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Drug delivery to the central nervous system by polymeric nanoparticles: What do we know?☆

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ABSTRACT

Nanoparticles enable the delivery of a great variety of drugs including anticancer drugs, analgesics, anti-Alzheimer's drugs, cardiovascular drugs, protease inhibitors, and several macromolecules into the brain after intravenous injection of animals. The mechanism of the nanoparticle-mediated drug transport across the BBB appears to be receptor-mediated endocytosis followed by transcytosis into the brain or by drug release within the endothelial cells. Modification of the nanoparticle surface with covalently attached targeting ligands or by coating with certain surfactants that lead to the adsorption of specific plasma proteins after injection is necessary for this receptor-mediated uptake. A very critical and important requirement for nanoparticulate brain delivery is that the employed nanoparticles are biocompatible and, moreover, rapidly biodegradable, i.e. over a time frame of a few days. In addition to enabling drug delivery to the brain, nanoparticles, as with doxorubicin, may importantly reduce the drug's toxicity and adverse effects due to an alteration of the body distribution. Because of the possibility to treat severe CNS diseases such as brain tumours and to even transport proteins and other macromolecules across the blood–brain barrier, this technology holds great promise for a non-invasive therapy of these diseases.

Contents

1. Introduction

The blood–brain barrier (BBB) represents an insurmountable obstacle for most drugs, including neurological drugs, cytostatics, antibiotics, etc. One efficient possibility to deliver drugs including peptides [1–3] and even macromolecules [4] across this barrier is the employment of polymeric nanoparticles. This possibility was recently summarised in a short review in this journal [5]. Unfortunately, previous reviews frequently cite similar references and highlight similar points, often for studies that are repetitive or incremental over time. Focus on increasingly important pharmacological effects achieved with nanoparticle-based delivery as well as studies regarding mechanisms of nanoparticle-

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mediated drug transport are not often analysed. This review seeks to achieve this goal.

The above mentioned reports [1–3] demonstrate that overcoating of drug-loaded biodegradable poly(butyl cyanoacrylate) nanoparticles with certain surfactants such as polysorbate 80 (Tween® 80) or poloxamer 188 (Pluronic® F68) yields significant dose- and time-dependent [6] pharmacological effects in the CNS after intravenous injection into mice and also rats, whereas all the controls, including drug solution, empty nanoparticles, polysorbate 80 solution, simple mixtures of the components, i.e. nanoparticles, drug, and polysorbate 80, or uncoated drug-loaded nanoparticles did not achieve such effects [7]. These results clearly showed that the drugs indeed were transported across the BBB by the polysorbate-coated particles [1,8]. Similar results were obtained by overcoating of the poly(butyl cyanoacrylate) nanoparticles with polysorbate 20, 40, or 60 whereas a large number of other surfactants were not able to achieve a delivery across the BBB [9]. Alternative to poly(butyl cyanoacrylate), polylactic acid and polylactic acid-polyglycolic acid copolymers [10,11] as well as albumin [12] and chitosan [13,14] also can be used as nanoparticle materials.

Given these *in vivo* results demonstrating efficiency with particle-based penetration of the BBB using translatable drug delivery methods, new important questions remain to be addressed. These include: 1) the mechanism of nanoparticle-mediated drug transport across the BBB, and, 2) closely related to this, the influence of the surface properties and of targeting ligands, 3) the amount of drug that can be transported by this pathway in order to achieve a pharmacological effect, and 4) the aspect of toxicity. This review attempts to critically evaluate past *in vivo* results with a deliberate effort to identify mechanisms that might lead to new delivery breakthroughs, as well as to highlight key features of particle-based systems and approaches that seem to penetrate the BBB.

2. Definition of nanoparticles and particle size influence

This review follows the classical definition of nanoparticles in the Encyclopedia of Pharmaceutical Technology [15] and in the Encyclopedia of Nanoscience and Nanotechnology [16] which was formulated already 40 years ago [17]:

> *Nanoparticles for pharmaceutical purposes are solid colloidal particles ranging in size from 1 to 1000 nm (1 μm) consisting of macromolecular materials in which the active principle (drug or biologically active material) is dissolved, entrapped, or encapsulated, or to which the active principle is adsorbed or attached.*

This definition deviates from the definition of physicists and material scientists who limit the upper size of nanoparticles to 100 nm. However, up to 1000 nm size appears to be of no important influence concerning uptake into cells of the reticuloendothelial system (RES), i.e. macrophages and endothelial cells, and also most other parts of the body. Schäfer et al. [18] demonstrated in vitro using electron microscopy that human macrophages endocytose nanoparticles independent of size, while Gao and Jiang [19] reported an only small, i.e. 20% increase in methotrexate delivery to the brain using 70 nm sized methotrexate-loaded poly(butyl cyanoacrylate) nanoparticles overcoated with polysorbate 80 after intravenous injection. In addition, this increase was not statistically significant in many parts of the brain. No differences in methotrexate brain delivery occurred between 170, 220, and 345 nm sized particles. This insignificant particle size influence can be attributed to the mechanism of nanoparticle uptake and of bound drugs into the brain (see next section).

It has to be kept in mind that the drug payload decreases with a reduced particles size. On the other hand, with sizes above 1000 nm in the micrometer range the danger of embolisation of the lung capillaries is increasing in size- and dose-dependent manner [20].

3. Mechanism of nanoparticle-mediated uptake of drugs into the brain

About eight possibilities exist for the mechanism of uptake of nanoparticles and of bound drugs into the brain that were proposed in an earlier review in this journal [2,21]:

1. An increased retention of the nanoparticles in the brain blood capillaries combined with an adsorption to the capillary walls. This could create a higher concentration gradient that would increase the transport across the endothelial cell layer and as a result enhance the delivery to the brain.
2. The polysorbate 80 used as the coating agent could inhibit the efflux system, especially P-glycoprotein (Pgp).
3. A general toxic effect on the brain vasculature.
4. A general surfactant effect characterised by the solubilisation of the endothelial cell membrane lipids that would lead to membrane fluidisation and to an enhanced drug permeability across the blood–brain barrier.
5. Opening of the tight junctions between the brain blood vessel endothelial cells. The drug could then permeate through the tight junctions in free form or together with the nanoparticles in bound form.
6. Endocytosis by the endothelial cells followed by the release of the drugs within these cells and delivery to the brain.
7. Transcytosis through the endothelial cell layer.
8. A combination of the above effects.

As discussed in detail in a recent review [22], the nanoparticle-mediated transport across the BBB seems to occur by endocytosis of the particles by the brain capillary endothelial cells after intravenous injection followed by nanoparticle transcytosis across these cells. Earlier reviews [2,3,21,22] already pointed out that mechanisms 1–6 appear to be of no major relevance: Creation of high drug concentration gradients by adherence of the nanoparticles to the inner surface of the blood capillary walls (mechanism 1) would not be sufficient for an effective and pharmacologically relevant drug transport across the endothelial cell layer since the diffusing drug still would have been subjected to the highly efficient efflux transporters such as Pgp in the luminal membranes of these cells. These efflux transporters also cannot be blocked by the presence of the 1% polysorbate 80 in the injected nanoparticle suspension because the pre-injection of polysorbate 80-coated empty nanoparticles 5 or 30 min before injection of a dalargin solution did not induce any pharmacological effects [23]. If efflux transporter inhibition would have been the underlying mechanism, these transporters would have been inactivated by the polysorbate bound to the empty nanoparticles which then would have enabled the drug flux across the endothelial cells. The fact that pre-injection of polysorbate-coated empty nanoparticles did not achieve such a transport of drug in solution into the brain also refutes mechanisms 3, 4. and 5, permeabilisation of the blood–brain barrier by toxic effects (mechanism 3) or by membrane solubilisation caused by the surfactant (mechanism 4) as suggested by Olivier et al. [24] and Calvo et al. [25] and also opening of the tight junctions (mechanism 5). This conclusion was further substantiated by electron microscopic studies [23,26,27], histological investigations [7,28], and toxicological experiments (see Section 10), which did not reveal any toxic effects at therapeutic levels. Additionally, a surfactant-induced permeability enhancement appears to be unlikely as no pharmacological responses were observed after injection of dalargin nanoparticles coated with other surfactants such as poloxamers 184, 338, 407, poloxamine 908, Cremophor® EZ, Cremophor® RH 40, and polyoxyethylene-(23)-laurylether (Brij® 35) [9]. The opinion that toxicity is not the mechanism for the nanoparticle-mediated drug transport across the BBB further was corroborated by the experiments of San et al. [29] and of Koziara et al. [30]. Moreover, the electron microscopic studies by Zensi at al. [26,27] and by Kreuter et al. [23] showed that the tight junctions (mechanism 5) did not open after intravenous administration of the nanoparticles. The latter result also is supported by the

findings that no major increase in the inulin spaces was observable in rat brain perfusion experiments [31]. Such an opening of the BBB could be achieved for instance by injection of hyperosmotic solutions and would increase the inulin spaces by a factor of 10–20.

