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Is there a clinical future for polymeric nanoparticles as brain-targeting drug delivery agents?

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Injectable nanosized carriers (5–250 nm) are actively studied as anticancer drug delivery agents for targeted drug delivery to the brain. Among these, polymeric nanoparticles (Np) have been studied since 1995, but only five of them recently started Phase I clinical trials, and none of these targets brain pathologies. To date, clinical trials for brain drug delivery have started for macromolecular- and nanocarrier-based systems in the treatment of brain tumors. This review, on the basis of the results obtained so far from preclinical studies, will critically consider the possibilities that polymeric Np have to reach the clinic as drug delivery agents for the brain, in comparison with other platforms.

Introduction

Injectable nanosized carriers (5–250 nm) are actively studied mostly as anticancer drug delivery agents. Their use is aimed at improving current disease therapies, owing to their ability to cross multiple biological barriers, protect the drug from degradation, release a therapeutic load in the optimal dosage range and enable the delivery of the therapeutic agent to a preferential site, realizing a targeted delivery. Moreover, nanopharmaceuticals offer the ability to extend the economic life of proprietary drugs and create additional revenue streams, thereby significantly affecting the drug commercialization landscape; because the drugs are already in clinical use, they have greater potential for reaching the clinic in a relatively short time.

There are over two-dozen nanotechnological therapeutic products that have been approved for clinical use to date (see Table 1) [1–3] and none of these targets the brain. Most of them address the delivery of antitumor drugs by means of the enhanced permeability and retention effect [EPR effect; the extravasation of the nanomedicine-associated drug into the interstitial fluid at the tumor site, exploiting the locally increased vascular permeability] [4].

By contrast, nanotechnological products raise a profound interest owing to the emergence of new classes of bioactive compounds such as those involved in RNAi pathways (e.g. siRNA and microRNA) or proteins. Owing to their characteristics, these new kinds of drugs need the development of nanotechnology platforms that can enable their delivery [5]. Several vehicles for systemic delivery of siRNA are currently being tested in animal studies [6–9].

Drug delivery to the brain is difficult, owing to the presence of the blood–brain barrier (BBB) (Fig. 1). Cerebral endothelial cells form complex tight junctions that effectively seal the paracellular pathway of brain entry; these cells interact with perivascular elements such as basal lamina and closely associated astrocytic end-feet processes, perivascular neurons and pericytes to form a functional BBB [10]. All the nanosized systems that can cross the BBB possess a targeting molecule or can bind blood molecules that are recognized by receptors present on brain endothelium, mediating endocytosis or transcytosis. Several endogenous BBB receptors have been considered for CNS-targeted drug delivery [e.g. receptors for iron transferrin (Tf), insulin (INS), glutathione, low-density lipoprotein (LDL), LDL receptor-related protein-1 and -2 (LRP-1, LRP-2)]. Drugs conjugated with ligands for these receptors [i.e. peptides or peptidomimetic monoclonal antibodies (mAbs)], or with other peptides discovered by phage-display, are actively studied [11–13] and recently started to be used for targeted delivery of nanocarriers. After transcytosis [14–18], the diffusion in brain extracellular space of most of the nanovectors under investigation, which have sizes >100 nm, has to be expected to be low because the width of this space in the healthy brain has been estimated to be 38–64 nm [19]. Then, nanosized carriers can be

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TABLE 1

The development status of carrier-based drug delivery systems for intravenous administration.

Carrier[a]	Main component	Product (platform)	Targeting moiety (target)	Company	Brain tumors: clinical phase (drug)	Market situation or clinical phase (indications) [drug]
Polymer-based						Several polymer-based drugs are on the market[b]
Polymer–drug conjugate	Polyaminoacids	CT2103, Xyotax		Cell Therapeutics	Phase III (paclitaxel)	
Peptide–drug conjugate		ANG1005 (EPiC)	Angiopep-2 peptide (LRP-1)	AngioChem	Phase II (paclitaxel)	
			VH0455Peptide (LDLR)	Vect-Horus	Preclinical	
		Trascend™	Melanotransferrin (LRP)	biOasis Technologies	Preclinical	
			Peptide RAP (LRP-2)	Raptor Pharmaceutical	Preclinical	
mAb-fusion proteins (Trojan horses)						
		AGT181	mAb against HIR	ArmaGen Technologies	Planned to enter Phase I soon (iduronidase enzyme)	
Nanosized drug carriers						
Polymeric Np	Cyclodextrin	CALAA01	Tf	Calando		Phase I (melanoma) [siRNA]
		IT101		Insert Therapeutics		Phase I (metastatic solid tumors) [camptothecin]
	Cyanoacrylate	Livitag®		BioAlliance Pharma		Phase II (hepatic tumors) [doxorubicin]
				Sichuan University, China		Phase II (hepatic tumors) [mitoxantrone]
	Polyester[c]	Bind014	Prostate-specific membrane antigen	Bind Biosciences,		Phase I (prostatic tumor) [docetaxel]
Liposomes[d,e]						Several drug-loaded liposomes are on the market not for brain diseases
	Lipids	2B3101	Glutathione (glutathione receptors)	To-BBB	Phase I, II (doxorubicin)	
		NLCPT11		University of California (USA)	Phase I (irinotecan)	
		Marqibo		Talon Therapeutics	Phase I–II (vincristine)	On the market (leukemia)
Albumin						
	Albumin	Abraxane		Abraxis Bioscience		On the market (metastatic breast cancer) [paclitaxel]

[a] Other carriers [polymers, dendrimers (among polymer-based carrier), nanogel, crosslinked BSA Np, SLN (among nanosized drug carriers)] are in preclinical research for CNS delivery; with the exception of polymeric drugs, no products based on these platforms are on the market (even for other indications).

[b] Currently, more than 14 polymer–drug conjugates are in clinical trials for other targets [1]; several compounds are clinically approved [2,3]. Over all, only one (PK2) is based on active targeting (the carrier contains a moiety that can establish specific interactions with the target cells).

