confirming the feasibility of these new Np for a brain gene therapy against glioma.

Experiments were carried out *in vitro* and *in vivo* in order to explain the CBSA-PEG-PLA Np mechanism of BBB crossing [85]. The hypothesis of a possible adsorptive-mediated transcytosis of CBSA-PEG-Np was confirmed by the result that free CBSA was be able to inhibit the passage of Np across the BBB.

A recent *in vitro* study on the fundamental features for a satisfactory passage across the BBB [86] analyzed the effect of the surface charge along with the inner composition of the maltodextrin-derived Np. The Np were prepared with or without a cationic ligand and the dipalmitoyl phosphatidyl glycerol as the inner core. The experiments carried out at 4°C showed a different localization of the Np according to the surface charge. Cationic Np were found mainly

Table 1. Summary of the methods, models and results concerning the magnetic-Np, nanogel, EX and B-72 and the surface-charge approaches.

Approach	Polymer	Drug	*In vitro*		*In vivo*		Ref.
			Model	Results	Model	Results	
Magnetic-nanoparticle							
	Cationic/neutral CS				i.v. rats bearing brain glioma	Higher brain accumulation	[63,69]
Nanogel							
	PEI/PEG	ODN			i.v. mice	Higher ODN brain localization Lower ODN spleen and liver accumulation Increased CNS delivery when coupled with ligand	[71-73]
EX and B-72							
	EW, B-72, B-78, T-80				Cerebral perfusion on Balb/c mice	Studies on BBB transport kinetics and mechanism	[75]
					Cerebral perfusion on Balb/c mice	Th coated Np allow increased BBB transport	[76]
			BBMEC [79]	Non-toxic effect on BBB	Cerebral perfusion on Balb/c mice	Non-toxic effects on BBB	[77]
		Px	U-118 HCT-15	Px maintained cytotoxic activity Lack of Px interaction with P-gp	Cerebral perfusion on Balb/c mice	Increased Px brain uptake	[80]
					Cerebral perfusion on Balb/c mice	Low amount of anionic Np did not affect the BBB permeability (10 µg/ml)	[81]
Surface charge based							
	Charged M-DX		Bovine brain capillary culture		i.v. mice	Increased BBB penetration	[82]
	Charged M-DX + DPPG		Co-culture of BCEC and glial cells	Caveolae pathway used by neutral Np for BBB crossing Transcytosis of cationic or neutral Np			[86]
	PEG-PLA + CBSA	6-Coumarin	BCEC (primary culture)	Increased cell uptake	i.v. mice	Increased BBB penetration in comparison with neutral bovine albumin	[83]
			Co-culture of BCEC and astrocyte	Absorptive mediated transcytosis of Np	i.v. mice	Increased BBB penetration Investigation on BBB transport mechanisms Demonstrated ABC considering PEGylated Np	[85]
		pORF-hTRAIL	C6 glioma cells (CCL-107)	Different localization in function of time	i.v. mice bearing i.c. C6 gliomas	*In vivo* apoptosis Significant delayed tumor growth	[84]

ABC: Accelerated blood clearance; B: Brij; BBB: Blood–brain barrier; BBMEC: Bovine brain microvessel endothelial cells; BCEC: Brain capillary endothelial cell; CBSA: Cationic bovine serum albumin; CNS: Central nervous system; CS: Chitosan; DPPG: Dipalmitoyl phosphatidyl glycerol; HCT: Human colorectal adenocarcinoma cell line; M-DX: Malto-dexstrin; ODN: Oligonucleotide; PEG: Polyethylene glycol; PEI: Polyethyleneimine; P-gp: P-glycoprotein; PLA: Polylactide; Px: Pacliataxel; T-80: Polysorbate 80; Th: Thiamine.

localized around the paracellular area, while neutral ones were localized mainly on the cell surface. Dipalmitoyl phosphatidyl glycerol-Np were found at both paracellular areas and the surface of the cells. When the temperature was increased up to 37°C, neutral and cationic Np had similar degrees of binding and uptake, which led to the conclusion that a transcytosis mechanism occurred. The treatment of the cells with filipin, a sterol-binding agent known to interfere with the caveolae-dependent pathway, displayed an inhibition of neutral Np trancytosis, indicating the involment of the caveolae pathway in the mechanism of transport of the Np across the BBB.