Consequently, mechanisms 6 and 7, endocytosis followed by transcytosis, are the underlying mechanisms for the transport of drugs across the BBB into the brain [22]. *In vitro* studies already clearly demonstrated the endocytotic uptake of polysorbate 80- or poloxamer 188-coated nanoparticles into several primary endothelial cells as well as into cell lines including mice [26] and rat endothelial cell lines [31] as well as primary bovine [32,33], porcine [34], and human endothelial cells [33]. Similarly, an *in vitro* uptake also was observed into neurons [35] and glioblastoma cell lines [36] including A172 human glioblastoma cells [37].

Further *in vitro* experiments demonstrated that apolipoproteins E and/or A-I (apo E or apo A-I) were adsorbed on the surface of poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80 or poloxamer 188 after their incubation in blood plasma [38,39]. For this reason, *in vivo* experiments were performed in which different apolipoproteins were adsorbed directly onto uncoated or onto polysorbate 80-precoated dalargin-loaded poly(butyl cyanoacrylate) nanoparticles prior to their intravenous injection, and the pharmacological responses (antinociceptive effects) were measured [40]. These experiments demonstrated that especially apo E and B (apo A-I was not included in these experiments) yielded high antinociceptive effects that were similar to polysorbate 80-coating alone and even higher after polysorbate coating plus apo E and apo B overcoating. Therefore, it was concluded that polysorbate 80 (or other polysorbates and poloxamer 188) act as an anchor for the apolipoproteins and that these apolipoproteins then interact with lipoprotein receptors on the brain capillary endothelial cells [2,3,22]. This hypothesis was then challenged by an experiment by Michaelis et al. [12] who used human serum albumin nanoparticles instead of the cyanoacrylate and bound apo E covalently to the nanoparticles using a NeutrAvidin-biotin linker. They showed that these apo E-modified nanoparticles yielded even more pronounced antinociceptive effects than the polysorbate 80-coated particles. Later, Kreuter et al. [41] achieved comparable effects by covalent attachment of apo A-I and apo B-100 to the serum albumin nanoparticles.

Apo A-I is able to interact with the scavenger receptor class B type I (SR-BI) [42,43] and apo E and B with the LDL receptor (LRP1) [44]. For this reason, interaction with these receptors, followed by endocytosis and transcytosis across the brain capillary endothelial cells appears to be the underlying mechanism for the drug delivery by surfactant-coated poly(butyl cyanoacrylate), polylactic acid, and albumin nanoparticles or of particles with adsorbed or covalently linked targeting ligands such as the apolipoproteins A-I, B, and E. The nanoparticles thus would mimic lipoprotein particles and act as Trojan horses.

This assumption is supported by the electron microscopic pictures of Zensi et al. [26,27] (Fig. 1). In these investigations again albumin nanoparticles with covalently linked apo E [26] or apo A-I [27] were used. Apo A-I or apo E were attached to the surface of these particles via a bifunctional Mal-PEG-NHS crosslinker. As a control HSA nanoparticles with poly-ethylene-glycol (PEG) chains and no Apo A-I or E on their surface were prepared by linking mPEG-SPA-5000 to the particle surface. Only the nanoparticles with covalently bound apolipoprotein A-I or apo E were found in brain capillary endothelial cells as well as in neurones, whereas no uptake into the brain was detectable with nanoparticles without apolipoprotein A-I or E. In order to verify the maintained integrity of the tight junctions [45], a perfusion with a cacodylate buffer containing 1% lanthanum (III) nitrate after nanoparticle injection was performed [26]. In addition to the animal experiments, the uptake of the albumin/ApoE nanoparticles into mouse endothelial (b.End3) cells and their intracellular localisation was demonstrated in *in vitro* experiments [26]. These findings indicate that nanoparticles with covalently bound apolipoprotein E or A-I are taken up into the cerebral endothelium by an endocytotic mechanism followed by transcytosis into the brain parenchyma.

In the brain the nanoparticles were observed in the neurons of mice and rats [26,27] already 30 min after their intravenous injection. As the extracellular spaces of the mammalian brain were estimated to have a width of about 38 to 64 nm [46], these nanoparticles with a size of about 250 nm could not be transported through these spaces. Begley [47] therefore suggested that once in the brain, the particles may follow the speedy cytoplasmic route through the tissue, because pharmacological responses often were observed within a very short time after intravenous injection of the delivery system. Because they link the BBB with more remote structures, astrocytes may provide such a cytoplasmic route through the brain [47].

The conclusions that can be drawn from the above studies of Zensi et al. [26,27] are corroborated by the results of Reimold et al. [48] in rats using rhodamine-123-, fluorescein-isothiocyanate-dextran-, or doxorubicin-labelled poly(butyl cyanoacrylate) nanoparticles. After the administration of polysorbate 80-coated particles by carotic injection, fluorescence could first be detected in cryosections of the brain in capillary lumina with a progressive shift to capillary endothelial cells at 30 min followed by a rather evenly spread distribution across the brain tissue at 60 min after administration. Sixty minutes after administration into the tail vein, the fluorescent particles could be assigned to endothelial cells, whereas after 2 h a rather evenly spread distribution across the

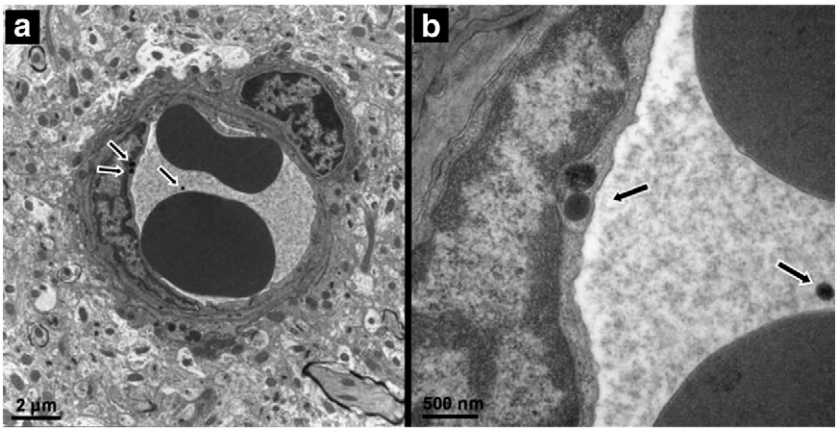

Fig. 1. Micrographs of the cortex region of SV 129 mice 15 min after injection of Apo E-modified human serum albumin nanoparticles. The nanoparticles (dark spheres indicated by arrows) were observed inside the endothelium cells (2a and b). Fig. 2a shows a cross section of a blood vessel in the cortex region with two nanoparticles in the endothelium cell and one nanoparticle still in the lumen of the vessel. Fig. 2b shows a higher magnification of the same micrograph.
Reprinted from Zensi et al. [26] with permission of the copyright holder, Elsevier, Amsterdam.

brain tissue was seen. No fluorescence in the brain was detectable with uncoated nanoparticles.

