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Perspective

Perspectives on Nanodelivery to the Brain: Prerequisites for Successful Brain Treatment

Yang Hu and Margareta Hammarlund-Udenaes*

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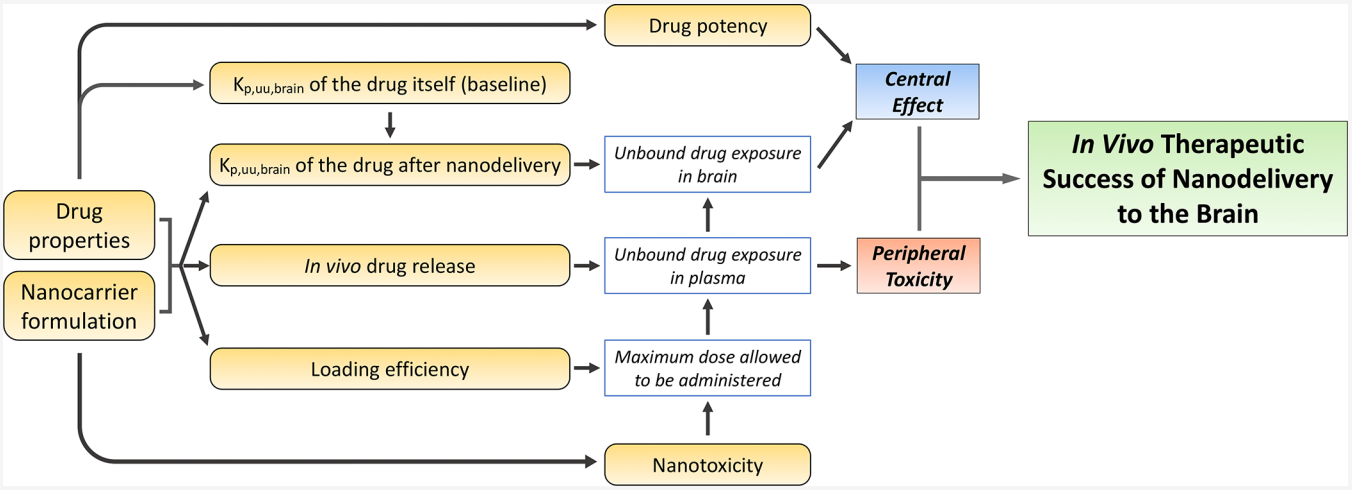

ABSTRACT: Nanocarriers (NCs) are promising tools to improve drug delivery across the blood–brain barrier (BBB) for more effective treatment of brain disorders, although there is a scarcity of clinical translation of brain-directed NCs. In order to drive the development of brain-oriented NCs toward clinical success, it is essential to understand the prerequisites for nanodelivery to be successful in brain treatment. In this Perspective, we present how pharmacokinetic/pharmacodynamic (PK/PD), formulation and nanotoxicity factors impact the therapeutic success of brain-specific nanodelivery. Properties including high loading efficiency, slow *in vivo* drug release, long systemic circulation, an increase in unbound brain-to-plasma concentration/exposure ratio ($K_{p,uu,brain}$), high drug potency, and minimal nanotoxicity are prerequisites that should preferably be combined to maximize the therapeutic potential of a brain-targeted NC. The PK of brain-directed NCs needs to be evaluated in a more therapeutically relevant manner, focusing on the released, unbound drug. It is more crucial to increase the $K_{p,uu,brain}$ than to improve the ability of the NC to cross the BBB in its intact form. Brain-targeted NCs, which are mostly developed for treating brain tumors, including metastases, should aim to enhance drug delivery not just to tumor regions with disrupted BBB, but equally important to regions with intact BBB where the drugs themselves have problems reaching. This article provides critical insights into how a brain-targeted nanoformulation needs to be designed and optimized to achieve therapeutic success in the brain.

KEYWORDS: *nanocarrier, brain delivery, therapeutic success, PK/PD, formulation, nanotoxicity*

INTRODUCTION

Chronic and acute central nervous system (CNS) disorders such as neurodegenerative diseases, neuroinflammation, primary and metastatic brain tumors, ischemic stroke, traumatic brain injury, etc., represent a growing medical problem globally.[1,2] To date, it remains very challenging to achieve effective treatment for these diseases, owing to the presence of the blood–brain barrier (BBB), which efficiently regulates the transport of endogenous and exogenous molecules between blood and brain.[3,4] Due to the tight junctions between the brain capillary endothelial cells and the extensively expressed efflux transporters, the BBB plays a pivotal role in protecting the CNS, preventing blood-derived toxic molecules from reaching the brain.[5,6] However, this protective nature of the BBB also poses an enormous challenge

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to neurotherapeutic agents, limiting their access to the brain targets at effective concentrations.

The limited success in developing BBB-penetrating drugs has promoted the innovation of various strategies to improve brain drug delivery. Among these strategies, nanocarriers (NCs), e.g., liposome, nanoparticle, micelle, nanoemulsion, nanocrystal, and dendrimer, have emerged as promising approaches that have received increasing research attention from both academia and pharmaceutical industry.[7,8] Although many nanoformulations for non-CNS therapies are widely used in clinical practice, clinical development of brain-directed nanoformulations is considerably lagging behind with no clinically approved CNS nanomedicines to date.[2,9] Furthermore, ongoing clinical trials of NCs specifically for CNS indications only account for 4% of the total numbers of trials of NCs (data extracted in August 2020) (Table 1). Together,

Table 1. Numbers of Ongoing Clinical Trials of Therapeutic and Diagnostic Nanomedicines[a]

	nos. of ongoing clinical trials	
type of NC	for all indications	for CNS indications
liposome	498	11
nanoparticle	141	11
dendrimer	1	0
nanocrystal	7	5
nanoemulsion	6	0
micelle	8	0
sum	661	27

[a]All data (not yet recruiting, recruiting, active, and enrolling by invitation cases) were extracted from ClinicalTrials.gov in August 2020.

these facts imply that current nanodelivery approaches may be inefficient in surmounting the BBB to an extent that significantly improves the therapeutic index compared to the drug itself. In order to drive the clinical translation, there is a strong need for a better understanding of nanodelivery to the brain, in particular of what the prerequisites are for nanodelivery to achieve clinical success in brain treatment, and how a nanoformulation should be properly designed and optimized.