3.5 The surfactant-based approach

Radiolabeled poly(butylcyanoacrilate) (PBCA) Np coated with a surfactant such as T-80 demonstrated their ability to cross the BBB when administered intravenously [87]. In fact, PBCA Np coated with T-80 and loaded with dalargin [88], loperamide [89], doxorubicin [90-92], some NMDA-receptor antagonists [93] and some peptides [94] were tested and the pharmacological effects were considered as proofs of the BBB crossing. Similar results were obtained in the presence of PEGylated PBCA Np [95].

Starting from the early studies in which a BBB crossing was demonstrated, several investigations were performed in order to understand the mechanism of Np entrance to the CNS. Initial studies indicate an endocytic mechanism when Np cross the BBB, with possible transcytosis [96], while further experiments conducted in the presence of inhibitors of phagocytosis (cytochalasin B) and inhibitors of pinocytosis (colchicines) suggested the involvement of a phagocytic mechanism. More recent studies carried out with PBCA Np with covalently linked T-80, showed the involvement of low-density lipoprotein (LDL) receptors in Np uptake [97,75]. In fact, Kreuter and colleagues clearly illustrated that only the Np preincubated with apolipoprotein E (ApoE) and B (ApoB) were able to cross the BBB after *in vivo* administrations [97]. It seemed that T-80 coated PBCA Np allowed for a stronger anchoring of ApoE and ApoB to the Np surface; thus, the Np mimic LDL particles and the uptake by endothelial cells seems to be mediated by their interaction with LDL receptors. Moreover, the ability of Np to cross the BBB seems to be also linked to the presence of apolipoproteins, such as A-I and A-IV, which allow the avoidance of the hepatic uptake prior to the contact with the ApoE receptors located on the BBB [98].

Notwithstanding these promising results, numerous criticisms have been raised concerning two aspects of the formulations. First, the role of the surfactant (T-80) and the possible effect of the polymer or its degradation products on the integrity and permeability of the BBB.

It has been shown that T-80 is able to inhibit P-gp [99-101] and to reverse multi-drug resistance [102]. It has been suggested that the surfactant may be delivered more efficiently to the brain endothelial cells if it is adsorbed to the Np surface. Kreuter and colleagues in fact suggested that blockage of the efflux system by T-80 may contribute to brain drug delivery of Np but that other mechanisms, such as endocytosis, would play a much more important role [96].

Second, questions have been raised on Np made regarding the role of the polymer, and in particular its toxicity and the possible involvement in the TJ opening, in T-80-coated PBCA Np. In fact, Kreuter and colleagues showed in earlier studies that the brain intravascular inulin spaces increased from 1.03 up to 2.05% [94,103] in an *in vivo* model. Notwithstanding, the increase in the inulin spaces, an antinociceptive effect was not created after an *in vivo* treatment with a simple mixture of T-80-coated PBAC Np and dalargin.

Olivier and colleagues suggested that the BBB crossing of T-80-coated PBCA Np was due to their toxicity towards the BBB [104]. A further study on the *in vitro* interaction of PBCA Np with endothelial cells showed a significant increase in sucrose flux across the cell monolayer, allowing a direct TJ opening by PBCA Np to be hypothesized.

However, Kreuter *et al.* recently stated that there is 'a little *in vivo* and *in vitro* evidence to suggest that a generalized toxic effect on BBB by T-80 PBCA Np is the primary mechanism for drug delivery to the brain' [105]. They verified the importance of the need to preadsorb the drug dalargin onto Np in order to obtain a pharmacological response, thus excluding a simple disruption of the BBB and suggesting a more specific mechanism of transport across the BBB. In this work, the authors proposed some explanations concerning the increase in inulin spaces, such as a recruitment of the cerebral capillaries, a stimulation of endocytosis in the endothelium, a modulation of the TJ permeability or a combination of such factors.