[c] Micelles based on the polyester PLA are commercially available for the treatment of breast cancer (Genexol-PM, Samyang)

[d] Tekmira, Alnylam (Cambridge, MA, USA), Marina Biotech (Bothell, WA, USA), Silence Therapeutics (London, UK) and Sirnaomics (Gaithersburg, MD, USA) are involved in Phase I clinical trials with lipid-based systems for the delivery of siRNA [155,156].

[e] Ten untargeted liposomal preparations are clinically approved and others are in clinical trials [2].

endocytosed by brain parenchyma cells [14–16,20] and, at the same time, the drug released in the extracellular space can be removed from the brain by the P-glycoprotein (P-gp) efflux system and by the high cerebrospinal fluid turnover rate [21]. Brain tumor cells overexpress Tf [22], LDL [23] and LRP-1 receptors [24], present also on the BBB. These receptors therefore represent attractive targets, actively explored, for brain-tumor-targeting drug carriers.

Brain-targeted nanocarriers proved to be capable of delivering drugs into the CNS. To date, clinical trials for CNS-targeted drug delivery started for glutathione-decorated liposomes and drug–protein conjugates such as ANG1005, and also for nontargeted polyglutamate-paclitaxel (CT2103, Xyotax) for the combination treatment of brain tumors (Table 1).

This review, on the basis of the results obtained so far in noninvasive, non-viral-based preclinical brain drug delivery research, will highlight the potentialities that polymeric nanoparticles (Np), studied since 1995 as drug carriers for brain delivery, have to reach in the future the clinic, in comparison with other, recently discovered, platforms.

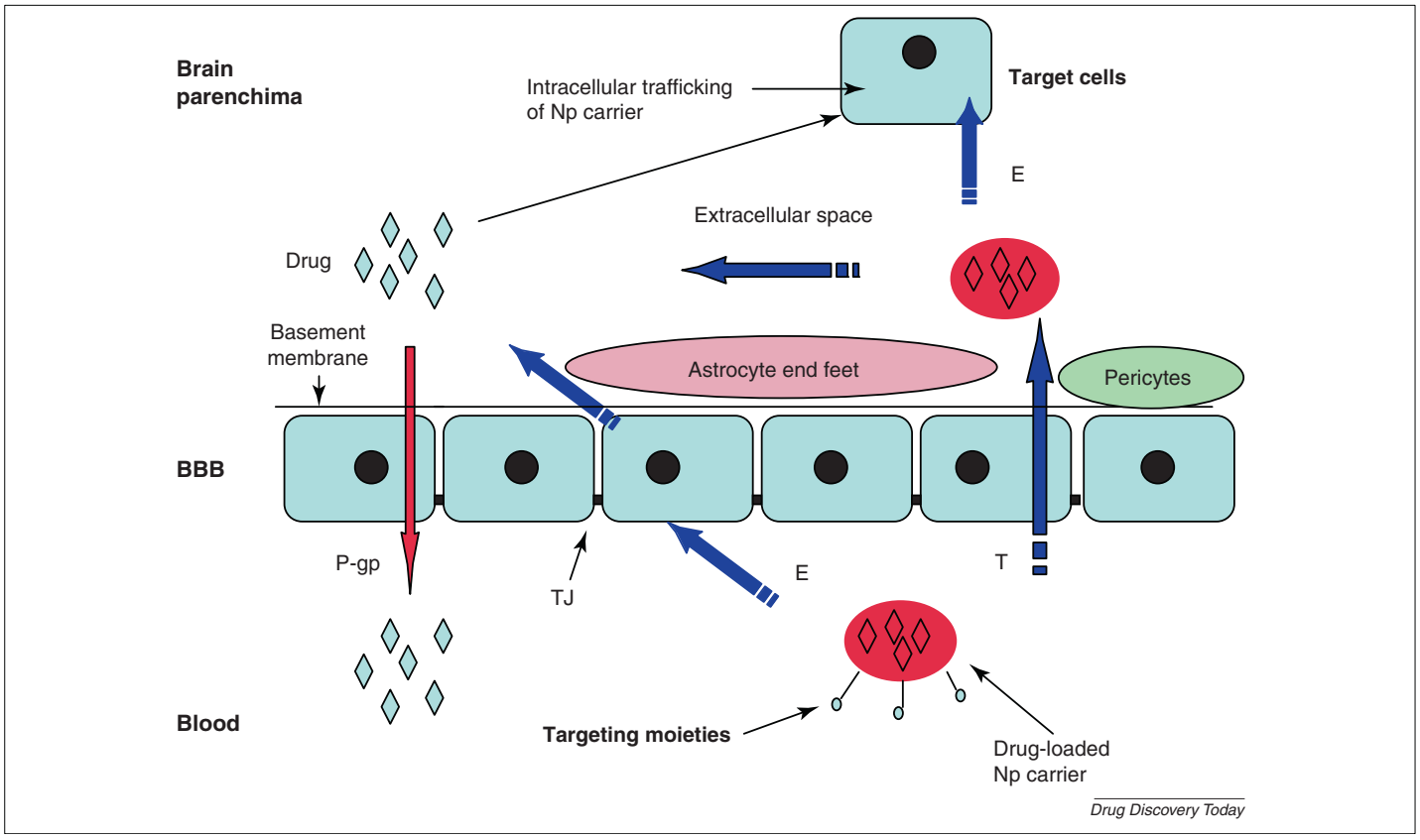

FIGURE 1

Np-mediated drug delivery to the brain in healthy conditions, with intact BBB. P-gp: P-glycoprotein-mediated drug efflux system. Blue diamonds: free drug; red ovals: Np containing the drug; E: endocytosis; T: transcytosis; TJ: tight junctions.