Another problem limiting the clinical applicability of brain-directed NCs is the lack of *in vivo* assessments in general. From all of the publications related to NC-mediated brain delivery in PubMed, *in vivo* evaluations were only involved in less than one-third of the articles (Table 2). When it comes to evaluating the performance of an NC *in vivo*, assessing

Table 2. Number of Published Articles (Excluding Reviews) in PubMed Searched with Certain Keywords (Data Extracted in August 2020)

	nos. of publications with or without the additional keyword "*in vivo*"	
searched keywords	without "*in vivo*"	with "*in vivo*"
"liposome" and "brain"	3078	603
"nanoparticle" and "brain"	6115	2014
"dendrimer" and "brain"	275	100
"micelle" and "brain"	644	165
"nanoemulsion" and "brain"	136	57
sum	10 248	2939

pharmacodynamics (PD) (e.g., measuring brain tumor growth) is preferred in most of the studies as an ultimate proof of successful delivery. However, PD measurements are unable to provide any quantitative and direct evidence of how much an NC improves drug delivery to the brain. The absence of a PD effect after NC administration does not necessarily reflect the lack of improvement in brain delivery. Instead, the NC may have increased the delivery, but not to an extent sufficient for a therapeutic concentration to be reached in the brain. Although pharmacokinetics (PK) and biodistribution studies of NCs are sometimes performed together with PD measurements, total drug concentrations in plasma and brain are often measured, which fails to provide any information on released, therapeutically, and toxicologically relevant entities. To date, quantitative assessments on how NCs may affect the released unbound drug remain extremely scarce, which limits the translational potential of brain-specific nanodelivery.[10−14] In fact, without the PK of unbound drug in plasma and brain, it is extremely difficult to evaluate the PK/PD relationships and the therapeutic index of an NC.

In this Perspective, we first briefly recapitulate the nanodelivery systems suitable for CNS drug delivery before systematically discussing the factors contributing to the *in vivo* therapeutic success of nanodelivery to the brain (Figure 1). We also discuss the necessity of performing *in vivo* quantitative studies for NCs, the mechanisms by which NCs interact with the BBB, and whether NCs should aim to improve drug delivery across disease-influenced BBB or healthy BBB. With these aspects discussed, this Perspective aims to provide critical insights on what needs to be considered for clinical success of treating devastating brain diseases and how the properties of a nanoformulation should be optimized in order to better design and develop NC-based brain treatments.

CURRENT STATE-OF-THE-ART FOR BRAIN-DIRECTED NANODELIVERY

Today, no nanoformulations that specifically aim at increasing drug delivery across the BBB are available on the market. However, there are many clinically approved nanomedicines (nontargeted) mainly for treating non-CNS diseases, especially various cancers.[9,15,16] It remains unknown whether or not these nanoformulations are also capable of improving brain delivery compared to the unformulated drug.

NCs that may be clinically useful for brain drug delivery today mainly include liposomes, albumin nanoparticles (NPs), and polymeric NPs. Liposomes have been widely used in clinical practice mainly for non-CNS indications since the first liposomal formulation was approved in 1995 (Doxil). Liposomes feature excellent safety profiles and the ability to encapsulate both hydrophilic and lipophilic therapeutic agents, including both small molecules and large biologics without the need to modify the compounds.[2,17] Currently, there are only a limited number of clinical trials in which liposomal formulations are investigated for treating brain diseases. In the majority of these trials, marketed nontargeted liposomal formulations are used either alone or in combination with other drugs. Several examples include liposomal irinotecan (Onivyde) for brain metastases (ClinicalTrails.gov: NCT03328884), liposomal cytarabine (DepoCyt) together with rituximab and methotrexate for CNS prophylaxis of lymphoma (ClinicalTrails.gov: NCT00945724), and liposomal amphotericin B (AmBisome) for cryptococcal meningitis (ClinicalTrails.gov: NCT03945448). The only brain-targeted

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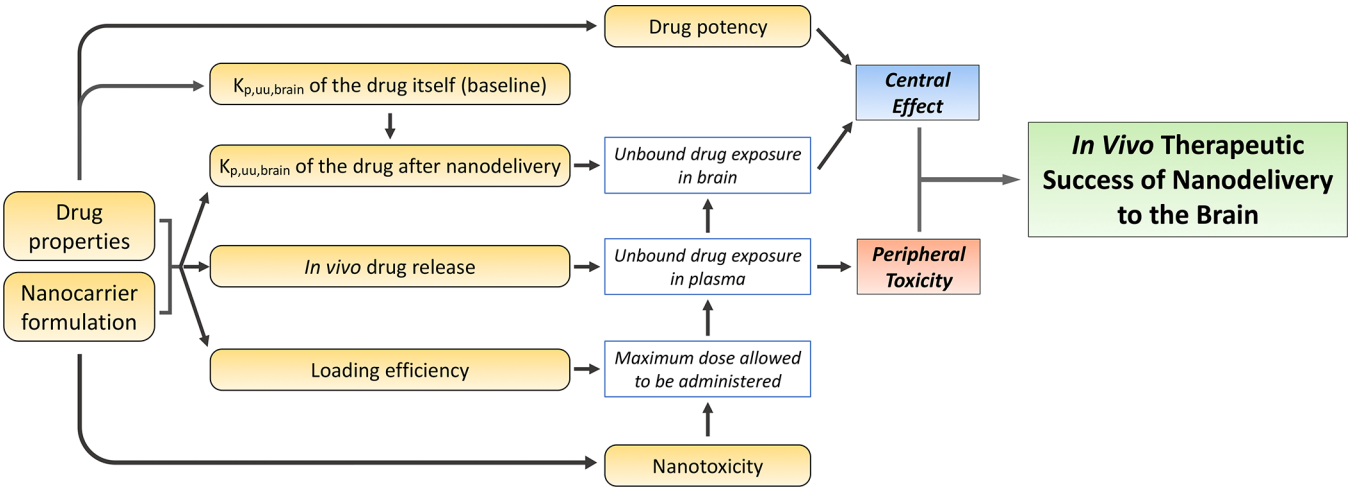

Figure 1. Schematic representation of factors contributing to the *in vivo* therapeutic success of nanodelivery to the brain. The NC formulation in conjunction with drug properties could impact loading efficiency, *in vivo* drug release, and $K_{p,uu,brain}$ of the drug. Whether or not nanotoxicity occurs is dependent on the NC formulation used. Drug-specific properties like $K_{p,uu,brain}$ and potency are important. The $K_{p,uu,brain}$ of the drug itself will determine whether and how much the brain delivery can be improved by nanodelivery. Both loading efficiency and nanotoxicity have an impact on the maximum dose allowed to be administered, which will further influence unbound drug exposure in plasma and brain. *In vivo* drug release will affect unbound drug exposure in plasma. The $K_{p,uu,brain}$ of the drug after nanodelivery will influence how high the unbound brain exposure could be. Unbound brain exposure, together with drug potency, will determine the drug effect in the CNS. Drug-induced peripheral side effects are associated with unbound drug exposure in plasma. It is the central effect and peripheral side effect combined that determine the therapeutic success of nanodelivery to the brain.

liposomal formulation that has been tested in clinical research is glutathione PEGylated liposomal doxorubicin (2B3−101) using glutathione (GSH) as a BBB-targeting ligand. 2B3−101 has completed a Phase I/IIa trial in patients with gliomas or brain metastases (ClinicalTrails.gov: NCT01386580) and is currently being investigated in a Phase II trial for treating breast cancer with leptomeningeal metastases (ClinicalTrails.gov: NCT01818713). In preclinical studies, a variety of BBB-targeting ligands including antibodies, peptides, proteins and small molecules have been investigated in combination with liposomes for improved brain delivery.[17,18] The enhanced pharmacological effects *in vivo* have been often shown as proof of delivery in these studies.