Sun *et al.* [106] investigated the role of T-80 in increasing the BBB crossing by the *in vivo* administration of PLA Np coated with T-80. The study was carried out by using surfactant-free Np or T-80-coated PLA Np, both with or without fluorescein isothiocyanate-dextran as the loading probe. The results showed the safety of PLA Np, which did not cause the death of animals in any case; moreover, the ability of only T-80-coated Np to reach the brain has been demonstrated by fluorescence studies of the brain. These findings seem to rule out the hypothesis of a direct toxicity of PBCA on the BBB endothelium, but rather suggests a fundamental role of T-80 in increasing the permeability of BBB to polymeric Np [106].

Another remarkable consideration, which may hinder the development of PBCA Np, is directly connected to the lack of FDA approval both for this polymer and for the surfactant (T-80) required for a good brain targeting result (**Table 2**).

3.6 PEG-approach

The rate of Np clearance from the blood is related to their size and surface characteristics; one possible approach to

Table 2. Summary of the methods, models and results concerning the surfactant based approach.

Approach	Polymer	Drug	*In vitro*		*In vivo*		Ref.
			Model	**Results**	**Model**	**Results**	
Surfactant-based							
	PBCA + T-80	Dalargin			i.v. rats	Pharmacological response (opioid activity)	[88]
			Brain blood vessel endothelial cells	Uptake of Np	i.v. rats	Pharmacological response (opioid activity)	[94]
		Loperamide			i.v. rats	Pharmacological response (opioid activity)	[89]
		Doxorubicin			i.v. in gliobastoma rats	Increased accumulation in tumor tissues and positive pharmacological response (anti-cancer activity)	[90-92]
		MRZ 2/576 (NMDA receptor antagonist)			i.v. rats	Prolonged duration of anticonvulsive activity	[93]
		Loperamide Dalargin			i.v. rats and i.v. in rat ApoE deficients	Antinociceptive effect of the drugs ApoE and B mediate the BBB crossing	[97]
		Dalargin	Bovine brain capillary endothelial cells and rat astrocytes	Permeabilization of the BBB model	i.v. mice	Potent antinociceptive effect Decreased locomotor activity Occasional mortality	[104]
		Dalargin	Bovine primary cerebral endothelial cells	No increase in paracellular transport of sucrose and inulin	i.v. mice	Antinociceptive effect only if dalargin is preadsorbed to PBCA Np	[105]
	PLA + T-80	FITC-dextran			i.v. mice	BBB crossing is related to the presence of T-80 bound to the Np surface	[106]

Apo: Apolipoprotein; BBB: Blood–brain barrier; FITC: Fluorescein isothiocyanate; Np: Nanoparticle; PBCA: Poly(butylcyanoacrilate); PLA: Polylactide; T-80: Polysorbate 80.

prolong the plasma half-life of Np is to render the surface more hydrophilic, therefore avoiding macrophagic attack.

The use of PEG is surely the most successful approach, widely used not only in nanoparticulate carriers but also generally in drug delivery systems [107] and in liposome-based therapy (**Table 3**) [32]. PEG can be covalently linked to other hydrophobic polymers [108]; for example, the conjugation of PLGA/PLA with PEG led to the development of block copolymers in which the covalent linkage of the hydrophilic PEG shell to the core of the Np avoids the possibility of PEG removal/desorption once in contact with biological fluids [109]. Different studies were carried out in order to understand the importance of the MW of PEG in avoiding plasma protein interactions; the best results were obtained in the presence of polymers having a MW range of between 2000 and 5000 Da [31].

Another important focus is connected with the surface of the PEG chain conformation on PLA-Np. Depending on the surface density, PEG blocks could be in a brush-like (elonged coil, high density) or mushroom-like (random coil, low density) conformation: brush-like PEG surface reduced the phagocytosis, while mushroom-like ones activated both the complement factors and macrophages [110-112].

Not only was PLGA conjugated with PEG to prolong Np circulation, but also several derivatives of PACA, such as poly(cyanoacrylate-co-hexadecylcyanoacrylate) (PHDCA), have been modified using the same method [95,113-120].