Blood–brain barrier and diseases

The ability of brain-targeted nanocarriers to deliver drugs into the CNS has been studied in healthy or disease-affected brains, particularly in the presence of brain tumors. It is unlikely that results obtained in an experimental setting can be translated in to other conditions, because it is known that the BBB itself varies in pathological conditions (e.g. in terms of receptor expression levels or function) [25–27]. In the presence of brain tumors (malignant gliomas and metastatic lesions to the brain from peripheral cancers such as lung, breast, renal, gastrointestinal tract and melanoma), tumor growth can disrupt the BBB but in the case of micrometastases or infiltrative gliomas the BBB could remain intact [28]. Although the size of gaps in the vascular endothelium (as measured in a variety of implanted tumors in mice) was found to be up to 600 nm in diameter [29,30], the size of those present in brain tumors is significantly smaller (~12 nm) [31]. This characteristic has been taken into account in the design of brain-tumor-targeting doxorubicin-loaded spherical polymers [doxorubicin-(poly(amidoamine)) (PAMAM) generation (G) = 5 dendrimers] with a diameter ~7 nm that can access the brain tumor through the 12 nm gaps but do not extravasate across the normal BBB [32]. Analysis of over 2000 brain metastases from two models (human 231-BR-Her2 and murine 4T1-BR5) showed partial compromission of the blood–brain tumor barrier (BBTB) permeability in over 89% of lesions, varying in magnitude within and between metastases. Uptake of [^{14}C]-paclitaxel and [^{14}C]-doxorubicin showed that cytotoxic concentrations are reached only in a small subset (~10%) of the most permeable metastases. Thus, the BBTB remains a significant impediment to standard chemotherapy and remains sufficiently intact in most experimental brain metastases to impair drug delivery significantly; this aspect, together with the pattern of tumor spread in glioma and micrometastases [28], reinforces the need for brain-permeable molecular therapeutics [28,33].

The following paragraphs will deal with the carriers that have been studied for the delivery of drugs to the CNS, and the results obtained will be compared with those obtained using polymeric Np, the oldest Np considered for targeted drug delivery to the brain, studied since 1995, but not yet present on the market.

Polymer–drug conjugates

Polymer therapeutics are a family of nanoscale entities in which the bioactive agent is not encapsulated, instead it is linked to a polymeric water-soluble biocompatible carrier to generate a polymer–drug conjugate. After the description of the EPR phenomenon, these conjugates are actively studied (Table 1) for the potential treatment of several types of cancer, and could also be used for the treatment of CNS inflammatory disorders.

Polyglutamate-paclitaxel (CT2103 or Xyotax; Cell Therapeutics) is the most clinically advanced polymer–anticancer-drug conjugate for the treatment, among several types of cancer, of brain tumors, and it has been developed to Phase III clinical trials [34] (Table 1).

Clinical trials on patients with brain tumors treated with CT2103 associated with temozolomide and radiotherapy showed

promising results [35]. A new-targeted polymeric drug has been recently reported that can inhibit tumor angiogenesis in a mouse model with intracranial human U87MG glioma. The polymeric drug includes a mAb for the Tf receptor as the targeting moiety, and carries an antisense oligonucleotide (AON) that blocks the synthesis of the tumor neovascular trimer protein laminin-411 [36].

Among macromolecular carriers, dendrimers of generation $G = 5$ (diameter ~7 nm) have been surface-decorated with ligands for BBB receptors (Tf, lactoferrin (Lf) and Angiopep-2) [37–39]. Although untargeted dendrimers rarely cross the BBB, the presence of the targeting moiety (the 19-amino-acid-long peptide Angiopep-2) increases their ability to cross the BBB up to 0.26% of the injected dose (ID)/g brain tissue [37]. Data, however, have been obtained without intravascular content corrections, to account for particles still present in the circulation at the time of sacrifice (brain perfusion or inclusion of a vascular marker), or capillary depletion corrections (by means of capillary depletion experiments) for the endothelium-associated particles [37–39]. The capillary-depletion technique [40] involves homogenization of the brain, followed by dextran density centrifugation to deplete the homogenate of its vasculature – care should be taken previously to remove particles loosely bound to the capillary surface, which might dissociate from the capillaries during centrifugation, by flushing the vasculature.

CNS-targeted peptide–drug conjugates

Recently, AngioChem (http://www.angiochem.com) developed a platform called EPiC (engineered peptide compounds technology for small and large molecules). This platform is based on the peptide Angiopep-2, capable of crossing the BBB [41,42], which targets the LRP-1 receptor present in the brain capillary endothelia and also in neurons, and which is upregulated in brain tumors [24]. In oncology, GRN1005 (formerly ANG1005), which binds three moles of paclitaxel per mole of Angiopep-2, entered Phase II trials in 2011, partnering with Geron (http://www.geron.com/) (Table 1), whereas paclitaxel, which showed promise in preclinical studies for glioblastoma multiforme, was ineffective in systemic delivery applications [43]. *In vivo* uptake of [^{125}I]-ANG1005 into vascularly corrected brain and brain metastases showed an improved brain delivery of the drug – it exceeded that of [^{14}C]-paclitaxel by 4–54 times (brain metastasis and healthy brain, respectively) [24].

The administration of ANG1005 to patients (who received more than six treatment cycles) showed no antibody production or immune reactions [44–46]. In Phase I clinical trials in humans, ANG1005 reached therapeutic concentrations in brain tumors and produced a significant antitumor response in patients with primary gliomas or secondary brain metastases who had failed prior standard therapy. Data from extracted tumors ($n = 6$) show concentrations of ANG1005 in tumors relative to plasma up to 379% [44]. By July 2011, the AngioChem (http://www.angiochem.com) pipeline generated another peptide–drug conjugate, ANG1007 (doxorubicin as a drug [47]), for brain tumor therapy.

This platform has been considered for macromolecular drugs (siRNA and peptides), besides low molecular weight compounds: ANG1011 contains Angiopep-2 conjugated with a histone deacetylase inhibitor (for the treatment of neurological disorders), whereas ANG2002 is a conjugate with the peptide neurotensin for the treatment of pain [48]. In the latter case, the transport rate across the BBB is ten times higher than for neurotensin alone, and the conjugate exhibits activity in several animal pain models. Other peptide conjugates are at the discovery stage: ANG1004 (EPiC Leptin, for the treatment of obesity) and ANG2008 (EPiC for glial-derived neurotrophic factor, GDNF, a potent neuroprotective agent for acute brain disorders such as stroke and Parkinson's disease).