Albumin NPs have also been extensively used in the clinic with Abraxane (nanoparticles albumin-bound paclitaxel) approved in 2005 by the FDA for cancer treatments.[2,19] Currently, a new nanoformulation, nanoparticle albumin-bound rapamycin (ABI-009), is being studied in multiple clinical trials for treating different CNS disorders, including high-grade glioma and glioblastoma, Leigh or Leigh-like syndrome, and surgically refractory epilepsy (ClinicalTrails.gov: NCT03463265, NCT03747328, and NCT03646240). To improve drug delivery across the BBB, albumin nanoparticles have been tested in many preclinical studies, either without a BBB-targeting ligand[20] or with a ligand like transferrin,[21] apolipoprotein (Apo) A-I, B-100, and E,[22,23] cell-penetrating peptide,[24] or antitransferrin/insulin receptor antibodies.[25,26] The improved brain delivery in these studies was shown based on *in vivo* brain distribution, pharmacological evaluation (e.g., antitumor efficacy), or visualization techniques like transmission electron microscopy.

Polymeric NPs are the most studied NCs in preclinical research. However, their clinical translation remains slow with only limited investigations in clinical trials, none of them focusing on brain delivery. The commonly used polymers are biodegradable and biocompatible including poly(butyl cyanoacrylate) PBCA, poly(lactic-*co*-glycolic acid) PLGA, and chitosan.[2] As summarized in several reviews, various moieties like cell-penetrating peptides, Apo E, angiopep-2, transferrin, and antitransferrin receptor antibody have been tested as BBB-targeting ligands conjugated on polymeric NPs, and brain-targeting effects have been shown from *in vivo* studies.[19,27,28] However, nanotoxicity remains a huge issue for polymeric NPs, potentially limiting their clinical translation.[19,29] When applying polymeric NPs for brain delivery, it is worth noting that nanotoxicity may lead to (temporary) BBB opening and potentially even result in neurotoxicity if intact NPs cross the BBB.[27,30]

There are also some other types of NCs involved in clinical studies. For example, gold nanocrystals (CNM-Au8) are currently being evaluated in multiple Phase II trials for the treatment of different CNS disorders such as multiple sclerosis, Parkinson's diseases, and amyotrophic lateral sclerosis (ClinicalTrails.gov: NCT03993171, NCT03815916, NCT03843710, NCT04098406, and NCT03536559). Another novel nanoformulation is bacterially derived nanocells encapsulating doxorubicin with tumor-targeting bispecific antibodies (EGFR(V)-EDV-Dox), which is being investigated in a Phase I trial for glioblastoma multiforme (ClinicalTrails.gov: NCT02766699).

WHAT FACTORS COULD IMPACT THE THERAPEUTIC SUCCESS OF NANODELIVERY TO THE BRAIN

Multiple factors could determine the *in vivo* therapeutic success of NC-mediated brain delivery through their influence on the maximum dose administered, unbound drug exposure in brain or plasma, central effect, and/or peripheral toxicity (Figure 1). These factors can be divided into three categories: PK/PD factors, NC formulation, and nanotoxicity.

PK/PD Factors. *The Unbound Brain-to-Plasma Exposure Ratio ($K_{p,uu,brain}$).* The most therapeutically relevant measure-

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ment of brain exposure is based on unbound drug concentrations. One way of evaluating these concentrations is to estimate the partitioning coefficient of the unbound drug across the BBB ($K_{p,uu,brain}$).[31,32] This parameter describes the ratio of target site exposure associated with a central effect to off-target site exposure (unbound plasma concentrations) related to a peripheral side effect. $K_{p,uu,brain}$ is the most important parameter in CNS drug discovery to evaluate drug candidates for brain action and can be used to estimate the dose needed for central action. Briefly, a $K_{p,uu,brain}$ around unity suggests predominant passive diffusion or similar efflux and influx transport at the BBB. If $K_{p,uu,brain}$ is below unity, active efflux is more efficient than active influx, while a $K_{p,uu,brain}$ higher than unity indicates that active influx dominates the transport at the BBB.[32,33]

$K_{p,uu,brain}$ is also a critical parameter to investigate and optimize when developing NC-based brain treatments.[34] By comparing the $K_{p,uu,brain}$ values of a drug with or without nanoencapsulation, the ability of nanodelivery to influence drug transport across the BBB could be quantitatively evaluated, without being confounded by other *in vivo* processes of the NC. The more the $K_{p,uu,brain}$ can be increased, the more therapeutically effective and less peripherally toxic the nanodelivery would be.

The $K_{p,uu,brain}$ of the drug payload itself plays a key role in the therapeutic success of nanodelivery to the brain. For drugs with active efflux at the BBB ($K_{p,uu,brain} < 1$), NCs could potentially increase their $K_{p,uu,brain}$ if the right formulation is chosen.[10,12] However, depending on how low the $K_{p,uu,brain}$ is for the drug itself, the magnitude of $K_{p,uu,brain}$ increase by nanodelivery required for therapeutic success may be different. For example, for a drug with $K_{p,uu,brain}$ of 0.1, a 10-fold increase in $K_{p,uu,brain}$ by nanodelivery would be adequate to elicit brain effect if the required therapeutic concentration in the brain is similar to the unbound plasma concentration. However, for a drug with $K_{p,uu,brain}$ of 0.01, a 100-fold increase in $K_{p,uu,brain}$ would be required from the NC if the therapeutically relevant concentration is at the same level as the unbound plasma concentration. In general, given similar potency and unbound plasma exposure, drugs with more efficient efflux at the BBB would pose a greater challenge for nanodelivery and require a higher increase in $K_{p,uu,brain}$ to achieve therapeutic success.