In vivo studies [95] demonstrated the ability of PEGylated ^{14}C-PHDCA Np to cross the BBB. Owing to their long-circulating characteristics, these Np penetrated into the brain to a higher extent than T-80- or poloxamine 908-coated or uncoated PHDCA Np. The PEGylated Np were found in the ependymal cells of the choroid plexuses, the epithelial cells of pia mater and ventricles, and, to a lower extent, the capillary endothelial cells of BBB. The internalization occurred without any modification of BBB permeability whereas T-80-coated Np owed, in part, their efficacy to BBB permeabilization induced by the surfactant.

Based on these findings, Couvreur and colleagues also proposed the PEG approach for the treatment of prion disease [113]. After the administration of Np in infected animals, a higher uptake by the target tissues (spleen and brain) occured in comparison with conventional non-PEGylated Np.

Another *in vivo* study was carried out [114] in order to quantify the accumulation of these Np into the brain of rats infected by allergic encephalomyelitis. As the PEGylated Np were found at a higher concentration in brain areas, the authors proposed two explanations for a BBB-crossing mechanism: the first consists of passive diffusion of the Np due to the increase in BBB permeability and the second of a possible transport of the macrophages containing the Np, which then infiltrate the inflammated tissues.

The *in vivo* experiments also took place in tumor models. By using a gliosarcoma as the model, PEG-PHDCA Np were shown to accumulate preferentially in the tumoral tissue [115]. These findings were explained by reduced plasma clearance with the consequent diffusion by extravasation, and due to the affinity of PEG-PHDCA Np for the endothelial cells of the BBB.

Relevant *in vitro* models [116-120] were recently developed in which structural characteristics of brain endothelial cells, such as the expression of P-gp, occludin and zona-occludens-1, were maintained in order to study the mechanism of Np brain entry [116,118]. A further improvement [118] on the study of the mechanism of PEG-PHDCA Np entry into the brain has been performed using different kinds of transport inhibitors, such as filipin and nystatin for the caveolae-mediated pathway or chlorpromazine and NaN_3, which interferes with clathrin and energy-dependent endocytosis. The results indicated the importance of this last pathway along with the involvement of LDL receptor-mediated pathway.

An important criticism concerning the PEG approach is directly linked to the effect of repeat administrations of PEGylated carriers.

As shown in Section 3.4, CBSA-PEG-PLA Np were used for enhancing BBB crossing. In these studies [121-129], Lu and colleagues analyzed the accelerated blood clearance phenomenon, first recorded with PEGylated liposomes, which undergo a rapid clearance from the circulation when repeatedly injected.

In fact, IgM, produced as a consequence of the injection of PEGylated liposomes, created an activation of complement with a corresponding accelerated clearance and enhanced hepatic uptake [130]. The same results were found in the presence of PEGylated CBSA-PLA Np.

On the basis of these results, it is surely desirable to investigate in detail the effect of multiple administrations of PEGylated nanosystems, to better understand the real effectiveness of those 'stealth' systems and to prevent any side effect deriving from their administrations.

3.7 Ligand-based approach

The ligand-based approach has become an interesting choice for more specific and selective drug delivery to the CNS district (**Figure 2** and **Table 4**). This approach is based on the covalent linkage of ligands to the polymers or to the Np in order to promote direct interaction with transport systems [131,132].

The ligands, which could be used in CNS drug delivery, have to be chosen with appropriate characteristics in order to take advantage of receptor-mediated trancytosis or receptor-mediated endocytosis. Possible ligands could be natural substrates, such as Tf, INS and thiamine but also synthetic or natural peptides.

3.7.1 Transferrin and OX-26 antibody

This approach is based on the conjugation of Np with Tf and INS, which were among the first proteins for

Table 3. Summary of the methods, models and results concerning the PEG-approach.