Other companies are developing peptide-targeted CNS-drug conjugates (Table 1). Among these, there is Vect-horus, biOasis Technologies and Raptor Pharmaceutical. Vect-horus (http://www.vect-horus.com/) identified a cyclic 8-mer peptide ligand for LDL receptor (LDLR), VH0445, active *in vivo* as a carrier for the delivery of a model drug (an opioid peptide). biOasis Technologies (http://www.bioasis.ca/) developed p97 (melanotransferrin; Transcend^{TM}) as a CNS drug carrier; in 2011 the initiation of a therapeutic program for treating the neurological effects of lysosomal storage disorders was announced. Intravenously administered iduronidase-conjugated p97 induces a 4-fold increase of enzyme levels in the brain, as compared with the level achieved by administrating the enzyme alone. The conjugates with doxorubicin (four to seven molecules of doxorubicin per molecule of p97) can accumulate in the brain (0.25% of the ID), with a reduction in cardiotoxicity, significantly prolonging the survival of animals bearing intracranial gliomas [49]. Raptor Pharmaceutical (http://www.raptorpharma.com/) developed a carrier (the peptide RAP, a ligand for LRP-2 receptor) useful for proteins or small molecules. In 2009, an agreement was signed with Roche for further development of this brain delivery technology.

Trojan horses

ArmaGen^{TM} Technologies (http://www.armagen.com/) developed a Trojan horse technology, represented by fusion proteins consisting of a mAb against the human insulin receptor (HIR) that does not interfere with INS binding and large protein molecules, to be delivered to the CNS. The technology is aimed at delivering multiple classes of biopharmaceuticals to the brain (http://www.armagen.com/) [12]. Several of these fusion proteins are in the pipeline at various levels of development (http://www.armagen.com/). Among these, ArmaGen^{TM} completed a pre-IND meeting with the FDA for a Phase I–II trial of intravenous AGT181, a mAb against HIR fused to iduronidase, the lysosomal enzyme that is missing in mucopolysaccharidosis type I that affects the brain [50] (Table 1). AGT190, fused to GDNF [51], was planned to enter Phase I in 2010 [50]; results are not available yet. The OX26 anti HIR mAb is 10-fold more active than the antihuman transferrin receptor (TfR) mAb as a delivery system across the BBB [52]. However, these systems are not active in the mouse, and have forced usage of a surrogate Trojan horse (engineered with anti-mouse TfR mAb) in the *in vivo* mouse models [12].

Despite *in vivo* demonstration of CNS pharmacological effects of large molecules attached to the anti-TfR mAb [12], concern was raised about the ability of an antibody to dissociate from its receptor, to be released with its cargo into the brain. Quantitative data concerning the ability to cross the BBB of the fusion protein between the anti-TfR mAb and erythropoietin, a potent neuroprotective agent, have recently been published. Two percent ID/g brain was found after 60 min, and capillary depletion experiments