It is very important to note that endocytotic processes by endothelial cells including brain capillary endothelial cells are quite rapid processes. As mentioned above, nanoparticles were observable in these cells of mice and rats already 15 min after i.v. injection [26,27], and at the same time significant pharmacological effects were occurring with nanoparticle-bound dalargin [6] or loperamide [8]. The rapid endocytotic uptake by these cells was not only observed with nanoparticles but also occurs with many macromolecules already in a time frame of a few minutes [49,50].

The transport of the nanoparticles across the blood–brain barrier appears to be circadian phase-dependent [6]: In that report mice were subjected to a 12 h:12 h light–dark circle. Dalargin solution did not at all effect the normal pain reaction and the circadian phase of the mice as shown by the hot-plate test. In contrast, a significant dose-dependent antinociceptive effect was achieved with dalargin-loaded polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles. Interestingly the maxima of the effects obtained with the nanoparticles were shifted by about 12 h compared to the normal circadian phase-dependent pain reaction of the mice, i.e. the maximal pain reaction with the nanoparticles was observed when then normal pain reaction was at its minimum and vice versa. This is indicative of a circadian-time-dependent fluctuation in the permeability – or the transcytosis capacity – of the small cerebral vessels [6].

4. Types of nanoparticle polymers

As stated in a recent review by Wohlfart et al. [3], an important major requirement for nanoparticulate brain delivery systems is that they are rapidly biodegradable, i.e. over a time frame of not more than a few days. Non-degradable particles such as fullerenes, metal particles, toxic systems such as quantum dots, or potentially risky needle-shaped delivery systems such as carbon nanotubes, which may have hazardous effects similar to asbestos, therefore, are not useful. Likewise, silica particles also appear to be not useful as they would import a very foreign material into the brain. Fumed silica has been used as an auxiliary material for oral application but appears to be not absorbed into the body in significant amounts. Silica nanoparticles also are not a good model system as their body distribution is totally different from carbon chemistry-derived materials [51].

For this reason, especially three types of nanoparticle materials (poly(alky cyanoacrylates) such as poly(butyl cyanoacrylate) (PBCA) or poly(isohexyl cyanoacrylate) (PIHCA), poly(lactic acid) (PLA) or its copolymer (lactide-co-glycolide) (PLGA), and human serum albumin (HSA)) appear to be the materials of choice [3]. Of these materials PBCA is the fastest biodegrading material. Grislain et al. [52] and Ambruosi et al. [53,54] showed in mice and rats using ^{14}C-labelled nanoparticles that after 24 h already approximately 80% of the injected poly(butyl cyanoacrylate) was eliminated from the body after intravenous injection. The reason for this rapid elimination probably is the low molecular weights of the poly(alkyl cyanoacrylates) in nanoparticulate form of around 2000–3000 Da [55–58] although a minimal portion of higher molecular weight compounds also may be present in the particles [57,58]. Degradation occurs by enzymatic cleavage of the ester bond of the alkyl side chain of the polymer [52,58,59] resulting in the formation of the low-toxic water-soluble products — poly(cyanoacrylate) acid and appropriate alcohol. Consequently, the molecular weights of the poly(alkyl cyanoacrylate) esters increase with increasing molecular weights [55], whereas their degradation rate decreases. This reduced degradation significantly decreases their toxicity [60]. For this reason, poly(isohexyl cyanoacrylate) was chosen for clinical trials and is now in clinical phase III [61]. The biodegradation rate was somewhat reduced by the binding of certain drugs such as doxorubicin [53,54].

Biodegradation of the poly(lactide-co-glycolide) nanoparticles also appears to occur by enzymatic degradation [62] due to their small size which is in contrast to larger polylactide particles and materials where hydrolytic degradation is the prevailing degradation mechanism. Poly(lactide-co-glycolide) nanoparticles seem to be degraded by lipases [62] whereas esterases seem to be responsible for the degradation of the cyanoacrylates [52,58,59].

The degradation rate of albumin nanoparticles probably also is quite rapid. Albumin nanoparticles prepared by heat denaturation were totally degraded within 3 days in macrophages [17]. Although the albumin particles used for brain drug delivery were crosslinked with aldehydes and although the brain represents a different environment, a rapid degradation also can be anticipated, and the observed toxicity of the latter particles was extremely low [26].

In addition to the above materials, chitosan may be another useful polymer [13,14]. The chitosan nanoparticles also are biodegradable and biocompatible, possess good stability, low toxicity, and can be manufactured by simple and mild preparation methods. These methods include production in form of microemulsions, ionotropic gelation, emulsification combined with solvent diffusion, polyelectrolyte complexing, emulsification cross-linking, complex coacervation, and solvent evaporation.

5. Influence of the surface properties

As discussed in Section 3, the surface properties of the nanoparticles play the paramount role for the ability of the particles to deliver drugs to the brain. Apart from polysorbate 80, also polysorbate 20, 40, and 60 [9,28] and poloxamer 188 [10,11] were able to achieve antinociceptive effects in mice after binding of dalargin following intravenous injection, whereas other surfactants such as poloxamers 184, 338, 407, poloxamine 908, Cremophor® EZ, Cremophor® RH 40, and polyoxyethylene-(23)-laurylether (Brij® 35) did not yield such effects. In another set of experiments, Calvo et al. [25] showed in mice and rats that after intravenous injection of PEGylated [^{14}C]-poly[methoxy poly (ethylene glycol) cyanoacrylate-co-hexadecyl cyanoacrylate] nanoparticles ([^{14}C]-PEG-PHDCA nanoparticles) the ^{14}C-concentration in different brain tissues also was enhanced considerably. This enhancement was significantly greater than after coating of [^{14}C]-poly(hexadecyl cyanoacrylate) nanoparticles ([^{14}C]-PHDCA nanoparticles) with polysorbate 80 or poloxamine 908. PEGylation of nanoparticles as with liposomes leads to the so-called stealth effect that is characterised by a significant reduction in liver uptake and increase in blood circulation time and distribution into other organs and tissues [63,64]. Adsorption of poloxamine on the surface of the nanoparticles yields similar effects [65–67] in a dose-dependent manner [68]. Surprisingly, coating with polysorbate 80 but also with poloxamine 908 led to lower brain concentrations than uncoated particles in both species [25]. In addition, a species-dependent influence of the surfactants on the brain concentrations was observed: in mice the brain concentrations of the [^{14}C]-PHDCA nanoparticles were higher after coating with polysorbate 80 than with poloxamine 908 whereas in rats this order was reversed. Another important finding was that after reduction of the nanoparticle dose while maintaining the same total polysorbate concentration, higher [^{14}C] brain levels were observed with the polysorbate 80-coated nanoparticles than with the PEGylated [^{14}C]-PEG-PHDCA particles.

The results with PEGylated nanoparticles were mirrored by an increased uptake of ^{14}C-labelled PEG-PHDCA and PHDCA nanoparticles into intracerebrally transplanted 9L glioblastomas after intravenous injection in Fischer rats [69]. The tumour concentrations of the PEGylated nanoparticles were about 3 times higher than with the normal PHDCA particles and about 5–6 times higher than in the adjacent brain areas. Interestingly in the tumour-bearing rats, the brain concentrations in the areas adjacent to the tumour as well as in the contralateral brain hemisphere also were increased significantly compared to normal animals without tumour, indicating a generally higher permeability in the diseased animals.