Approach	Polymer	Drug	*In vitro*		*In vivo*		Ref.
			Model	**Results**	**Model**	**Results**	
PEG							
	PHDCA-PEG	^{14}C radiolabeled-PHDCA or fluorescent polystyrene coating			i.v. mice and rats	Increased entry into the CNS of T-80 or P-908-coated PHDCA-PEG Np with respect to uncoated PHDCA Np No alteration of BBB parameters Interaction with endothelial cells	[95]
		^{14}C radiolabeled-PHDCA			i.v. rats as model of prion diseases	Higher uptake by the spleen and the brain (target tissue of prion diseases)	[113]
		^{14}C radiolabeled-PHDCA			i.v rats infected of EAE	Higher localization in cerebral parenchyma compared with P-908-coated Np Higher CNS localization in pathological situation in which BBB permeability is compromised and infiltrated by macrophages BBB transport mechanisms: passive diffusion and macrophage uptake in inflammatory lesions	[114]
					i.v. rats bearing 9L gliosarcoma	Increased entry both in tumor brain regions and in normal brain regions	[115]
			RBEC	Different patterns of intracellular capture, in function of surface composition			[116]
			Co-culture of RBCE and rat astrocytes	Higher passage of Np probably due to endocytosis			[117]
			RBEC	Adsorption of Apo E and Apo B-100 on the Np surface Involvement of LDL_R in BBB crosssing			[118]
			RBEC	Endocytosis as BBB transport; mechanism mediated by LDL_R			[119]

Apo: Apolipoprotein; BBB: Blood–brain barrier; CNS: Central nervous system; EAE: Experimental allergic encephalomyelitis; LDL_R: Low density lipoprotein receptor; Np: Nanoparticle; P-908: Poloxamine-908; PEG: Polyethylene glycol; PHDCA: Poly(cyanoacrylate-co-hexadecylcyanoacrylate); RBEC: Rat brain endothelial cells; T-80: Polysorbate 80.

Table 4. Summary of the methods, models and results concerning the ligand-based approach.

Approach	Polymer	Drug	*In vitro*		*In vivo*		Ref.
			Model	Results	Model	Results	
Ligand-based							
OX-26 mAb	PEG-PLA					Physico-chemical characterizations of immuno-pegylated Np. No *in vivo* or *in vitro* experiments	[143,144]
	PEG-Cs	FITC-labeled			i.v. mice	(A-B_Tec) labelled Np presence into the brain parenchyma	[145]
	DSPE-PEG-LNc		Tf cell line	Specific binding of LNc to TfR			[146]
Tf	PEG-Albumin	AZT			i.v. mice	Enhanced uptake of Tf-anchored Np in the brain tissues	[147]
Tf and INS	Nanogel	ODN	BBMEC	Significant transport across the BBB	i.v. mice	No adverse toxic effects Increase brain accumulation of ODN	[73]
Th	EW, B-72, B-78, T-80				Cerebral perfusion on Balb/c mice	Enhanced Np brain delivery due to interaction of Th-Np with the Th-T at the BBB	[76]
Peptide-derivatized	PLGA + gluco-heptapetide				Cerebral perfusion (rats) i.v. rats	BBB crossing assessed by fluorescent microscopy Accumulation of Np in cerebral areas after cerebral perfusion Presence of Np in cerebral areas after systemic administration	[168]
		Loperamide			i.v. rats	Prolonged analgesic effect due to loperamide delivered to the CNS	[173]
		Rh-123			i.v. rats	Delivery of Rh-123 to the CNS Decreased liver uptake of Rh-123 loaded Np	[173]

A-B_Tec: Avidin-biotin technology; AZT: Azidothymidine; B: Brij; BBB: Blood–brain barrier; BBMEC: Bovine brain microvessel endothelial cells; CNS: Central nervous system; Cs: Chitosan; DSPE: Distearoylphosphatidylethanolamine; EW: Emulsifying wax; FITC: Fluorescein isothiocyanate; INS: Insulin; LNc: Lipid nanocapsule; mAb: Monoclonal antibody; ODN: Oligonucleotide; PEG: Polyethylene glycol; PLA: Polylactide; Rh: Rhodamine; T-80: Polysorbate 80; Tf: Transferrin; TfR: Transferrin receptor; Th: Thiamine; Th-T: Thiamine transporter.

which it was suggested the binding to the brain microvessels and a transcytosis mechanism for the passage through the BBB [133-135]. Tf mediates the transport of iron to the brain by means of its interaction with Tf receptor (TfR) [136]. Tfr undergoes receptor-mediated trancytosis and is highly expressed by brain capillaries [137]. However, Tf is not an ideal brain delivery vector as it is nearly saturated with endogenous Tf, present in the bloodstream at a concentration of 25 μM [138]. As an alternative, there has been much proof-of-concept success in using antibodies against the TfR. The mouse mAb against the rat TfR, OX-26, has been the most extensively studied [138-140]. This antibody binds to an extracellular epitope of TfR distinct from the Tf binding site, thus preventing the competition for binding sites between the drug targeting vector and the natural ligand [140-142].