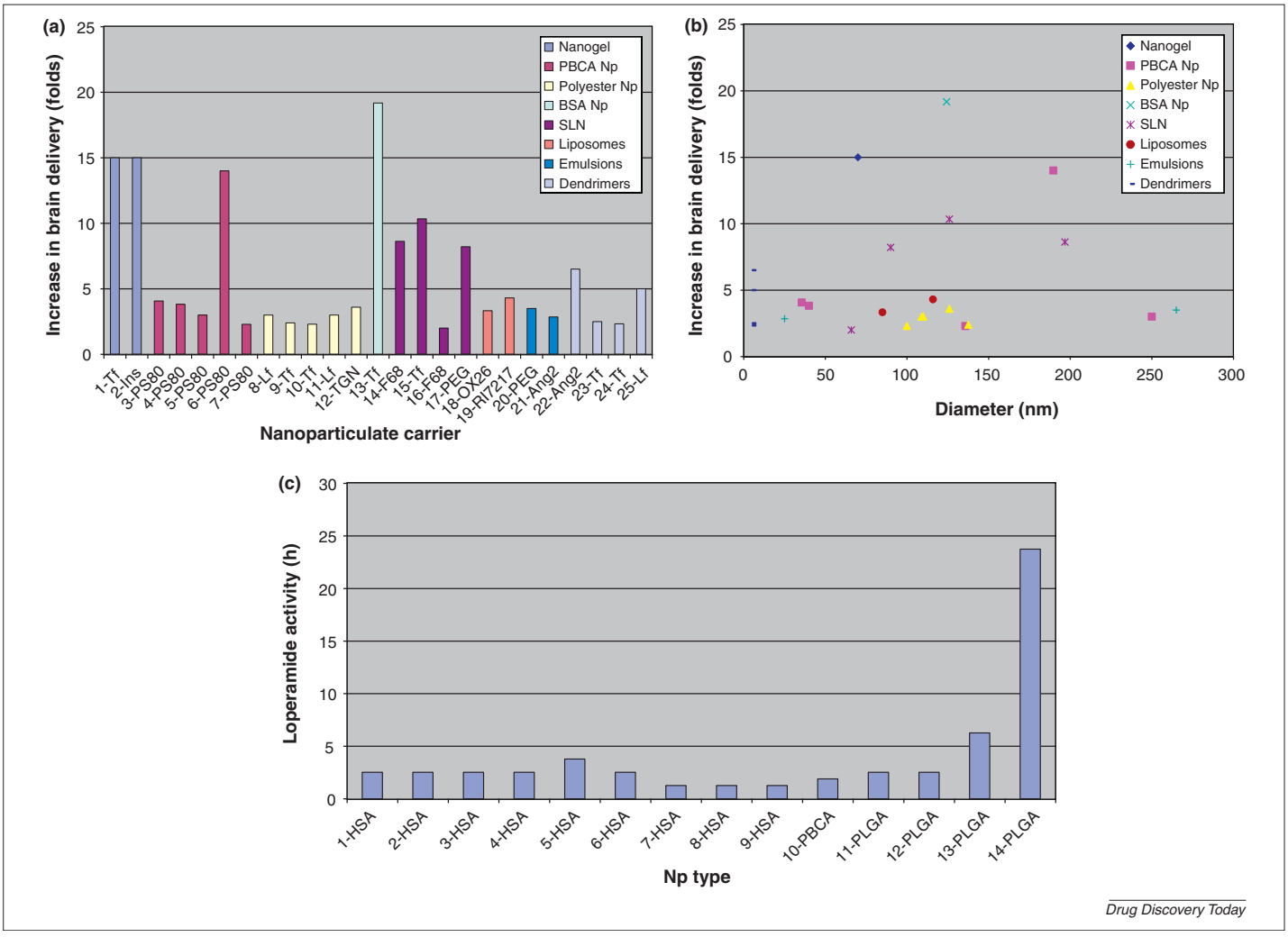

FIGURE 2

Ability of nanocarriers to deliver a cargo to the healthy brain. (a) Fold-increase in drug delivery to the brain obtained in presence of targeted nanocarriers. Carriers are: nanogel (entries 1 and 2), PBCA Np (entries 3–7), polyesters (entries 8–12), bovine serum albumin (BSA) Np (entry 13), solid lipid Np (SLN) (entries 14–17), liposomes (entries 18 and 19), lipid-based systems (entries 20 and 21), dendrimers (entries 22–25). (b) Plot of the nanocarrier size versus the fold-increase in drug delivery to the brain. The diameter of the PAMAM G5 dendrimer was assumed to be 7 nm, on the basis of literature data for underivatized G5 PAMAM dendrimers. Carriers are the same as in (a). (c) Loperamide delivery to the healthy CNS by means of nanocarriers. The pharmacological nociceptive activity exerted by loperamide, evaluated by tail flick or hot-plate tests, was considered as an index of brain delivery, and expressed in time (h) of effect duration. Entries 1–9 refer to HSA Np surface-decorated with different targeting moieties or with the same targeting moiety and a different linker (Tf, insulin, mAb anti-TfR, mAb anti-INSR, ApoE, ApoA1, ApoB100; loperamide dose: 0.7 mg/kg) [86–89]; entry 10, PBCA/PS80 (loperamide dose 3.6 mg/kg) [107]; entry 11, PLGA/poly(vinyl alcohol) (PVA)/PS80 [108]; entry 12, PLGA/PVA/Pluronic F68 (loperamide dose 7 mg/kg) [108]; entry 13, PLGA/F68 Np surface-decorated with simil-opioid peptide (loperamide dose 2.7 mg/kg) [109]; entry 14, PLGA/F68 Np surface-decorated with simil-opioid peptide and *N*-acetylneuraminic acid (loperamide dose 2.7 mg/kg) [110].

showed that 69% of the protein was actually present in the brain parenchyma and not associated to the vascular endothelium [53]. Similar results have been obtained more recently for avidin anti-TfR mAb fused to the Aβ1-40 peptide [54]. By contrast, in support to the concern reported above, it was shown that reducing the affinity of an antibody to TfR enhances its receptor-mediated transcytosis across the BBB [55] and the cargo (an antibody against BACE-1) is active *in vivo* in reducing the amount of brain amyloid-β levels after a single systemic dose [56].

Nanosized drug carriers

In general, the percentages of the ID found in the brain after targeting of nanocarriers are highly variable, and range from 0.01% to 0.5% in the case of intravascular content correction, although values up to 15% ID can be found in the absence of the correction [57]. However, there is not much data available.

Then, among nanosized carriers, we evaluated: (i) the efficiency of the targeting moieties and (ii) the effect of size on drug delivery. The parameter that has been considered in this review to describe the efficiency of the carriers that target the brain is the fold increase in brain delivery, measured as the ratio between the amount of drug present in the brain after an i.v. (intravenous) administration of drug embedded in the targeted nanocarrier and that obtained after the i.v. administration of the drug in solution. Alternatively, in the absence of this value, it has been considered the brain drug level obtained following i.v. administration of drug embedded in an untargeted carrier (Fig. 2a,b). This ratio was calculated by the authors of this review from the data reported

in the referenced articles. Carriers are: nanogel [58], poly(butyl cyanoacrylate) (PBCA) Np [59–63], polyester Np [64–68], bovine serum albumin (BSA) Np [69], solid lipid nanoparticles (SLN) [70–73], liposomes [74,75], lipid-based systems [76,77] and dendrimers [37–39].

Experiments considered have been conducted, with few exceptions, without capillary depletion and/or intravascular content corrections, to take the amount of cargo present in brain capillaries into account. Indeed, therapeutically effective drug delivery to the brain could also be obtained by accumulation inside brain capillary endothelial cells, providing enough time for transport of the drug but not the vehicle across the BBB [78], or simply by drug transfer rather than Np transcytosis [79]. Moreover, brain pharmacokinetics of the Np and the assessment of BBB integrity during the experiments are seldom performed. Thus, data obtained can be considered only as approximate values.

The most active carriers (i.e. with an increase in brain delivery >7 times taken as cut-off) are: nanogels, flexible hydrophilic polymer gels of nanoscale size, made of cross-linked poly(ethylene glycol) (PEG) and cationic poly(ethylene imine) (PEI) chains (PEG-PEI-Tf and PEG-PEI-INS) loaded with oligonucleotides, BSA-PEG-Tf Np loaded with azidothymidine (AZT), PBCA/polysorbate 80 (PS80) Np loaded with doxorubicin, SLN/PS80/Tf Np loaded with quinine, SLN-PEG Np loaded with doxorubicin and SLN/Pluronic F68 (F68) Np loaded with camptothecin. On the basis of the results, it is not possible to draw any conclusion about the ability of the targeting moieties to deliver a cargo inside the CNS. It is difficult to translate data obtained with targeting moieties conjugated to a given Np carrier to another Np carrier. Each carrier–drug conjugate should be considered as a new entity, made of polymers and other components such as surface-active agents, targeting moieties and embedded drug. Each component can modify the Np characteristics and hence biodistribution and pharmacokinetics (see for example the effect of the loaded drug [80–82]). Thus, experiments with 'model drugs' or fluorescent probes could be of limited predictive value regarding the therapeutical application of the carrier in the presence of a 'real' drug.