However, these nanoparticles loaded with doxorubicin did not increase the survival of Fisher rats bearing the same tumour, 9L, after intracranial transplantation [70]. Biodistribution studies revealed that the binding of the doxorubicin to the nanoparticles decreased the tumour accumulation of the particles by a factor of 2.5 which may be the cause for the lack of efficacy of these particles against 9L. This reduction in the tumour accumulation also could be the result of an alteration of the surface charge of the nanoparticles as adsorbed drug may alter this charge [53,54,70]. In contrast to the PEGylated nanoparticles in the 9L tumour, doxorubicin bound to poly(lactide-co-glycolide) nanoparticles overcoated with poloxamer 188 [10,11] or poly(butyl cyanoacrylate) nanoparticles overcoated with polysorbate 80 [7,28,71] or poloxamer 188 [39] yielded a pronounced tumour reduction and a prolonged survival with incidence of total tumour remission in rats with intracranially transplanted glioblastoma 101/8. This tumour histologically and morphologicly resembles human grade IV glioblastomas exhibiting a similar diffuse, highly proliferative growth pattern, and considerable necrotisation [7,28]. In this context it has to be mentioned that Huang et al. [72] in contrast to the above mentioned results by Calvo et al. [25] found no difference in brain uptake between stealth and non-stealth [^{14}C]-labelled poly methoxyethyleneglycol cyanoacrylate-co-n-hexadecyl cyanoacrylate (PEG-PHDCA) nanoparticles in mice after intravenous injection, although the blood circulation time of the stealth particles again was significantly prolonged. Stealth properties alone, therefore, do not appear sufficient for enabling a nanoparticle-mediated transport into the brain.

These results underline the importance of the surface properties for brain drug delivery by intravenous injection. However, not only the properties of the coating surfactant but also the core polymer, the adsorbed or incorporated drug, and the stabilizers used during nanoparticle production such as dextran, poly(vinyl alcohol), albumin, lecithin and other surfactants play a major role in the ability and efficacy of this mode of delivery [10,11]. While polysorbate 80 was very efficient after coating of the poly(butyl cyanoacrylate) nanoparticles, comparable or even superior to poloxamer 188, the latter surfactant was considerably more efficient after coating of the poly(lactide-co-glycolide) nanoparticles as shown with doxorubicin in glioblastomas as well as with loperamide in the antinociception experiments [10]. The latter findings were further supported by studies by Kulkarni and Feng [73] who observed that the brain uptake of poly(D,L-lactide-co-glycolide) nanoparticles loaded with coumarin-6 was higher after coating with poloxamer 188 or 407 than with polysorbate 80 but lower with TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate) or polyvinyl alcohol.

6. Targeting ligands

In Section 3 the importance of apolipoprotein A-I, B, or E adsorptive binding or covalent attachment for the nanoparticle-mediated drug delivery to the brain was described. These ligands interact either with the scavenger receptor class B type I (SR-BI) (apo A-I) [42,43] or with the LDL receptor (LRP1) (apo E and B) [44]. However, there exist a lot of other receptors that transport peptides and large molecules across the BBB [74–76]. One possibility is to use the transferrin receptor for the delivery of drugs to the brain using nanoparticles [77–79]. Transferrin or anti-transferrin receptor monoclonal antibodies (OX26 or R17217) were covalently coupled to the human serum albumin nanoparticles using the NHS-PEG-MAL-5000 linker [80]. Loperamide was used as model drug since it normally does not cross the blood–brain barrier and was bound to the nanoparticles by sorption. Loperamide-loaded human serum albumin nanoparticles with covalently bound transferrin or the OX26 or R17217 antibodies induced very significant antinociceptive effects in the tail-flick test in ICR (CD-1) mice after intravenous injection, demonstrating that transferrin or these antibodies covalently coupled to HSA nanoparticles are able to transport bound loperamide and possibly other drugs across the BBB. Control loperamide-loaded human serum albumin nanoparticles with IgG2a antibodies yielded only insignificant marginal effects.

These finding were supported by Chang et al. [81] who found a significantly increased uptake of fluorescent dye-loaded poly(lactide-co-glycolide) nanoparticles to which transferrin was adsorbed into intracranially transplanted F98 glioblastomas in rats in comparison to bovine serum albumin-coated nanoparticles.

Likewise the insulin receptor can be used [82]. Insulin or an anti-insulin receptor monoclonal antibody (29B4) were covalently coupled to the human serum albumin nanoparticles, using the same NHS-PEG-MAL-5000 linker and loperamide as the model drug. Covalently bound Insulin or the anti-insulin receptor 29B4 antibodies induced similar antinociceptive effects in the tail-flick test. Pre-injection of a solution of the 29B4 anti-insulin receptor antibody 30 min before injection of the loperamide-loaded human serum albumin nanoparticles with covalently bound insulin totally inhibited the loperamide transport across the BBB, demonstrating that the insulin receptor indeed is involved in this transport. The mechanism of the loperamide transport, therefore, appears to be similar to the apolipoprotein E-mediated transport [22], namely receptor-mediated endocytosis followed by transcytosis. The amount of 100 μl 29B4 anti-insulin receptor antibody was sufficient to either block all insulin receptors in the body, or there may be a special affinity of this antibody to the insulin receptors in the brain.

In addition to antibodies or natural substrates for certain receptors, also peptides such as Angiopep (Thr-Phe-Phe-Tyr-Gly-Gly-Ser-Arg-Gly-Lys-Arg-Asn-Asn-Phe-Lys-Thr-Glu-Glu-Tyr) [83] or H-2N-Gly-l-Phe-d-Thr-Gly-l-Phe-l-Leu-l-Ser-O-ß-d-glucose-CONH2 [84] which is similar to synthetic opioid peptides can be employed. Angiopep-conjugated poly(ethylene glycol)-co-poly(ε-caprolactone) copolymer nanoparticles were used for the targeting of paclitaxel to U87 MG gliomas transplanted into the striatum of nude mice taking advantage of the low density lipoprotein receptor related protein (LRP) receptor that is not only over-expressed on the BBB but also on glioma cells [85]. The anti-glioblastoma efficacy of paclitaxel-loaded Angiopep-conjugated nanoparticles thus was significantly enhanced compared to paclitaxel or paclitaxel-loaded nanoparticles without Angiopep.

Gly-l-Phe-d-Thr-Gly-l-Phe-l-Leu-l-Ser(O-β-d-glucose)-CONH2 bound to poly(D,L-lactide-co-glycolide) nanoparticles also enabled the delivery of loperamide into the central nervous system (CNS) after intravenous administration and yielded prolonged antinociceptive reactions as shown by the hot plate test [86]. Another approach may be the attachment of the sequences 12–32 (g21) of leptin via the avidin–biotin system to poly-(lactide-co-glycolide) nanoparticles [87].

Alternatively, the Thr-His-Arg-Pro-Pro-Met-Ser-Pro-Val-Trp-Pro protein may be used to target the transferrin receptor although the gold nanoparticles that were used in the respective study by Prades et al. [88] due to their lack of biodegradability probably are not a suitable material for brain delivery.

These results indicate that different receptors in the brain could be employed for the delivery of drugs across the BBB. It is possible that any ligand for which a receptor exists on the brain capillary endothelial cells may be used for this purpose. On the other hand, since most of these receptors are ubiquitously expressed, there is the danger of non-specific adverse effects resulting in potential limitations of this approach.

7. Quantification of the in vivo uptake into the brain

Several *in vivo* techniques have been developed to study and measure the uptake of CNS compounds into the brain which were discussed in detail by van Roy et al. [89]. With these techniques, various parameters can be determined after drug administration, including the blood-to-brain influx constant (Kin), the permeability-surface area (PS) product, and the brain uptake index (BUI). For nanoparticles the best methods appear to be capillary depletion [90] and microdialysis: The

latter two techniques not only allow the determination of the amount of drug that is distributed within the whole brain but also enable the quantification of the drug that indeed has passed the blood–brain barrier and appears in the brain parenchyma.

For capillary depletion the brain is excised after sacrifice of the animal and then is gently homogenised in a mortar. The homogenate then is centrifuged and the drug is analysed in the supernatant which represents the brain parenchyma as well as in the pellet representing the cell debris content including the parenchyma. Microdialysis using special devices allows the dynamic determination of the drug concentration in the brain. However, this method cannot be used if the compound is adsorbing too much to the dialysis system as it is the case for instance with doxorubicin.