For drugs that already show active uptake at the BBB ($K_{p,uu,brain} > 1$), NC encapsulation will very likely not further increase their brain uptake, but rather reduce the $K_{p,uu,brain}$ and, therefore, therapeutic performance. This is exemplified by two recent studies showing that encapsulation in PEGylated liposomes and lipid core nanocapsules significantly decreased the $K_{p,uu,brain}$ of diphenhydramine and quetiapine.[11,35]

Potency. The therapeutic potency and $K_{p,uu,brain}$ of a CNS drug combined determine whether the drug will be pharmacologically effective in the CNS without being toxic in the periphery. Given similar $K_{p,uu,brain}$ values, drugs with higher therapeutic potency can more easily elicit brain effect since the required therapeutic concentration is lower compared to less potent drugs. Some highly potent CNS drugs, like risperidone and paliperidone, can still exert their effect in the brain even if they penetrate the BBB to a limited extent.[36]

From our previous studies, the increase in $K_{p,uu,brain}$ resulting from nanodelivery was found to be maximally 15-fold for methotrexate.[10,12−14] Although 15-fold represents a large improvement, it is not guaranteed that nanodelivery can increase the $K_{p,uu,brain}$ to the same or even larger magnitude for any given drug. Therefore, high therapeutic potency is a prerequisite for successful nanodelivery to the brain, as it will increase the possibility of attaining therapeutic concentrations in the CNS, even if the NC would not drastically improve $K_{p,uu,brain}$. A good example to show how drug potency limits the therapeutic success of nanodelivery is an earlier study on DAMGO, a low potent opioid peptide.[13] Although the $K_{p,uu,brain}$ of DAMGO was doubled from 0.05 to 0.1 when delivered with glutathione PEGylated liposomes, the unbound brain concentration of DAMGO was still below the therapeutic level, although the maximally possible NC dose was administered.

Low toxic potency in the periphery is also a prerequisite for successful nanodelivery to the brain, especially when the NC is not able to substantially increase $K_{p,uu,brain}$. This is because, with lower toxic potency in the periphery, the maximum tolerated drug dose will be higher. As a result, the NC can be given at a higher drug dose to achieve desired therapeutic concentrations in the brain.

Half-life. A favorable feature of NC encapsulation is the possibility of prolonging plasma half-life by, e.g., coating the NC with a hydrophilic molecule like polyethylene glycol (PEG). A longer half-life is achieved by the slow release from the NC, as well as by minimal systemic elimination of the intact NC. After administration of a nanoformulation, the half-life of the released, unbound drug is extended with broader and flatter PK profiles, with a decreased peak concentration (C_{max}) but a similar area under the curve (AUC) compared to the unformulated drug. Given that the central effect of the drug is AUC-driven and the peripheral toxicity is C_{max}-driven, the prolonged half-life by nanoencapsulation was proven to increase the therapeutic index by reducing peripheral side effects.[34] If the PD effect is driven by the unbound drug concentration in the brain, a prolonged drug half-life will allow brain action to last longer compared to the unformulated drug.

The ability of NCs to protect payloads from degradation in plasma and prolong circulation time could be particularly important for biologic payloads like peptides and small interfering RNAs (siRNAs). After systemic administration in free form, these macromolecules often undergo rapid elimination or degradation in blood circulation, exhibiting unfavorable PK profiles with plasma half-lives of just a few minutes, which greatly limits their therapeutic potential in the CNS.[37,38] Formulating these biologics in NCs has been proven to be effective in solving their stability issue *in vivo*. For example, encapsulation in liposomes dramatically increased the half-life of DAMGO (6.9 h vs 9.2 min of free DAMGO).[39] A similar finding was also shown for siRNA when formulated in PEGylated liposomes, with extended half-life compared to unformulated siRNA.[40] In fact, we have previously found that a CNS drug with a shorter half-life in itself will benefit more therapeutically from NC encapsulation.[34] Therefore, for CNS-acting peptides and siRNAs with extremely short circulation times, nanodelivery holds the potential to tremendously improve their therapeutic performance. Another issue is whether nanoencapsulation will also improve uptake across the BBB, which would further improve the gain of the formulation. However, according to our simulations, in this case, a significant improvement is the protection from degradation and prolonged half-life in plasma.[34]

NC Formulation. In the current nanodelivery field, too much attention is paid to designing innovative NC formulations and characterizing their *in vitro* properties like

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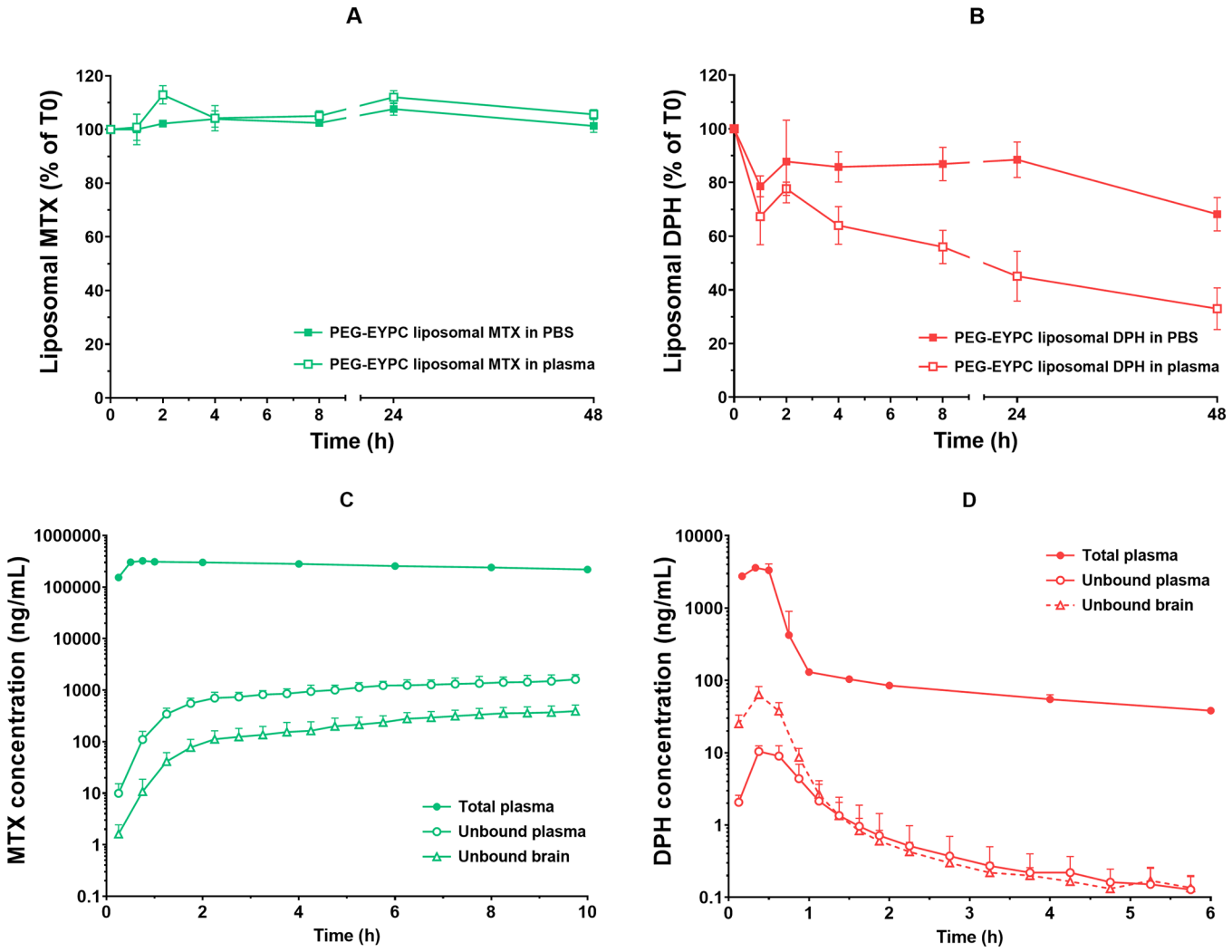