Accordingly, the only comparative study on the ability to target a drug carrier (liposome) to the CNS by different targeting moieties (Tf, RI7217, a mAb against TfR, COG133, an apolipoprotein-E (ApoE) mimetic peptide, Angiopep-2 and CRM197, which is a mutated, non-toxic form of diphtheria toxin) showed that many targeting ligands that have been described for brain targeting do not target the brain *in vivo* when coupled to a liposomal delivery vehicle (see, for example, Angiopep-2) [75].

Based on the available data, we have generated a plot of fold increase in brain delivery, measured as described above, relative to Np dimensions, to highlight the role of the nanocarrier size in the efficiency of brain delivery (Fig. 2b). We selected diameter as a parameter for Np characterization because it is important for biodistribution and/or pharmacokinetics, and it is always assessed, although its determination is not always carried out in a proper way [83], and it is always conducted in water or buffers. In this way, the interaction of Np with proteins and the possible impact of this interaction toward aggregation into larger structures are entirely neglected [84].

Size requirements do not appear to be strict for CNS delivery, because diameters of carriers able to increase the amount of drug delivered to the CNS are between 7 and >200 nm; this result is in disagreement with those obtained in a systematic experiment, in which it was shown that methotrexate was delivered to the brain by Np smaller than 100 nm at best (again, without capillary depletion experiments) [85].

Looking at the pharmacological activity of the model drug loperamide, loaded on HSA Np (entries 1–9 of Fig. 2c) [86–89], it can be seen that at the same drug dose the pharmacological effect is independent of the targeting moiety (Tf, INS, mAb anti-TfR, mAb anti-HIR, apolipoproteins), and also of the Np diameter (size ranges from 150 to 340 nm; data not shown).

A preparation (entry 2, Fig. 2c) consisted of human serum albumin (HSA) Np surface-decorated with OX26, an antibody to rat TfR, which is not an effective brain delivery vector in mice [90]. Surprisingly, the activity of the loaded drug, tested on mice, is the same as that reported for the other differently targeted HSA or PBCA Np; this effect remains to be explained.

Because data reported in Fig. 2 have been obtained in the healthy brain and with a model drug, it is difficult to predict if the delivered 'real' drug will be able to elicit pharmacological activity. In fact, after crossing the BBB, the drug can be released and becomes available for the interaction with a membrane receptor (as in the case of the opioid loperamide) but, at the same time, the drug and/or drug embedded into the carrier can be endocytosed by secondary cell targets (the actual target of the drug) and activity will depend on the subsequent intracellular trafficking.

Liposomes

Liposomes are a class of nanosized drug carriers characterized by the presence of one or more amphiphile bilayers surrounding an aqueous core. Liposomes have been used for increasing the therapeutic index of a wide range of antineoplastic agents. Ten untargeted liposomal drugs are available on the market today, and only one of these (Doxil®/Caelyx®) is a long-circulating PEG-surface-decorated liposome (loaded drug is doxorubicin) [2] (Table 1). This formulation achieves an increased drug concentration in tumor tissue owing to its long-circulating characteristics and the EPR effect, in parallel with a lower volume of distribution, with a consequent reduction of therapy-associated cardiotoxicity [91]. In the case of brain tumors in humans, high intratumoral accumulation of PEGylated liposomal formulation was observed (13–19 times higher in the glioblastoma and 7–13 times higher in metastatic tumors, as compared with the normal brain tissue) [92,93]; but only modestly promising results for untargeted PEG-doxorubicin liposomes have been obtained in Phase II trials in patients with recurrent glioblastoma [94–97].

For the treatment of tumors outside the brain, attempts are underway to combine tumor-specific targeting with longevity-sponsored EPR accumulation. Thus, various antitumor antibodies are used to modify PEGylated drug-containing liposomes and enhance their therapeutic effect via better delivery inside cells, for example by receptor-mediated endocytosis [98,91,99,100]. However, targeting ligands did not appear to be able to improve the tumor specificity of liposomes; the rate-limiting step for the tumor localization being extravasation from the vasculature – the mAb-antigen interactions have no role in facilitating this process [101].

In the field of drug delivery to the brain by means of nanocarriers, the company To-bbb is developing glutathione-decorated liposomes (glutathione is the targeting moiety for the BBB) (http://www.tobbb.com/). The most advanced product is 2B3101, a glutathione-PEGylated liposomal doxorubicin hydrochloride (Doxil®) for enhanced delivery of doxorubicin to brain cancer cells. This compound proved to be more efficacious in treating the intracranial U87 tumor (mouse model) than unconjugated liposomes. To-bbb initiated a Phase I/II clinical trial in 2011 for patients with solid tumors with or without brain metastases and in patients with brain metastases of breast cancer(http://www.tobbb.com/) (Table 1). 2B3-201, glutathione-PEGylated liposomal methylprednisolone for the treatment of inflammation in the brain, is at an early stage of development. Glutathione-decorated liposomes loaded with peptides and small molecules have been tested in several pain models and in viral encephalitis.

Polymeric nanoparticles and solid lipid nanoparticles

These carriers include solid lipid Np (SLN) and polymeric Np such as polyester-based Np [poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) (PLA)] and polycyanoacrylates, such as PBCA, containing surface-active agents such as F68 and PS80 (Table 1).

These Np have been surface-decorated with peptide CNS-targeting moieties or covered by PS80 (for PBCA and PLGA Np), a compound that is thought to adsorb ApoE from plasma, the actual targeting moiety [102]. The same mechanism has been hypothesized for SLN [103] and PEG-poly(hexadecylcyanoacrylate) (PHDCA) Np [104]. Conflicting data exist in the literature concerning the effect of PBCA Np on tight junctions of the BBB, reviewed in [105]. Recently, however, it was shown that the application of PS80-coated PBCA Np leads to a reversible disruption of the barrier after 4 h – porcine *in vitro* model of BBB; the transendothelial electrical resistance was monitored and the opening of tight junctions was confirmed by [^{14}C]-sucrose and fluorescein isothiocyanate (FITC)-BSA permeability studies [106]. This raised concerns about the safety of their use.

Brain drug delivery ability of the nanocarriers has been studied in two main ways: (i) in animal models of human brain tumors; and (ii) in the healthy animal brain.