One of the most relevant works on the synthesis of Np conjugated with antibodies is the recent preparation of PEGylated immunonanoparticles [143,144], made by PEG-PLA and coupled with OX-26 mAb; moreover, polymers that are different from PLA were also used. For example, chitosan nanospheres conjugated with PEG bearing OX-26 mAb were shown to possess an enhanced BBB transport [145]. After an *in vivo* administration (mice), the authors established the presence of fluorescently labeled Np in the brain areas, outside the intravascular compartments. This technology was applied to the transport of a caspase inhibitor (peptide Z-DEVD-FMK), which is able to significantly reduce the death of the neuronal cell after an ischemic attack.

PEGylation in association with the use of antibody OX-26 has also been applied in the preparation of lipid nanocapsules by Benoit's group [146]. The ability of these immunonanocapsules to be targeted via the TfR was successfully assessed with *in vitro* experiments on cells overespressing TfR.

Another study [147] in which the Tf ligand-based approach has been applied concerns engineered PEGylated albumin Np encapsulating azidothymidine, a water-soluble antiviral drug. The surface of these Np was modified by anchoring Tf; *in vivo* studies (fluorescence microscopy) demonstrated an enhancement of Np accumulation in the brain tissues.

3.7.2 Insulin

The INS receptor (INS-R), like the TfR, is found on the luminal membrane of brain capillary endothelial cells [148,149] and can undergo receptor-mediated transcytosis across the BBB endothelium [150].

The INS-R consists of two α-subunits and two β-subunits [151]: the α- and β-subunits are joined by disulfide bonds to form a cylindrical structure. When INS binds, a conformation change takes place allowing tyrosine kinase activity and subsequent receptor internalization.

When considering the ligand-based approach, the use of the INS as an endogenous ligand is surely not suitable because of its rapid degradation in the bloodstream (serum half-life of only 10 min) and the possible interference with the natural INS balance [152]. However, like the TfR, antibodies recognizing the INS receptor have been used as BBB-targeting vectors. Extensive research using the 83 – 14 mouse mAb against the human INS-R as a receptor-mediated transport delivery vector has been performed, mainly in primates (Rhesus monkey) [153].

This approach was also applied to nanogel [73]. In order to target the nanogel to the CNS, the use the PEG technology coupled with the avidin-biotin method have been applied for the attachment of Tf or INS; in particular, the biotin-modified nanogel was loaded with ODN and formed an ODN-loaded biotin-nanogel complex, which was reacted with the preformed complex of avidin and biotinylated Tf or INS. This study was built on *in vitro* experiments on bovine brain microvessel endothelial cells and *in vivo* tests on mice. The mechanisms of rhodamine-labeled nanogel transport in endothelial cells, the toxicity of nanogel and ^{3}H-nanogel or ^{3}H-ODN biodistribution in different organs (including the brain), have been investigated. The *in vitro* and *in vivo* results suggested that INS or Tf modified nanogels were non-toxic. Furthermore, the permeability increased 12-fold if compared to the free ODN; thus nanogel carriers can protect ODN from rapid clearance by peripheral tissues and increase ODN transport to the brain.

3.7.3 Thiamine

Thiamine (Th), also known as vitamin B1, is a water-soluble micronutrient that is essential for normal cell function, growth and development. It is able to cross the BBB via endogenous specific transport system [154-156]. Based on BBB thiamine transport capacity [154,155,157], this nutrient transporter has been suggested as a brain drug delivery vector [158].

The effectiveness of using the Th ligand in tumor-targeting via Np was studied *in vitro* [159]. Th-coated gadolinium NP made of emulsifying wax was specifically associated with human breast cancer cells that expressed the thiamine transporters THTR1 and THTR2.