Figure 2. Different *in vitro* and *in vivo* release of PEG-EYPC liposomal formulation encapsulating methotrexate or diphenhydramine. After incubation in phosphate-buffered saline (PBS) and rat plasma at 37 °C up to 48 h, (A) PEG-EYPC liposomal methotrexate had excellent stability *in vitro* with minimal drug release. (B) Instability of PEG-EYPC liposomal diphenhydramine was found with faster drug release in plasma than in PBS. The concentration−time profiles of unbound drug concentration in brain interstitial fluid (open triangles) and plasma (open circles) and total drug concentration in plasma (filled circles) after 30 min intravenous infusion of (C) PEG-EYPC liposomal methotrexate or (D) PEG-EYPC liposomal diphenhydramine. In line with the *in vitro* findings, PEG-EYPC liposomal methotrexate was notably stable in systemic circulation with a long half-life and sustainable drug release, reflected by PK profiles of total and unbound drug in plasma. A very different biphasic PK profile of total diphenhydramine in plasma was observed after PEG-EYPC liposomal diphenhydramine was administered. The fast decline in the early period indicates a fast diphenhydramine release from the liposomes early after administration, which correlates with the *in vitro* results (redrawn with permission from the publishers[11,12]).

size, charge, morphology, *in vitro* release, and cellular uptake, which are, of course, important to evaluate. However, all of these *in vitro* characterizations are of less value if not connected with *in vivo* assessments. In fact, the NC formulation in conjunction with the drug properties could simultaneously impact multiple *in vitro* and *in vivo* properties, including loading efficiency, *in vivo* drug release, and $K_{p,uu,brain}$, which ultimately determines the opportunity of achieving therapeutic success.

The composition of an NC (e.g., containing different phospholipids) and the type of NC (e.g., liposomes vs nanoparticles), together with the drug properties, will determine the drug loading efficiency. For instance, the loading efficiency of methotrexate was lower in PEG liposomes with hydrogenated soy phosphatidylcholine (HSPC) than in egg-yolk phosphatidylcholine (EYPC) counterparts.[12] While liposomes can obtain a loading efficiency of more than 90% when using a remote loading method,[41] polymeric NPs normally allow approximately 10% of the drug to be encapsulated.[19] The loading efficiency of diphenhydramine in PEG-EYPC liposomes is much lower than that of methotrexate in the same formulation.[11,12] An NC formulation with higher loading efficiency would meet the required therapeutic concentration/exposure in the brain more easily, as the maximum drug dose allowed to be given is higher with the same volume administered. As exemplified from the above-mentioned DAMGO case, high loading efficiency of an NC is particularly important when delivering drugs with low potency to the CNS. Improving the loading efficiency solely is, however, inadequate for improving the therapeutic index and has to be combined with additional changes in NC properties (release rate or $K_{p,uu,brain}$) to obtain this goal.

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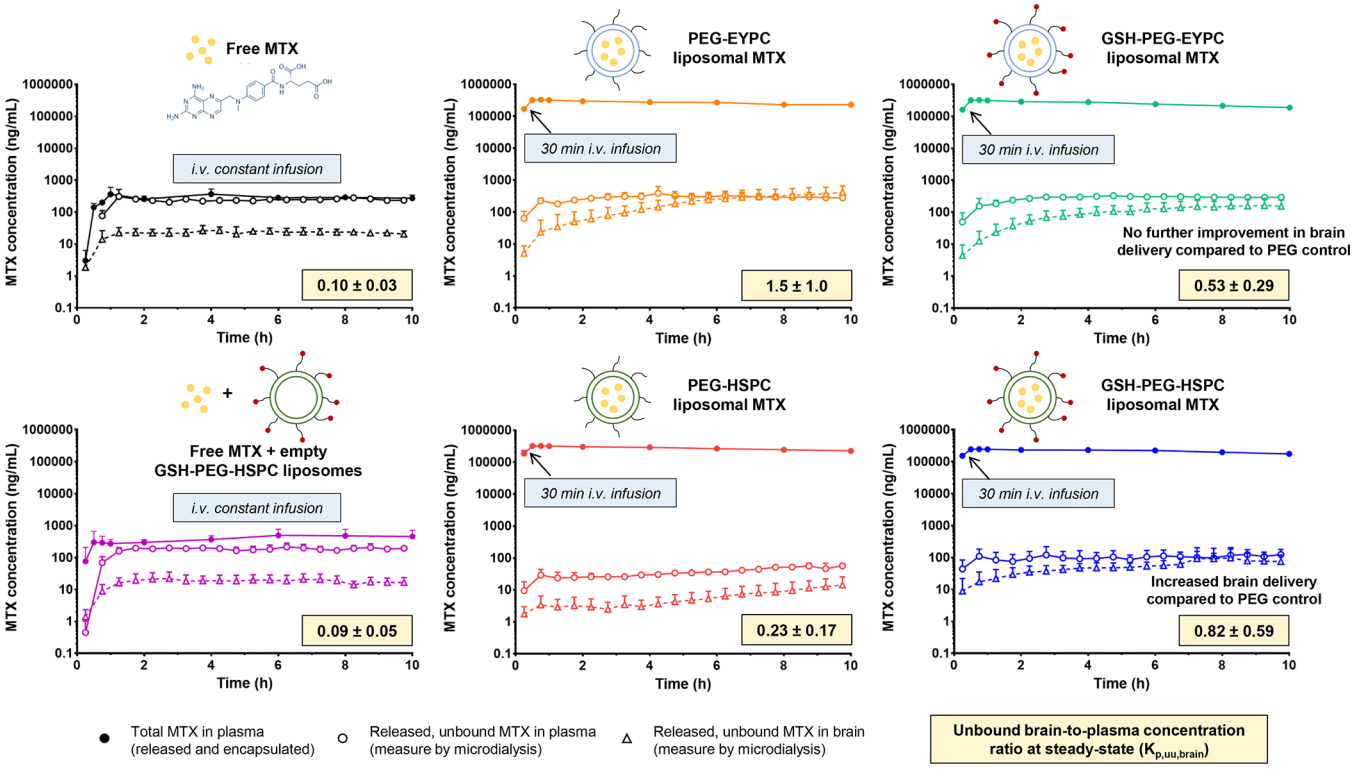

Figure 3. Unbound brain-to-plasma concentration ratios at steady state ($K_{p,uu,brain}$) and observed concentration−time profiles for the unbound drug concentration in brain interstitial fluid (open triangles) and plasma (open circles), and total drug concentration in plasma (filled circles) after intravenous administration of free methotrexate, free methotrexate + empty liposomes, and different liposomal formulations[10] (with permission from the publisher).