8. *In vitro–in vivo* correlation, route of administration

In vitro no fundamental difference appeared in the ability of brain endothelial cells to take up polysorbate 80-coated nanoparticles between cells of mammalian origin including human [33], bovine [33], porcine [34], rat [33], and mice endothelial cells [26,44]. However, some important differences can be observed between *in vitro* and *in vivo*: *In vivo* only single particles were observed in the brain capillary endothelial cells whereas *in vitro* in the mouse cell line b.End3 cells [26] as well as in hCMEC/D3 (unpublished results) cells multiple particles could be seen within larger vesicles suggesting vesicular fusion and a different intracellular pattern of trafficking of the particles in the *in vitro* and the *in vivo* experiments. Exocytosis of the nanoparticles was never observed at the basal border of the cells in the cell cultures. This may have been due to the presence of the collagen-coated glass support or a result of altered cell trafficking *in vitro*. When injected intravenously into intact mice or rats, the nanoparticles were internalised into the endothelial cells singly by coated pits but did not accumulate within a vesicle [26,27]. The particles were transcytosed *in vivo* and appeared to be able to avoid the lysosomal compartment within the endothelium.

In this context it is also noteworthy that some differences between rats and mice occurred in the experiments of Calvo et al. [25]: As mentioned above, a species-dependent influence of the surfactants on the brain concentrations was observed: In mice the brain concentrations of the poly(hexadecyl cyanoacrylate) nanoparticles were higher after coating with polysorbate 80 than with poloxamine 908 whereas in rats this order was reversed.

A frequent misconception is to conclude from results obtained with one drug or marker to another or from one administration route to another. For drugs in solution peritoneal (i.p.) injection may to a certain extent allow deductive conclusions about their body distribution and especially about their action. This is definitely not the case with particulate materials such as nanoparticles. First of all, the intraperitoneally injected nanoparticles will be captured by peritoneal macrophages and, at least partly, distributed into the lymphatic system [91]. Moreover, injection into the peritoneum, an unlikely route for administration in humans, provides contact with a different body fluid medium which in turn can significantly alter the nanoparticle surface properties. A typical example for such erroneous conclusions is a report by Geldenhuys et al. [92] who produced paclitaxel- as well as coumarin-6-loaded PLGA nanoparticles coated with glutathione and measured the coumarin-induced fluorescence in brain after i.p. injection. They then hypothesized that the paclitaxel would distribute into the brain in a similar pattern as the coumarin. However, in addition to the injection route, as discussed above not only the nanoparticle polymer and the coating material but also the incorporated or adsorbed drugs can significantly alter the biodistribution. Drug adsorption or even incorporation can considerably change the surface properties including hydrophobicity and surface charge [53,54] which determine the interaction with blood and its components. The core properties may be changed by drug dispersed in the polymer matrix in form of a solid solution or solid dispersion [93], and the degradation rate may be altered. For this reason, fluorescent or other markers may not necessarily predict the fate of a carrier system or its ability to enable drug delivery to the brain.

Moreover, frequently the differences in brain concentration between coated and uncoated nanoparticles or to control solutions may be very small [92], thus they provide no evidence of the possibility that these particles can achieve pharmacologically or therapeutically relevant effects.

9. Drugs that were transported across the BBB

A considerable number of drugs so-far have been transported into the brain across the blood–brain barrier using nanoparticles. These drugs include anticancer drugs, analgesics, cardiovascular drugs, protease inhibitors, several macromolecules, and others. The majority of these drugs are listed in Table 1 which is an extended and updated version of a table in a recent review by Wohlfart et al. [3]. In principle nanoparticles seem to enable the brain drug delivery of all sorts of compounds if a binding to the particles is possible and certain receptors responsible for transcytosis of endogenous substrates can be addressed. Of course the usefulness of this approach is restricted by the amount of drug that is required to achieve the desired therapeutic effect. Permanent, frequent continuous intravenous injections of higher drug doses are not possible due to the necessary quantities of nanoparticle polymers. For this reason, this approach probably is limited to the treatment of acute diseases such as stroke, brain tumours, and the delivery of effective substances that require infrequent treatments such as enzyme replacement therapy.

9.1. Brain tumours

One of the most promising areas of nanoparticle-mediated brain delivery is the treatment of brain tumours. These tumours, especially malignant gliomas, belong to the most aggressive human cancers, and the prognosis for patients still remains very unfavorable. Although these tumours are characterised by a rapid proliferation, diffuse growth, and invasion into distant brain areas in addition to extensive cerebral edema and high levels of angiogenesis, the disruption of the blood–brain barrier (BBB) remains a local event, which is evident in the tumour core but absent at its growing margins. For this reason, anticancer drugs can penetrate into necrotic tumour areas, while the drug levels in peritumoural regions were reported to remain low or non-detectable [125,126]. Very efficient anticancer drugs such as doxorubicin cannot cross the intact BBB and reach only the necrotic and not the peritumoural areas.

In addition to transcytosis through the brain endothelial cells two other possibilities make the nanoparticles promising carriers for the treatment of brain tumours. One is the possibility of tumour uptake via the enhanced permeability and retention (EPR) effect. This effect is occurring in many types of tumours that are characterised by a defective hypervasculature and an incomplete lymphatic drainage system [127]. Glioblastomas also seem to possess this property [54,128]. Secondly, in contrast to normal human brain, astrocytomas fail to express or express a non-functional form of occludin. Moreover, fenestrations and increase in the number and size of pinocytic vacuoles have been reported [129].

Gulyaev et al. [102] showed a considerable doxorubicin concentration of 6 μg/g in the brain after intravenous administration of 5 mg/kg doxorubicin bound to poly(butyl cyanoacrylate) nanoparticles whereas no doxorubicin was found in the brain after injection of doxorubicin in solution without or with polysorbate 80 or bound to the nanoparticles without polysorbate 80-coating. Wohlfart et al. [90] later confirmed by the method of capillary depletion that the doxorubicin indeed was transported across the blood–brain barrier and appeared in the brain parenchyma. In accordance with these results Steiniger et al. [7] observed a significantly extended survival time of rats with intracranially

Table 1
Drugs bound to nanoparticles for brain delivery.
Adapted and extended from a review by Wohlfart et al. [3].