Based on these findings, the authors tested labeled Th-coated Np to evaluate the *in vivo* brain uptake after *in situ* brain perfusion in comparison with blank Np [76]. The results showed a nonlinear, delayed, brain uptake after 45 s. Prolonging the time up to 120 s, the brain uptake was linear for both coated and un-coated Np with a transfer rate for Th-coated Np greater than the control. The administration of free Th (100 nM) produced the inhibition of the uptake of Th-coated Np. These data suggested that Th-coating of Np can be a successful technology to enhance brain delivery of Np owing to the interaction of surface Th on Np with the Th transporter at the BBB. The enhanced permeability across the endothelial barrier could be due to a facilitated transport mechanism or to the increase of passive diffusion produced by an increase of the concentration gradient at the BBB interface.

3.7.4 *Peptide-derived nanoparticles*

Very recently, the author's group developed a new strategy for Np brain targeting by using peptide-derived PLGA [160].

Some categories of opioid peptides have been shown to penetrate the BBB [161]; moreover, the glycosilation of peptides [162] has been widely used as a method to penetrate the BBB, as described for enkephalin analogs [163], vasopressin analogs [164], deltorphin and dermorphin glycopeptide analogs [165,166] and other peptides [167].

Hence it was decided to use peptides that are able to cross the BBB as delivery agents for the CNS [168]. The synthetic opioid peptide MMP-2200 (H_2N–L-Tyr-D-Thr-Gly-L-Phe-L-Leu-L-Ser–O–β-D-lactose–$CONH_2$) was considered the lead [169] and the peptide H_2N–Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu–L-Ser–O-β-D-glucose-$CONH_2$ was synthesized. The Tyr present at the N-terminus of MMP-2200 was substituted with Phe in order to avoid a potential opioid effect [170]. This glycopeptide was conjugated with PLGA to prepare modified polymers able to form Np or with a PLGA modified by a trifunctional linker to obtain increased Np surface coverage by the peptide [168,171].

The Np were prepared by the nanoprecipitation method, obtaining Np showing both the ligands for CNS targeting and the marker of fluorescence on their surface [172]. After administration, using *in situ* rat brain perfusion technique, the peptide-decorated Np were found to be able to cross the BBB, while PLGA Np were not able to reach the CNS. The administration of the same Np by a systemic route [168] demonstrated that these Np are able to bypass the hepatic uptake, thus reaching the brain.

Recent studies [173] were performed to assess the ability of these peptide-decorated Np to act as drug carriers. Loperamide, an opioid not able to cross the BBB, was loaded into the Np and administered through a rat tail vein; the evidence of loperamide delivery in the brain was achieved by the hot plate test (nociception assay). Loperamide-loaded Np (2.7 and 1.8 mg/kg) produced a high antinociceptive activity at 240 min after their administration. The effect, which lasted over 450 min, could be reversed by the injection of naloxone, confirming the involvement of the opioid receptors in the antinociceptive activity.

Since MMP-220 and other opioid peptides cross the BBB by adsorbption-mediated endocytosis owing to their amphypathic character [166], it is possible to hypothesize a similar mechanism for the BBB crossing of the author's peptide-decorated Np.

4. Conclusion

In the past an increasing number of studies have been performed in order to better develop therapies against brain diseases. The major obstacle for good cerebral treatments is the inability of some drugs to cross the BBB, hence not being bioavailable for the interaction with brain targets.

This challenge has been approached using different strategies, consisting of direct drug delivery or a temporary BBB opening. The drawback of these approaches is that they are too invasive for the patients. Thus, the colloidal approach represents one of the most interesting solutions.

Among colloids, polymeric Np have been rightly considered as promising carriers for CNS drug delivery, due to their potential both in encapsulating drugs, hence protecting them from excretion and metabolism within the body, and in delivering active agents across the BBB without inflicting any damage to the barrier.

Different polymers have been used and different strategies have been applied; among the polymers, PLGA, chitosan, PBCA and PHDCA have been used to prepare Np. A remarkable strategy is the use of different polymeric Np (such as the magnetic Np or the nanogels) or waxes (such as emulsifying wax or Brij 72), both to perform more specific and selective CNS drug delivery.