The *in vivo* drug release properties will naturally be different depending on the NC formulation as well as the payload drug. To illustrate, methotrexate was released faster from EYPC-based than from HSPC-based liposomal formulations, reflected by significantly higher unbound-to-total plasma concentration ratios of methotrexate from PEG-EYPC compared to PEG-HSPC formulations.[10,12] Furthermore, when encapsulating in PEG-EYPC liposomes, diphenhydramine was released much faster compared to methotrexate based on both *in vitro* and *in vivo* findings (Figure 2).[11,12] In a simulation study, the *in vivo* drug release was found to be strongly associated with therapeutic performance due to its influence on peripheral side effects.[34]

Likely, the most important factor for improving the therapeutic index is how the NC formulation is capable of increasing the $K_{p,uu,brain}$ of a drug, as this will give a distribution advantage and improve the central effect without necessarily influencing peripheral toxicity. As an example of *in vivo* differences between NC formulations, it was found that, while PEG-EYPC liposomes substantially increased the $K_{p,uu,brain}$ of methotrexate, formulations based on HSPC did not affect the $K_{p,uu,brain}$ at all.[12,14] Furthermore, glutathione (GSH), as a BBB-anchoring ligand conjugated to PEG liposomes of methotrexate, showed a brain-targeting effect only when it was combined with the HSPC-based but not the EYPC-based formulation (Figure 3).[12]

Based on our experience, the *in vivo* performance of NC formulations are very difficult to predict from *in vitro* experiments. Therefore, aiming to maximize the therapeutic potential in the brain, the NC formulation should be carefully optimized to possess several favorable features (ideally combined) including high loading efficiency, slow *in vivo* release rate, and large enhancement in $K_{p,uu,brain}$.

As formulation and drug properties combined decide *in vitro* and *in vivo* properties of an NC, it is unrealistic to expect that one nanoformulation would universally be suitable to deliver any given drug to the brain. Depending on the drug to be encapsulated, the NC formulation needs to be specifically designed and optimized. In the current nanodelivery field, a common approach to test and visualize whether an NC can improve brain delivery is *in vivo* fluorescence imaging. This approach involves loading an NC with a fluorescent dye. After administration of a dye-loaded NC, fluorescence intensity is detected in the whole body of a living small animal or brain sections by a sensitive camera.[42,43] However, this method is problematic since improved brain delivery of the dye does not necessarily guarantee a similar improvement of the actual drug that the NC aims to deliver.[44] It was also shown that the brain distribution of different fluorescent dyes varied when delivered with the same NPs.[44] Therefore, it is crucial to view the NC and drug to be delivered as an integrated system, analyzing the actual drug, not a drug surrogate.

Nanotoxicity. *In vivo* safety concerns about nanomaterials like polymers, especially after repeated administration of NCs, remain a key factor limiting NCs' human use.[45,46] The potential toxicities associated with the constituted nanomaterials (so-called nanotoxicity) include acute and/or chronic peripheral immunogenicity, (temporary) BBB disruption, and even neurotoxicity. If any nanotoxicity occurs, there would be dose limits for the nanomaterials. Consequently, the NC may not be administered at the required drug dose to reach therapeutic exposure in the brain.

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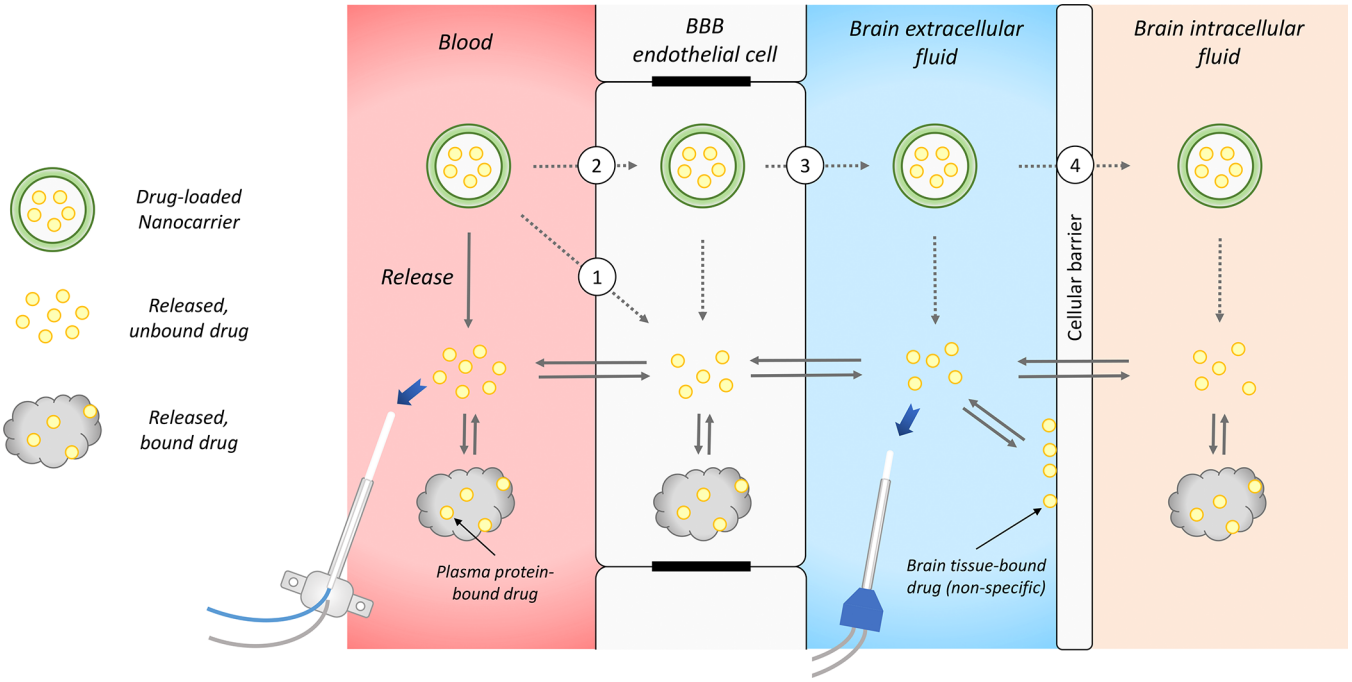