Drug	Type of action	Core polymer	Surface modification	Size [nm]	Surface charge [mV]	Reference no.
Amphotericin B	Antifungal drug	PLA-PEG	PS80*	114–133	n/r****	[94]
BCNU	Anticancer drug	PLA	Transferrin	120	−35	[95]
Breviscapin	Cerebrovascular drug	PLA[a]	P188**	127	−34 to −56	[96]
				320		
Camptothecin	Anticancer drug	Poly-ε-caprolact.-PEG	PEG	130–280	0 to −20	[97]
Caspase-3 inhibitor	Caspase-3 inhibitor	Chitosan-PEG	PEG + anti-transferrin antibody	650	+18 to +20	[14]
Coumarin-6	Dye	PLGA-PEG	PS 80*	194	−23	[73]
			P188**	188	−21	[73]
			P407***	196	−20	[73]
					−	
			PEG	200	22 to +2	[98]
			Glutathione	230–246	n/r****	[91]
			Phage-display peptide	104–121	−18 to −24	[99]
Dalargin	Analgesic drug	PBCA	PS 80*	230	n/r****	[1,6]
Dexamethasone	Steroidal drug	PLGA	Alginate hydrogel	400–600	n/r****	[100]
Dopamine	Anti-Parkinson drug	Chitosan	None (i.p. inject.)	98–148	+27 to +34	[101]
Doxorubicin	Anticancer drug	PBCA	PS 80*	270	n/r****	[7,28,39,102]
		PLGA	P188**	240–470	−11 to +16	[10,11]
Endomorpin-1	Analgesic drug	PBCA	PS 80*	*27*[a]	n/r****	[103]
Gemcitabine	Anticancer drug	PBCA	PS 80*	112	n/r****	[104]
Green fluorescent protein	Fluorescent dye	PBCA	PS 80*	150	n/r***	[105]
Horseradish peroxidase	Enzyme	PBCA	PS 80*	150	n/r****	[106]
Kyotorphin	Analgesic drug	PBCA	PS 80*	195–289	n/r****	[107]
Loperamide	Opiate receptor agonist	PBCA,	PS 80*	290	–, −35, −13	[8,10]
		HSA,	Apo E3, A1, B100	218–240		
		PLGA	P188**	178/290	−17 to −25	[10]
		PLGA	(R)-g7 peptide	139–182	−36 to +31	[85,107]
		PLGA-PEG	PEG	150		[98]
Methotrexate	Anticancer drug	PBCA Chitosan	PS 80**	*87*[a]–318	n/r****	[19]
			PS 80*	125–263	+15 to +31	[108]
MRZ 2/576	NMDA reception antagonist	PBCA	PS 80*	251	n/r****	[109]
Nerve growth factor (NGF)	Growth factor	PBCA	PS 80*	250	n/r****	[4]
Obidoxime	Acetylcholinesterase reactivator	HSA	Apo E	230	n/r****	[34]
Paclitaxel	Anticancer drug	Poly-ε-caprolact.-PEG	Angiopep	90	n/r****	[83,110]
		PLA-PEG	Glutathione	230–246	n/r****	[90]
		Poly-ε-caprolact.-	Aptamer	156	−33	[111]
		PEG	PEG	73	−4	[112]
Rivastigmine	Anti-Alzheimer's drug	PBCA	PS 80*	*41*[a]	−35	[113]
		Chitosan	PS 80*	*47*[a]	+30 to +33	[114]
Ritonavir:	Protease inhibitor	PLA[a]	Trans-activating transcriptor peptide	175	+2	[115]
Saquinavir	Protease inhibitor	PBCA,	PS 80*	192–184	Medium negative	[116]
Sulpiride	Atypical antipsychotic drug	PLA[a]	Maleimide PEG	329	−39 to −19	[117]
Superoxide dismutase	Radical scavenger	PLGA	None (Infarcted animal)	291	−25	[118]
Tacrine	Anti-Alzheimer's drug	PBCA	PS 80*	*36*[a]	−40	[119]
		Chitosan		*41*[a]	+35 to +37	[120]
Temozolomide	Anticancer drug	PBCA	PS 80*	136	−25	[121]
Tubocurarine	Muscle relaxants	PBCA	PS 80*	230	n/r****	[122]
TGF-ß antisense oligonucleotide	Antisense oligonucleotide	PBCA	PS 80*	n/r***	n/r****	[123]
Valproic acid	Anticonvulsant and mood-stabilizing drug	PBCA	PS 80*	n/r****	n/r****	[124]

Abbreviations:
PLA = poly(lactic acid).
PEG = poly ethylenglyco.
PLGA = poly(lactide-co-glycolide).
Poly-ε-caprolact. = poly-ε-caprolactone.
PBCA = poly(butyl cyanoacrylate).
HSA = human serum albumin.

[a] These sizes seem to be unusually small. They are determined by electron microscopy and therefore appear to have shrunken during drying and the high vacuum in the microscope. Normally poly(butyl cyanoacrylate) nanoparticles produced by the method of Kreuter et al. [1] possess a diameter of 200–300 nm. Drug loading can influence, mostly increase the diameter.

* PS80 — polysorbate 80.
** P188 — poloxamer 188.
*** P407 — poloxamer 407.
**** n/r — not reported.

transplanted rat 101/8 glioblastomas after three intravenous injections of 1.5 mg/kg of doxorubicin bound to the polysorbate 80-coated nanoparticles and a long-term survival of >180 days in 20–40% of the animals. As mentioned in Section 5, this tumour – in contrast to C6, Rg-2 or 9L – histologically and morphologically resembles human grade IV glioblastomas [7,28]. Necropsy of the long-term survivors did not reveal any signs of tumour growth. The efficacy of these particles was further substantiated by histological and immunohistochemical analysis [28]. Besides the important inhibition of the tumour growth, these nanoparticles drastically decreased the extent of necrosis

and inhibited microvascular proliferation which was indicative of an antiangiogenic mode of action of this preparation [3,28]. The extension to four (4 × 1.5 mg/kg) and five (5 × 1.5) injections [130], based on the fact that 7.5 mg/kg doxorubicin bound to nanoparticles was well tolerated, further improved the therapy of this brain tumour.

Similar or even better anti-tumour effects in this glioma model were obtained with doxorubicin bound to poly(lactide-co-glycolide) nanoparticles coated with poloxamer 188 [10,11].

Enhanced brain concentrations also were observed with methotrexate [19] or temozolomide [121] bound to polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles in the brain of rats after their intravenous injection. The increase in brain concentrations by a factor of 2 in comparison to the solutions, however, was only marginal compared to the enhancement obtained with doxorubicin.

The anti-tumour activity of gemcitabine bound to the polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles was investigated in rats 14 days after C6 glioblastoma implantation into the brain [104]. A 20% increase in medium survival time from day 21 to 25 was observed. The C6 glioblastoma also was transplanted intracranially into mice, and paclitaxel was bound to methoxy poly(ethylene glycol)–poly(ε-caprolactone) nanoparticles [112]. The mean survival times with the latter preparation increased by 40% compared to a paclitaxel injection and by 20% compared to non-PEGylated (ε-caprolactone) nanoparticles. A 75% increase in medium survival time from 21 to 37 days resulted with the U87 MG glioblastoma implanted into the right striatum of nude mice using the methoxy poly(ethylene glycol)–poly(ε-caprolactone) nanoparticles to which Angiopep was covalently bound [84]. An enhanced accumulation of Angiopep poly(ε-caprolactone) nanoparticles in the glioma and infiltrating margin was observed by real time fluorescence imaging [110].

Convection enhanced delivery (CED) [131] which is based on continuous infusion of drug via intratumoural or intraparenchymal catheters was used for camptothecin loaded to amphiphilic β-cyclodextrin, poly(lactide-co-glycolide), or poly-ε-caprolactone nanoparticles manufactured by the nanoprecipitation method [97]. The 6-O-Capro-β-cyclodextrin nanoparticles significantly reduced the growth or lethality of 9L gliomas and increased the medium survival by 27% compared to the untreated group.

The combination chemotherapy of implantation of a 3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-loaded wafers combined with the intracarotid perfusion of BCNU-loaded poly(D,L-lactic acid) nanoparticles coated with transferrin was tested in C6 glioma-bearing rats [95]. The combination chemotherapy showed a stronger inhibitory effect and prolonged the average survival time of rats by 164% compared to the controls.

9.2. Alzheimer's disease

Alzheimer's disease is a neurodegenerative disorder of the elderly and represents the most prevalent form of dementia. The cognitive decline associated with this disease drastically affects the social and behavioral skills of these patients. As the delivery of many potential anti-Alzheimer drugs also is restricted by the BBB, a delivery by nanoparticles also may be of great interest [132,133]. A number of such drugs have been bound to nanoparticles for this purpose including rivastigmine [113,114], tacrine [119,120,134], quinoline [135], piperine [137], and curcumin [133]. The drugs were bound to poly(butyl cyanoacrylate) [110,116,135], chitosan [113,129,134], and poly(lactide-co-glycolide) nanoparticles. In the case of the piperine solid lipid nanoparticles were employed. The binding to nanoparticles significantly altered the body distribution of the bound drugs and increased their brain concentration.

The curcumin nanoparticles were conjugated with a targeting moiety-Tet-1 peptide. A 12-amino acid peptide which has an affinity to neurons and possess retrograde transportation properties [137]. This peptide can interact specifically with motor neurons and is capable of retrograde delivery in the neuronal cells. Curcumin has properties that are potentially beneficial for the treatment of Alzheimer's disease, such as anti-amyloid and anti-oxidant activity. However, these nanoparticles were only tested *in vitro* but were able to destroy the amyloid aggregates, exhibited anti-oxidative properties, and were non-cytotoxic [137].

The piperidine solid lipid nanoparticles consisting of glycerine monostearate and Epikuron® also were investigated in force swimming tests [136]. In order to develop an artificial Alzheimer model, albino rats were treated with ibotenic acid. The resulting immobility was significantly improved after 7, 14, and 28 days only with the polysorbate 80-coated solid lipid nanoparticles and not with any of the controls. In addition, the polysorbate-coated nanoparticles reduced the superoxide dismutase values, increased the acetylcholenesterase values and showed superior results than donepezil. Histopathology studies revealed reduced plaques and tangles [136].