Specific ligands are commonly used in order to increase the specificity of drug delivery to the CNS, decreasing the loss of the drug over the peripheral blood circulation and improving the efficacy at the target site. Novel synthetic peptides derived from opioids, along with the use of antibodies or ligands for endogenous receptors, seem to be a very promising strategy, allowing CNS drug delivery that couples the CNS targeting with controlled release.

Different rationales drove the other approaches; in particular, the use of positively charged Np could be explained on the basis of their interaction with the negative charges of the BBB, while the use of surfactant (such as Polysorbate-80) and PEG is connected with the possible endocytic mechanism of BBB crossing and with the aim of prolonging the Np circulation half-life.

5. Expert opinion

The studies reviewed in this paper have shown that Np can be useful carriers for the targeting of drugs to the CNS. Np could bypass the defensive BBB and also the defensive mechanism of the body, which brings about the sudden removal of the nanoparticulate systems from the circulation.

Moreover, a good knowledge of the polymer, of its degradation rate, toxicity and finally its capacity to cause adverse immunological responses is the basis for successful Np. Thus, the polymers used in the preparation of Np for CNS drug delivery have to be approved by the FDA for their good safety qualities. At present, PLA-PLGA is one of the few approved polymers, together with chitosan or other natural polymers. To maintain the integrity of the BBB and to prevent inhibitory activity against P-gp, all the excipients used in the preparations of Np have to be non-toxic.

For the future clinical use of this drug delivery system the following problems must be solved. Even if the toxicity

of Np could be the same as that of the polymers used (bulk material), the potentially injurious Np as whole entities need to be investigated. Studies based on histopathology, haematology and clinical assays could help in detecting the effects of an exposure to Np, in addition to correlated studies on the Np distribution in different organs. Moreover, *in vivo* experiments should be targeted in order to determine the potentially toxic effects linked to a repeated administration of Np.

The FDA proposed the creation of a central NanoBank of particles available to research workers, which summarized all the Np-based experiments. The availability of this kind of information is one of the most important requirements of the FDA for nanoparticulate systems, as demonstrated by a very recent study on the 'Toxicology of Nanoparticles used in Healthcare' [174]. Hence, the FDA has begun to consider Np not only as drug carriers (like what happened for the other delivery systems, such as capsules, pellets and so on) but also as nanotherapeutics themselves, and owing to this, they need a proper nomenclature and in-depth studies about pharmacokinetic parameters, bioavailability and toxicity, which are now required for a new drug entity. Very recent studies have been directed to the effect of multiple administrations of PEGylated liposomes and PEGylated Np. The results obtained, which showed a fast blood clearance of the PEGylated nanosystems, should be taken into consideration with a view of the chronic administration of nanocarriers. Finally, considering the impact of the size, shape, chemistry, surface properties and degradation rates of both Np and all the polymers used on human health, new detailed guidelines are certainly a fundamental need.

On the other hand, the advantages of these nanosystems in CNS drug delivery seems to be real as demonstrated by *in vitro* and *in vivo* experiments. The efficacy of different strategies for crossing the BBB drew different researchers to focus their creative wit; linking a surfactant to a polymer and creating systems acting as Trojan horses, or taking advantages of more specific mechanism to target these carriers to the CNS by means of specific ligands linked to the Np surface. All of these approaches and strategies are good starting points for the improvement of drug delivery to CNS.

In conclusion, the need for a successful carrier for CNS drug delivery could be based on 'stealth' qualities, such the ability to escape macrophagic attack and not to be recognized by the immune system. Secondly, a 'healthy' carrier should be not toxic and should be easily biodegraded. At the same time the 'technological-pharmaceutical' properties, such the ability of the Np to protect the drug from elimination or metabolism, along with the control or modulation of the rate of drug release should be possible. Finally the ability of Np to deliver the drugs in the right site (CNS), crossing the BBB and thus rendering the action of the embedded drugs more specific and selective, represent properties that are key requirements to be able to name these Np 'smart' CNS drug delivery systems.

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