Figure 4. Potential *in vivo* "fate" of brain-directed NCs and the critical role of microdialysis in evaluating the *in vivo* performance of nanodelivery to the brain. After administration of an NC in blood, the drug payload will release from the NC. Once the drug is released, it will behave based on its own properties, being transported across the BBB and cellular barrier and also binding to plasma protein, brain cellular membrane, and intracellular components. NCs may contribute to improved brain drug delivery through several proposed mechanisms: (1) NCs interact and fuse with the BBB endothelial cell membrane and then release the drug to the endothelial cells. (2) NCs are endocytosed into BBB endothelial cells, followed by drug release within the endothelial cells. (3) NCs are transcytosed across the BBB, before releasing the drug in brain extracellular fluid. (4) Transcytosed NCs are further internalized into brain cells, after which the drug is released intracellularly. Microdialysis separates the released, unbound drug from the drug remaining in the NC, enabling continuous quantifying therapeutically and toxicologically relevant drug entities over time, as described by the blue arrows.

To minimize potential nanotoxicity, it is important to choose the proper types of NC and safe nanomaterials. Liposomes have better safety features compared to other types of NCs and are normally nontoxic in both CNS and periphery, as they are composed of biocompatible lipids.[47] From a functional perspective, liposomes do not seem to influence the BBB integrity as coadministering empty liposomes with unformulated drugs did not impact their $K_{p,uu,brain}$ compared to administering unformulated drugs alone.[10,12,14] In order to produce polymeric NPs with acceptable safety profiles, it is essential to use biocompatible and biodegradable polymers like PLGA, although the potential toxicity, particularly long-term toxicity, of polymeric NPs remains elusive. As combining NCs with BBB-targeting ligands may be required to enable CNS-targeted delivery, it is also critical to ensure that these ligands are not immunogenic and will not lead to BBB disruption or neurotoxicity. In this regard, endogenous molecules with known safety and compatibility properties like glutathione and transferrin may be better choices as targeting ligands compared with exogenous moieties like antitransferrin receptor antibodies or synthesized cell-penetrating peptides.

Increasing the loading efficiency and $K_{p,uu,brain}$ of a brain-directed NC may also lower the risk of nanotoxicity. In both cases, the NC can be administered at a lower excipient dose and thereby possibly decrease the risk of toxicity.[2]

HOW TO EVALUATE THE *IN VIVO* PERFORMANCE/SUCCESS OF NANODELIVERY TO THE BRAIN

Currently, there are still methodological issues regarding how to properly evaluate the performance/success of nanodelivery to the brain *in vivo*. PD measurements like nociceptive tests, behavior tests, tumor growth, and survival rate can be used as the ultimate proof of whether brain drug delivery benefits from nanoencapsulation. However, for any brain-targeted NC developed toward clinical application, evaluating PD solely is not optimal and has to be combined with PK assessments in order to accurately describe the PK/PD relationships for both effectiveness and safety.

When it comes to evaluating PK of brain-targeted NCs, most of the studies focus on determining total drug concentrations (encapsulated plus released) in plasma and whole brain tissue.[48] However, this is insufficient if the purpose is to provide information on possible improvements in brain delivery. After the administration of an NC, there are three drug entities in plasma: NC-encapsulated drug, released plasma protein-bound drug, and released drug in the unbound form (Figure 2). If the NC can cross the BBB in intact form, there would be three similar entities in the brain interstitial fluid (ISF) as well (Figure 4). Measuring only the total drug is obviously not able to differentiate the NC-encapsulated drug (normally with very high concentration) from the released, unbound drug being the therapeutically/toxicologically relevant moiety.

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Another limitation associated with analyzing whole brain tissue is that only one terminal brain samples can be taken from one individual. As a result, the time aspects of brain delivery cannot be examined without substantially increasing the use of animals. Furthermore, the contamination of NC-associated drug in the brain tissue either from the residue blood or from NC bound to endothelial cells (if the residual blood is completely removed through perfusion) may confound the quantification of the drug that has actually entered the brain.

Microdialysis is a valuable and probably the best tool for PK evaluation of nanodelivery to the brain, as long as the delivered drug is microdialysable and the study design is proper.[10−14,35,49] The unique feature of a microdialysis probe is that it has a semipermeable membrane, thus allowing only the unbound drug concentrations to be measured continuously. Therefore, microdialysis is able to separate the released, biologically active entity from the encapsulated drug and the released, protein-bound drug as the biologically inactive entities. By combining microdialysis with regular blood sampling, processes like *in vivo* drug release and drug transport across the BBB can be quantitatively and separately assessed over time.

The major limitation of microdialysis is that it cannot be applied to lipophilic drugs, as these drugs tend to stick to microdialysis tubings and probes and therefore compromise the reliability of the measurements.[50] Therefore, when trying to quantitatively evaluate the nanodelivery of lipophilic drugs to the brain, other techniques are needed.

The ultrafiltration method with a stable isotope tracer can be useful in evaluating unbound drug concentrations in plasma after administration of a nanoformulation, irrespective of the lipophilicity of the drug payload.[51,52] However, the usefulness of ultrafiltration in assessing unbound drug levels in whole brain tissue is limited. This is because the required homogenization of brain tissue prior to ultrafiltration may destroy the intact NCs that potentially enter brain parenchyma and release the encapsulated drug, thereby leading to an overestimation of unbound drug concentrations in the brain.

Cerebral open flow microperfusion (cOFM), as a novel *in vivo* technique for continuous sampling of brain ISF, can be useful in measuring brain drug concentrations after administration of nanoformulations. As cOFM allows unfiltered and nondialyzed sampling in brain ISF without certain cutoff, it overcomes the limitations of microdialysis and can be theoretically used to study all substances regardless of their lipophilicity.[53,54] However, since cOFM samples are unfiltered, they include both unbound drug and NC-encapsulated drug, if intact NCs cross the BBB. They need to be further differentiated using, i.e., ultrafiltration, in order to determine drug concentrations in each entity.[55] Therefore, cOFM, if combined with other separation techniques, would provide similar information on unbound drug concentrations in the brain as microdialysis, and would also offer additional mechanistic insights on whether intact NC could cross the BBB by potentially analyzing the separated NC-encapsulated drug entity. Overall, despite the complexity of analytical procedures, cOFM sampling combined with ultrafiltration might potentially be applied to quantitatively and mechanistically evaluate nanodelivery of lipophilic drugs to the brain. However, this combination is not yet tested.

In general, the crucial role of microdialysis in separating the released, unbound drug concentrations from the NC-encapsulated drug over time is irreplaceable, as there are yet no other techniques proven to achieve this goal.