Nerve growth factor also was bound to polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles and improved the memory in an Alzheimer model (PAR test, see next section) [4].

However, since Alzheimer is a chronic disease, it may require frequent injections that result in the accumulation of the nanoparticle polymer material, which would obliterate this approach as discussed in Section 11.

9.3. Delivery of large peptides

Peptide and protein drugs hold great promises for the treatment of various neurodegenerative diseases. The delivery of these drugs across the BBB mostly is even more difficult than with the majority of small molecules and therefore represents a major challenge. A comprehensive overview on the current understanding of the transport mechanisms at the BBB with promising strategies to enhance the delivery of peptide and protein drugs over this barrier is given by Brasnjevic et al. [138].

One possibility to transport large peptides across the BBB is the employment of nanoparticles. Poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80 enabled the delivery of nerve growth factor (NGF, MW ~ 130 kDa) into the brain of outbred and of C57BL/6 mice after intravenous injection [4]. The poly(butyl cyanoacrylate) nanoparticles yielded considerably enhanced NGF levels in the brain with a peak after 45 min whereas the injection of the NGF solution in saline or in polysorbate 80 solution as well as uncoated nanoparticles did not alter the NGF concentration in the CNS. These results were accompanied by pronounced and prolonged pharmacological effects: The polysorbate 80-coated nanoparticles with bound NGF were able to totally reverse the scopolamine-induced amnesia and improved the recognition and memory in an acute amnesic mouse model as shown by the passive avoidance reflex (PAR) test (Fig. 2). Moreover, in a number of Parkinson's disease models these particles significantly reduced the basic symptoms of Parkinsonism such as oligokinesia, rigidity, and tremor [4].

The polysorbate 80-coated nanoparticles also were loaded with either horseradish peroxidase (HRP ~ 44 kDA) or enhanced green fluorescent protein (EGFP ~ 29 kDa) and intravenously injected into normal as well as into brain-injured rats [103]. The nanoparticle-delivered HRP or EGFP was hardly detectable 45 min after injection in the brains of normal rats, but a small amount of EGFP was noticeable in these brains 48 h after administration, which was further confirmed by immunolocalization using anti-EGFP antibodies. In brain-injured rats, however, the nanoparticle-delivered HRP or EGFP was widely distributed near injured sites 45 min after nanoparticle injection. Similar results showing extravasation in the vicinity of injured brain sites were obtained with fluorescent polystyrene nanoparticles of a size of 20 nm [139] when the fluorescence intensity in the microdialysates was measured during cerebral ischemia/reperfusion. Under normoxic condition no nanoparticles extravasated into the brain, whereas cerebral ischemia and reperfusion induced a transient accumulation of

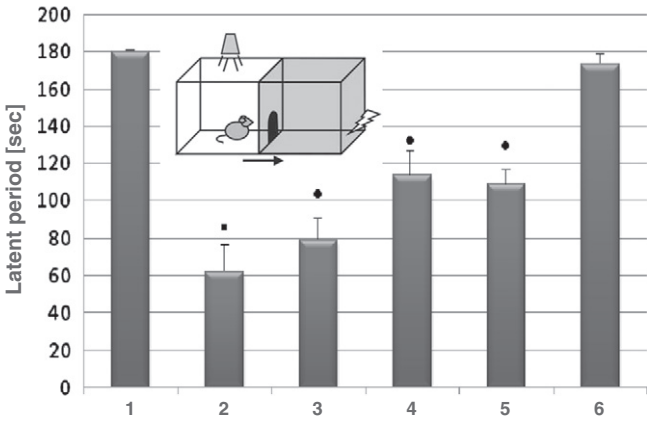

Fig. 2. Mean latent period in the passive avoidance reflex test (PAR test). In this test the animals are placed in a two chamber cage in the side with bright lighting. Rodents such as mice and rats avoid the light and rapidly disappear into the dark side where they then can be exposed to an electric shock [152]. After one week of training the latent time of the animals of remaining on the lighted side is expanded, in the displayed experiment to about 180 s. After subcutaneous injection of scopolamine the animals forget and return to a latency period of 60 s. Additional intravenous injection of NGF adsorbed on PBCA nanoparticles coated with PS 80 (group 6) reverses the latency period to approximately 175 s. The following formulations were used in this figure: 1: 0.9% NaCl solution intravenously (passive control); 2: scopolamine (1.5 mg/kg s.c.) (control of amnesia); 3: scopolamine (1.5 mg/kg s.c.) and NGF solution (5 μg/mouse i.v.); 4: scopolamine (1.5 mg/kg s.c.) and NGF in 1% PS 80 solution (5 μg/mouse i.v.); 5: scopolamine (1.5 mg/kg s.c.) and NGF adsorbed on PBCA nanoparticles (5 μg/mouse i.v.); 6: scopolamine (1.5 mg/kg s.c.) and NGF adsorbed on PBCA nanoparticles coated with PS 80 (5 μg mouse i.v.). Levels of significance: ▪ statistically reliable difference from groups 1 and 6 ($p \leq 0.01$); ● statistically reliable difference from groups 1 and 6 ($p \leq 0.05$).
Adapted from Kurachmaeva et al. [4] and reprinted from Wohlfart et al. [3] with permission of the copyright holder, Elsevier, Amsterdam.

extracellular nanoparticles in the brain. In a further brain injury model, i.e. rat focal cerebral ischemia–reperfusion, superoxide dismutase (SOD), a free radical scavenger, was bound to poly(D,L-lactide-co-glycolide) nanoparticles to neutralise the deleterious effects of the production of reactive oxygen species (ROS) after cerebral ischemia [118]. Animals receiving the nanoparticles showed a 65% reduction in infarct volume, whereas an equivalent dose of SOD in solution increased it by 25% over a saline control, indicating the protective effect of the encapsulated enzyme. Nanoparticles alone or just mixed with SOD in solution were ineffective in reducing infarct volume, with results similar to saline control. The SOD-loaded nanoparticles maintained BBB integrity, thereby preventing edema, reduced the level of ROS formed following reperfusion, and protected neurons from undergoing apoptosis. Animals treated with these particles yielded a greater survival than those with saline control (75% vs. 0% at 28 days) and later regained most vital neurological functions [118].

These results demonstrate that nanoparticles also can enable the transport of peptides and macromolecular substances to the CNS following intravenous injection and may improve the therapy of neurodegenerative diseases.

10. Toxicity

The toxicity of empty as well as doxorubicin-loaded poly(butyl cyanoacrylate) nanoparticles and of doxorubicin control solutions was investigated in a number of studies in normal and also in glioma 101/8-bearing rats [7,140–142]. Doses up to 400 mg/kg of empty nanoparticles did not cause any mortality within the period of observation (30 days) nor did they affect body weight or weight of internal organs after intravenous injection. Higher doses cannot be administered intravenously by bolus injection because of biological [143] and technical limitations. Single intravenous injections of doxorubicin formulations caused dose-dependent mortality and weight loss. No significant difference in toxicity occurred between healthy and tumour-bearing animals [140].

In addition to single injections, multiple intravenous injections (3 or 4 × 1.5 mg/kg) were performed [141,142] to assess body and internal organ weight, hematological parameters, blood biochemical parameters, and urinalysis. Pathomorphological evaluations including macroscopic and histological investigations of heart, lung, spleen, testes, and liver revealed a considerably decreased toxicity in heart and testes of the animals treated with the doxorubicin bound to nanoparticles compared to its solution confirming earlier results by Couvreur at al. [144,145]. The lower toxicity of the nanoparticulate formulations of doxorubicin most probably is explained by the altered biodistribution of the drug mediated by the nanoparticles [3]. Especially the reduced cardiotoxicity is of great importance as this toxicity is the limiting factor in the tumour therapy with doxorubicin. In addition, a lower hepatotoxicity compared to the solutions occurred with the nanoparticles although higher liver concentrations were determined in pharmacokinetic studies [102]. Probably binding to nanoparticles also leads to a different distribution of the doxorubicin within the liver increasing the Kupffer cell uptake thus making it less accessible to the more sensitive hepatocytes, thereby reducing its toxicity [142].