The ability of the nanoparticulate carriers to act as drug carriers to the CNS in healthy conditions (intact BBB) has been examined mainly by the evaluation of the pharmacological effect exerted by the model drug loperamide, an opioid unable to cross the BBB; its delivery into the brain can be easily determined by means of nociceptive assays (tail flick or hot-plate test). Results are reported in Fig. 2c (entries 10–14) [107–110].

Historically, PLGA and its various derivatives have been the center focus for developing microparticles (diameters from 1 to 250 μm, whereas PLGA Np studied for i.v. brain drug delivery are ~200 nm) encapsulating therapeutic drugs in controlled release applications, owing to their advantages over the conventional devices (i.e. extended release rates up to days, weeks or months, high biocompatibility and ease of administration) only via intramuscular or subcutaneous injection. To date, nine of these products are available on the market for sustained drug release [111]. Moreover, polyester Np have been studied, on the basis of their prolonged antigen release, for the development of single-shot vaccines [112]. These polyester Np were also shown to ensure sustained drug release (up to 14 days) into cells in culture [113,114], when applied topically to the vaginal mucosa [115] and in airway epithelia for the gene treatment of cystic fibrosis [116].

In the case of brain-targeted Np, a sustained pharmacological activity has been observed in only two cases (Fig. 2c); however, the loperamide antinociceptive effect lasted a few hours at best [109,110]. This effect could not only be caused by a sustained release but also modified pharmacokinetics and/or biodistribution of Np or the inhibition of the P-gp efflux system (not evaluated), because loperamide is a substrate of P-gp [117]. Although the effect of PLGA- and Np-targeting moieties on the P-gp efflux system has never been studied, it is well known that Pluronics [118] and in particular the F68 [119] used for the preparation of these Np [109,110] (the amount present on Np is unknown) are potent *in vitro* inhibitors of the P-gp efflux system.

In the case of TAT(a cell-penetrating peptide)-decorated PLA-poly(vinyl alcohol) (PVA) Np loaded with ritonavir [120], sustained brain drug levels were observed up to 14 days, then ritonavir levels slowly decreased. Because the ritonavir dose was 45 mg/kg and Np loading was 18.3% (w/w), it can be calculated that Np have been administered at a high dosage, ~250 mg/kg, which is clinically unrealistic. It is possible that the use of such a high Np dose modifies pharmacokinetics and biodistribution, with respect to conventional Np dosages in experimental settings, which are lower at least by one order of magnitude [121].

Polymeric Np have also been tested in other brain pathologies that require the delivery of proteins [62,122]. The use of various stabilization approaches led to some success in increasing protein stability, but liposomes and the more mechanically stable polymersomes (self-assembled vesicles of amphiphilic block copolymers) seem to be the most promising choice for the development of nanosized carriers for proteins [123].

Experimental *in vivo* data showed that several drug-loaded brain-targeted Np prolong survival in tumor-bearing rats: doxorubicin-loaded cyanoacrylate Np [108,124–130] (with a reduction in cardiac and testicular toxicity versus doxorubicin in solution [131,132]), polyesters [108,133] and also other Np such as albumin Np [134] and polymersomes [135,136]. The reduction in cardiotoxicity exerted by doxorubicin loaded into nanocarriers could be explained by the reduced cardiac vascular permeability. Even if it was predicted that particles of 50 nm could cross the capillary barrier present in the heart, Np of 15–30 nm diameter were not found in the heart after i.v. injection; so far, only the delivery of Np of 7 nm to cardiomyocytes after i.v. injection has been confirmed [137].

Even in the absence of a comparative study, it seems that PBCA/PS80 Np can deliver a quantity of a therapeutic agent to brain tumors lower than that delivered by the EPiC platform: the delivery of paclitaxel via ANG1005 was 161-fold greater for normal brain tissue and >12-fold greater for brain metastases at 0.5 h after administration [24]. In comparison, experiments conducted with doxorubicin-loaded PBCA/PS80 Np (healthy brain) showed brain parenchyma values of 1.6 μg/g after 2 h from the point of administration [59], an increase of approximately ten times as compared with doxorubicin in solution.

Another undisclosed lipid-based nanotechnology product at an advanced stage of preclinical development, LipoBridge® (Genzyme Pharmaceuticals; http://www.genzymepharmaceuticals.com/) is claimed to be able to deliver a huge amount of cargo

into the brain, by means of a temporary and reversible opening of the BBB. This mode of action, however, raises serious concerns for risks of infections or toxicity exerted by the uncontrolled passage into the brain of circulating infectious agents or toxic molecules and cells.

Toxicology concerns

Not much attention has been paid to the potential immunogenicity of injectable drug delivery systems. When a drug elicits an immune response this can have important clinical implications, such as acute reactions and reduced drug efficacy. Data on antibody responses against drug delivery systems are available mainly for liposomes, but the possibility exists that antibodies can be formed against other nanocarriers including polymeric Np and all the components of the loaded vector: carrier materials, drugs, targeting ligands and linkers. Polymers, by definition, contain repeat units that can trigger B-cell activation directly and, hence, antibody production. Because the immune system has evolved to recognize particulate materials as foreign, there is a probability that nanodrugs might be seen as potential dangers and eliminated. The high uptake by the RES, observed for several types of Np carriers, confirms this possibility [138].

Experiments to assess complement activation, hypersensitivity reactions and blood compatibility have never been performed for CNS-targeted nanocarriers. Accelerated clearance is another major problem of particulate drug efficacy. Intravenously administered Np can be readily 'opsonized' (covered with serum proteins such as complement factors or immunoglobulins) and thereby phagocytosed by monocytes and macrophages and accumulated into the RES, mainly in the liver and spleen, leading to rapid removal from the systemic circulation. If the drug carried by the Np is not aimed at these organs and/or cells, this poses a double disadvantage: (i) the drug is sequestered and does not reach its target, thus losing efficacy; (ii) the drug goes into liver and spleen macrophages and, probably, kills them, thus causing immunosuppression or failure of the detoxifying systems. Therefore, although targeting other organs appears particularly challenging [139], the Np appear to be more interesting for selectively directioning their drug load to the liver and spleen.