$K_{p,uu,brain}$ INCREASE MORE THERAPEUTICALLY IMPORTANT THAN NC TRANSCYTOSIS

The possible mechanisms by which NCs could improve drug delivery across the BBB have been summarized in several excellent reviews.[27,45,56] The major mechanisms proposed include (Figure 4): (1) NCs interact with the BBB endothelial cell membrane, followed by membrane fluidization with the NC, thereby facilitating drug penetration into the endothelial cells and then the brain; (2) NCs are endocytosed into the endothelial cells, after which the drug is released within the cells and delivered into the brain; (3) NCs are transcytosed in intact form across the endothelial cells, before releasing the drug in brain ISF; (4) NCs are internalized into brain cells and release the drug intracellularly. NCs may influence drug transport across the BBB in a more complex manner than expected, involving multiple above-mentioned mechanisms simultaneously. However, based on current methods/models, it remains very challenging to explore whether the actual mechanism involves any or several of the four proposed ones. Although *in vitro* cellular models may be useful for a mechanistic understanding of how NCs facilitate drug delivery at the BBB, it is difficult to confirm the mechanisms based on *in vivo* models.[2] Fluorescence or electron microscopy may serve as useful tools to analyze *in vivo* samples, visualizing if NCs are within endothelial cells or they have crossed the BBB.[23,57]

It is our opinion that NCs do not necessarily have to cross the BBB in the intact form in order to improve brain delivery and therapeutic effect. This is exemplified by earlier studies where nontargeted PEG liposomes that are considered to be incapable of penetrating the BBB by themselves could drastically increase the brain uptake ($K_{p,uu,brain}$) of methotrexate.[10,12] For a drug that has an intracellular site of action in the brain, an increased $K_{p,uu,brain}$ by nanodelivery can also help elicit higher intracellular concentrations and thereby PD effects. This is because the poor BBB penetration, rather than limited intracellular distribution, is often the major reason for unsuccessful treatment.[36] Once enough drug is delivered into the brain ISF, it will be more likely to exert the PD effect intracellularly, since many drugs have intracellular-to-extracellular concentration ratio values around unity.[36,58]

From a safety perspective, it may even be preferable that an NC could improve the $K_{p,uu,brain}$ of the drug payload without entering the brain in its intact form, as this will reduce the risk of neurotoxicity associated with the nanomaterial. In the current field of nanodelivery to the brain, a biocompatible way of thinking is generally lacking. There are many studies in which nanotherapeutics were directly injected into the brain (mainly tumor) through, i.e., convection-enhanced delivery to circumvent the BBB or were given intravenously combined with BBB opening techniques (e.g., focused ultrasound plus microbubbles), aiming at facilitating NC accumulation in the brain parenchyma.[59−62] However, although increased brain accumulation of intact NC may elevate unbound drug concentration at the site of action, nanomaterial-induced neurotoxicity remains a huge concern, which may ultimately limit the applicability of any nanodelivery involving BBB bypassing or disruption.

Therefore, when developing an NC-based brain delivery system, more focus should be put on investigating how much

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an NC could increase the uptake across the BBB, rather than if the NC could in itself enter the brain.

NCS SHOULD AIM TO IMPROVE DRUG DELIVERY ACROSS NOT JUST THE TUMOR-AFFECTED BBB

Currently, NCs have been mainly developed to deliver oncologic drugs, normally poor BBB-penetrants, to the CNS for the treatment of brain tumors. It is well-known that various pathological conditions, including brain tumors, can disrupt the integrity and function of the BBB.[63−65] However, the BBB disruption in primary tumors like glioblastoma multiforme or brain metastases is heterogeneous depending on tumor region and individual tumor.[66,67] Brain primary and metastatic tumors are highly infiltrative and, therefore, need to be treated as whole brain diseases. Therapeutic levels of chemotherapeutic drugs may be successfully delivered to the tumor core, where the BBB is disrupted.[68,69] However, at the tumor rim as well as in regions where the tumors just start to grow, the BBB mostly remains intact.[70,71] As a result, the treatment at these regions can be ineffective, since anticancer drugs normally have poor penetration across the intact BBB. Ultimately, the failure to effectively deliver oncologic drugs to all regions where brain tumor cells are present will become a major reason for unsuccessful treatment.[66] Thus, when delivering antitumor drugs with NCs for treating brain cancers including metastases, it is equally important to improve drug delivery to the tumor regions with a BBB disruption as well as to the regions with a healthy BBB. Therefore, in preclinical evaluations, it is crucial to show the ability of NCs to enhance the delivery of an anticancer agent also to a healthy brain.

CONCLUSIONS AND OUTLOOK

For a brain-targeted NC, the prerequisites for successful brain treatment while having minimal peripheral toxicity include high loading efficiency, slow *in vivo* drug release, long systemic circulation, a large increase in $K_{p,uu,brain}$, high drug potency, and minimal nanotoxicity. These properties should preferably be combined in one nanoformulation in order to maximize the therapeutic performance in the CNS. The therapeutic potential of a brain-directed NC can be determined by multiple factors including the improvement in $K_{p,uu,brain}$ by nanodelivery, NC-driven modulation of drug half-life, the potency of the drug payload, *in vivo* drug release properties, loading efficiency of the NC, NC formulation (affects all above-mentioned factors), and drug- or nanomaterial-induced toxicity.

From therapeutic and safety perspectives, it is more critical to elevate $K_{p,uu,brain}$ for a brain-targeted NC than to enhance the BBB-crossing of the NC in intact form. This is not only because $K_{p,uu,brain}$ is the parameter directly and quantitatively linked to CNS therapeutic effect versus peripheral toxicity (drug-induced) but also because the intact NC transcytosed into the brain will increase the risk of neurotoxicity. When developing an NC-based treatment for brain tumors, it is crucial to show that the NC is capable of improving drug delivery not just across the tumor-affected BBB but equally important across the healthy BBB to ensure effective treatments of all tumor sites.

It is our opinion that scientists from the nanoformulation, PK/PD, and toxicology fields should work collaboratively, understanding the prerequisites for nanodelivery to the brain and how to properly design and optimize a brain-directed nanoformulation. With all of this combined, we believe that the clinical success of nanomedicine-based CNS treatments will be achieved in the future.

AUTHOR INFORMATION

Corresponding Author

Margareta Hammarlund-Udenaes − *Translational PKPD Research Group, Department of Pharmacy, Faculty of Pharmacy, Uppsala University, SE-751 23 Uppsala, Sweden*; Phone: +46-18-4714300; Email: mhu@farmaci.uu.se; Fax: +46-18-4714300

Author

Yang Hu − *Translational PKPD Research Group, Department of Pharmacy, Faculty of Pharmacy, Uppsala University, SE-751 23 Uppsala, Sweden*; orcid.org/0000-0002-8702-6654

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.molpharmaceut.0c00881

Notes

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