Steiniger et al. [7] obtained similar toxicological results. Autopsy of the whole body in healthy animals in this study revealed an empty gastrointestinal tract only in all animals treated with doxorubicin. Healthy animals treated with doxorubicin solution also showed slight signs of lung edema. These changes were not observed in animals treated with doxorubicin bound to the nanoparticles. Indication of short-term neurotoxicity, such as increased apoptosis in areas distant from the tumour, increased expression of GFAP or ezrin on distant astrocytes or degenerative morphological changes of neurons, were entirely absent in nanoparticle-treated animals on day 12 as well as in long-term survivors. In addition, there was no indication of chronic glial activation in areas distant from the tumour site in long-term surviving rats. Moreover, long-term survivors did not exhibit any obvious neurological symptoms [7].

11. Conclusions

Nanoparticles were shown to enable the delivery of a great variety of drugs into the brain after intravenous injection in animals. These drugs include analgesics, anti-Alzheimer's drugs, cardiovascular drugs, protease inhibitors, and most importantly, anticancer drugs and several macromolecules. At the moment, it appears that any drug or larger biologically active compound or complex can be transported across the BBB if it can be efficiently bound to the BBB-transcytosable nanoparticles and is released within the brain in a therapeutically relevant concentration and time profile [3].

The mechanism of the nanoparticle-mediated drug transport across the BBB appears to be receptor-mediated endocytosis. Receptors that so-far were employed include the scavenger receptor class B type I (SR-BI) [42,43], the LDL receptor (LRP1) [44,83–85], the transferrin receptor [77–81], and the insulin receptor [82]. Possibly any ligand may be used for this purpose if a specific transport receptor exists for this ligand on the brain capillary endothelial cells. The respective targeting ligands can be bound to the nanoparticle surface by covalent attachment or by the adsorption of plasma components such as apolipoproteins A-I, B, or E from the blood following the intravenous injection of nanoparticles coated with certain surfactants. The choice of the appropriate surfactant depends on the nanoparticle polymer [10,11,39] but may additionally be influenced by the loaded drug as this can influence the nanoparticle's surface properties [53,54,70].

The definition of nanoparticles for pharmaceutical and medicinal purposes should not be restricted to 100 nm but instead include sizes within the entire nanometer size range as there appeared to exist only

an unimportant influence of the particle size on drug transport into the brain at least down to 70 nm [19]. In any case, nanoscale drug delivery systems that are on the market possess sizes well above the 100 nm limit, and quantum effects that take place below this size do not seem to influence nanoparticle-mediated drug delivery [146].

A critical and important requirement for nanoparticulate brain delivery systems is that they are rapidly biodegradable, i.e. over a time frame of a few days. For this reason poly(butyl cyanoacrylate) (PBCA) or poly(isohexyl cyanoacrylate) (PIHCA), poly(lactic acid) (PLA) or its copolymer poly(lactide-co-glycolide) (PLGA), human serum albumin (HSA), and possibly chitosan appear to be the materials of choice. Non-degradable particles such as fullerenes or metal particles such as gold or iron particles, toxic systems as quantum dots, or potentially risky pointed, i.e. needle-shaped delivery systems such as carbon nanotubes, which may have hazardous effects similar to asbestos, therefore, cannot be used for this purpose.

Among the above polymers poly(butyl cyanoacrylate) is the most rapidly degrading polymer. Poly(isohexyl cyanoacrylate) was similarly efficient for the treatment of the glioblastoma 101/8 in rats [147] but seems to be even better tolerated due to its slightly slower degradation rate [60]. For this reason, poly(isohexyl cyanoacrylate) was chosen for clinical trials, and doxorubicin-loaded nanoparticles made of this material now in clinical phase III [61] for the treatment of hepatocellular carcinomas. It has to be noted that in these trials the particles were not coated with polysorbates or poloxamer 188 as they were not intended for brain delivery.

A major reason of bringing the doxorubicin-containing nanoparticles into clinic as previously with liposomes was the reduction of the pronounced cardiotoxicity of this drug. This toxicity reduction also was observed with the nanoparticles that were used for the delivery of this drug to the brain [140–143]. It is possible that similar toxicity-reducing effects may be achieved with other drugs. Additionally, by binding to nanoparticles a reduction in the required drug dose may be possible because nanoparticles can alter the body distribution [20] and may increase the concentration of bound drugs at target sites such as solid tumours [52,145], areas of inflammation [149], or in HIV-infected vs. non-infected macrophages [150,151]. As a consequence a lower drug dose will be necessary.

It has sometimes been argued that the amount of drug that reaches the brain through the use of nanoparticles is too low. If approximately 1% of the dose reaches the brain, when considering that the mass of the brain in mice and rats represents approximately 1% of the total body mass (in humans 2%), this amount is significant.

In contrast to the substantial doxorubicin concentration increase in the brain after injection of the nanoparticles [90,102], with a number of other drugs the brain drug concentration only increased by a factor of 2 to 5 [4,19,121]. However, although with dalargin [148] an only 2-fold or in the case of NGF [4] just a 6-fold increase in the brain drug concentrations were achieved, the pharmacological effects with these drugs nevertheless were substantial. Therefore, even smaller brain concentration increases may yield noteworthy pharmacological effects.

Nevertheless, nanoparticle-mediated drug delivery into the brain following intravenous injection is restricted by the amounts of drug that are required to achieve the desired therapeutic effects. Because of this administration route and the required quantities of nanoparticle polymers, drugs that need permanent frequent injections cannot be used for the nanoparticle delivery approach, which limits its usefulness to the treatment of acute diseases and to the delivery of very effective substances that require infrequent treatments.

Although the mechanism of the nanoparticle uptake into the brain endothelial cells, receptor-mediated endocytosis and formation of caveoli [26], obviously a rapid process [49,50], has been largely elucidated, the further fate in the brain should be a main focus of future research. Begley [47] suggested a “superhighway” that transports the nanoparticles very efficiently within the brain parenchyma and could explain the rapid pharmacological responses. However, this definitely requires exhaustive investigations. Furthermore, biochemical investigations should be carried out in addition to the morphological studies. During these investigations it has to kept in mind that relevant differences may exist between *in vivo* and *in vitro* results not only because the particle concentrations mostly are much higher *in vitro* than *in vivo* leading to very different cell reactions and effects but also that the cellular environment apparently plays a major role in particle processing.

Last not least, all *in vivo* brain research with nanoparticles so-far has been done in rodents, and rodent species differences indeed were observed [25] (Section 5), although this difference may not be due to BBB differences and more likely is resulting from different pharmokinetics of the particles after injection (D. Begley, King's College London, personal communication). Correspondingly, Zensi et al. [26,27] found no relevant difference in their electron microscopic study of the brain between mice and rats after i.v. injection of nanoparticles. In addition, *in vitro* no important differences in nanoparticle uptake or processing were observable between mice [26] and rat endothelial cell lines [31] as well as primary bovine [32,33], porcine [34], and human endothelial cells [33]. Nevertheless, the *in vivo* studies also should be extended to other mammal species. Moreover, since glioblastomas are such terrible rapidly progressing and so-far largely untreatable tumours, clinical studies also are warranted in the next future.

To finalise, this review agrees with the suggestion by Wohlfart et al. [3] that the ability to deliver drugs that are not BBB permeable by simple intravenous administration is representing a major breakthrough. It is surprising that the pharmaceutical industry has not more frequently taken advantage of these brain drug delivery systems including liposomes although they are around for almost 20 years and hold great promise for the treatment of severe CNS diseases such as glioblastomas. This lack of innovative initiative is not necessarily shedding a positive light on the so-called research oriented pharmaceutical industry [3].

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