Clinical data on the immunogenicity of drug carrier systems are scarce, given to the fact that few products reach the clinical stage. Because most of the drugs embedded into Np are anticancer drugs the hope is that they will not be highly immunogenic and capable of triggering a significant antibody response. First, because cancer patients receiving these drugs are already immunocompromised and therefore unlikely to have a strong immune reaction and, second, because anticancer drugs are highly toxic for immune cells and this immunosuppressive capacity will limit antibody formation. However, PEGylation of Np, done to avoid immune recognition, is no guarantee that the drug delivery system will be immunologically safe [138,140–142].

Another concern about the use of polymeric Np is related to polymer degradation time. Bulk polyester copolymers are metabolized slower than polycyanoacrylates (without considering the fact that the degradation time also depends on the copolymer composition) [143,144]. Although the half-life of PBCA/PS80 Np (200–250 nm diameter) into neurons *in vitro* was 27 min [19], studies conducted on drug-loaded PLGA/PVA and PLA/PVA Np into human vascular smooth muscle and human breast carcinoma cells showed slower degradation times (7–14 days), suggesting that the Np might act as an intracellular depot [113,114]. Because the effect of loperamide in the CNS lasted at best a few hours, and biodistribution studies proved the disappearance of PLGA Np from the brain after 24 h [110], more studies are needed to determine the fate of Np in CNS cells, and to assess the possibility of accumulation of Np components other than the drug and consequently their possible toxic effects.

Toxicity studies performed with PLGA/PVA Np *in vitro* (Caco-2 and HeLa human tumor cell lines) and *in vivo* (mice orally administered with Np) showed an absence of toxicity [145], whereas repeated administration of cyanoacrylate Np showed reversible toxic effects on rat hepatocytes [146,147]. However, it is becoming clear that it cannot be assumed that, just because the Np are not killing a cell, cells are actually unaffected by the Np presence. Indeed, there is increasing evidence that several of the methods usually adopted to determine the toxic effects of Np on cells might not be appropriate to detect Np-induced perturbation of cell functions. Nanotoxicity studies should also be focused on the assessment of intracellular transport disturbances and other cellular processes induced by Np at appropriate time points, depending on the phases of Np degradation, and should also include the assessment of the intracellular degradation and cellular excretion of Np [148]. Importantly, these studies should be conducted on primary neurons rather than on transformed cell lines.

Concluding remarks

In the past, a wealth of studies has been performed to reveal the best way of delivering drugs to the CNS, mainly for the treatment of brain tumors.

Targeted delivery by nanocarriers can increase the amount of drug delivered to the brain (Fig. 2a,b) but, from the data available, the percentages of injected drug dose found in the brain after targeting with nanocarriers is $<1\%$, and the main proportion is localized in the liver [75,80,149]. Moreover, although untargeted liposomal drug formulations are on the market, only five polymeric Np (http://www.bioalliancepharma.com/; http://www.bindbio.com/) [150–152]and one polymeric micelle formulation (http://www.samyang.com/) are in clinical trials (and not for brain drug delivery) (Table 1).

Few data are available regarding the fate of the polymer and possible toxicity on neuronal cells [153], the immunogenicity of polymers in the form of Np [154], blood compatibility, capture by RES (drug sequestration, off-tissue toxicity for RES and consequent immunosuppression) and accumulation in the brain. The main part of Np comprises the polymer (the drug loading is usually $\sim$10%): in the case of repeated Np administrations, the accumulation of polymers (in particular polyesters) inside the CNS is a concrete possibility, and long-term toxicity studies in this sense have never been conducted; no convincing data are available about the pharmacokinetics of these carriers in the brain. However, drug delivery to the CNS by polymeric Np is the most studied (preclinical researches in academia, ten entries among 25; Fig. 2a,b), whereas companies only introduced (targeting)peptide–drug conjugates or targeted liposome platforms into clinical trials (Table 1). This could be owing to the fact that polymeric Np appear to have a series of concerns that could seriously hamper

their rapid adoption as a reliable therapeutic option. At present, other drug-loaded platforms such as EPiC or liposomal formulations (ANG1005, 2B3101, respectively; Table 1) seem to have better chances of resulting in clinically useful approaches.

Lipid-based systems, such as SLN, by contrast, proved to be promising platforms for the parenteral delivery of various therapeutic agents [103], on the basis of the data reported in Fig. 2a,b, as well as for brain drug delivery. Moreover, four companies have announced Phase I programs for assessing safety and tolerability of systemic delivery of siRNA by means of lipid-based systems (the only exception is represented by CALAA01; Table 1) – none of these targets the brain [155,156] (Table 1).

At the same time, innovative, basic research is evolving in the field of drug delivery to the brain toward the development of:

(i) Biocompatible platforms for siRNA delivery to the brain. Targeted exosomes, natural transport nanovesicles of 40–100 nm diameter, secreted by numerous cell types [157] have been recently described and shown to be promising [158] for brain delivery of siRNA. This class of therapeutic agents suffers from high sensitivity to enzymatic degradation, poor cellular uptake and rapid renal and liver clearance, limiting their *in vivo* applications. After intravenous administration into mice, nonspecific uptake in other tissues was not observed (i.e. muscle, spleen, liver, kidney and heart), and no immunological reactivity was evident. Importantly, exosomes can be re-administered repeatedly without loss in delivery efficiency, thus without raising an immune response. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA and protein knockdown of BACE-1 (a therapeutic target in Alzheimer's disease) in wild-type mice.

(ii) New targeting agents: because it remains difficult to target the nanodrug specifically to the brain endothelial cells (some results have been reported for selective targeting of inflamed BBB [159]) and to target specific cells present in brain parenchyma to enhance therapeutic effects while limiting side effects in nontarget cells [160–162].

Although the latter point will have little impact on the Np platforms considered for brain delivery, the former, together with the growing interest on lipid-based nanocarriers, enables us to hypothesize that the future of brain-targeted drug delivery will not be based primarily on polymeric Np alone.

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