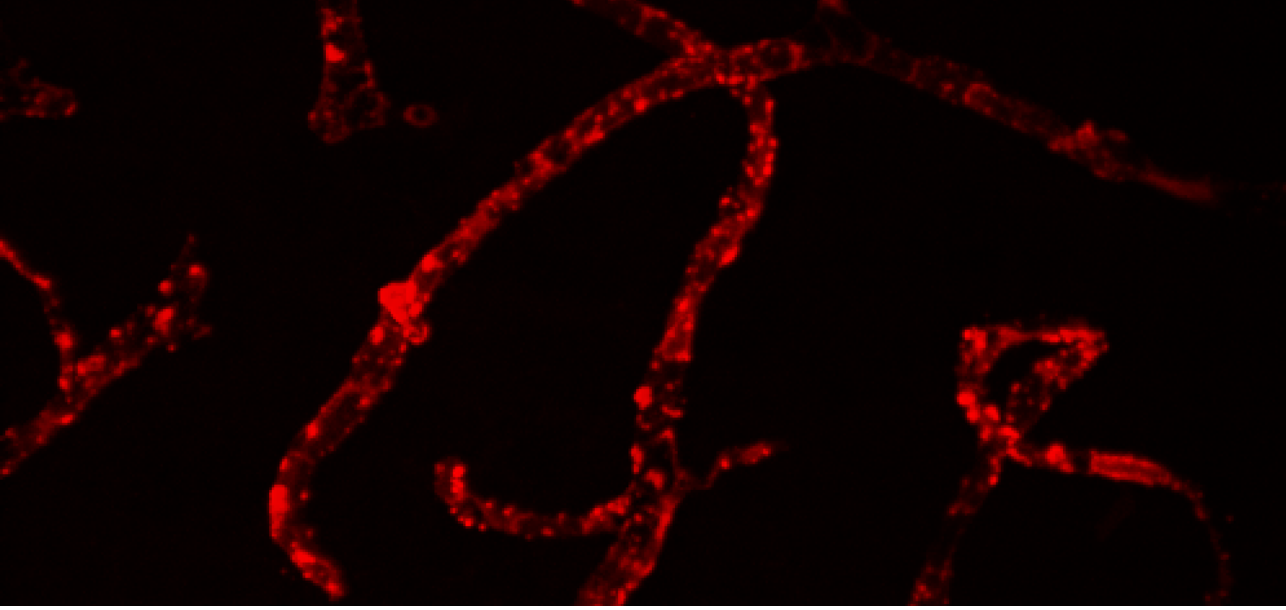
On the use of the transferrin receptor as a target for brain drug delivery

Johnsen, Kasper Bendix

DOI (link to publication from Publisher):
10.5278/vbn.phd.med.00108

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record



ON THE USE OF THE TRANSFERRIN RECEPTOR AS A TARGET FOR BRAIN DRUG DELIVERY

BY
KASPER BENDIX JOHNSEN

DISSERTATION SUBMITTED 2017

AALBORG UNIVERSITY
DENMARK

ON THE USE OF THE TRANSFERRIN RECEPTOR AS A TARGET FOR BRAIN DRUG DELIVERY

PHD DISSERTATION

by

Kasper Bendix Johnsen

AALBORG UNIVERSITY
DENMARK

Dissertation submitted 14th December 2017

Dissertation submitted: 14th December 2017

PhD supervisor: Prof. Torben Moos
Aalborg University

Assistant PhD supervisor: Prof. Thomas Lars Andresen
Technical University of Denmark

PhD committee: Assoc. Prof. Cristian Pablo Pennisi (chairman)
Aalborg University

Ph.d. Per-Ola Freskgård
F. Hoffmann-La Roche Ltd.

Professor Jørgen Kjems
Aarhus University

PhD Series: Faculty of Medicine, Aalborg University

Department: Department of Health Science and Technology

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-111-8

Published by:
Aalborg University Press
Skjernvej 4A, 2nd floor
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2018

CURRICULUM VITAE

I started my time at Aalborg University in 2009 as a student in Medicine with Industrial Specialization. My interest in brain diseases and drug delivery was founded already in 2010, where me and my group mates did our first student project regarding the inflammatory process in Parkinson's disease. Based on this project, I was invited by Professor Torben Moos to become a spare-time researcher in the Laboratory of Neurobiology working on immune cell entry and iron handling in Parkinson's disease.

Besides this spare-time research, me and my study group's interests moved in the direction of brain cancer and microRNA dysregulation. This interest was originally sparked by a student project in early 2011, where the conditioned medium of adipose-derived stem cells was found to inhibit the growth on glioblastoma multiforme cells in culture – a result and presentation of it, which was awarded the Best Poster Presentation Prize at the 22nd European Students' Conference in Berlin in 2011. Based on this award, me and my study group could apply for external funding and subsequently perform a very well-funded Bachelor's project regarding the microRNA content in adipose-derived stem cell-conditioned medium, and its effect on brain cancer cell growth. This work also included the writing and publication of a now well-cited, systematic review on microRNA dysregulation in glioblastoma multiforme.

For my Master's degree, written under the supervision of Associate Professor Meg Duroux and then PhD student Michael Henriksen in the Laboratory of Cancer Biology, I chose to work on yet another derivation from the project on conditioned medium, namely the content of exosomes and other extracellular vesicles, and how these endogenous nanoparticles could be used as drug delivery vehicles to transport microRNA-regulating medicines to brain tumors. While this work did not end in a new curative treatment for glioblastoma multiforme, it resulted in a huge amount of knowledge regarding nanoparticle characterization and drug delivery in general. In addition, it led to contributions in four published papers (two as first author) regarding either the dysregulation of microRNAs in glioblastoma multiforme patient, or issue pertaining to the use of exosomes as drug delivery vehicles.

Meanwhile, my work with Professor Torben Moos had progressed to include the use of liposomes for brain drug delivery, especially regarding how these (non-targeted) liposomes could accumulate in specific brain regions after experimental induction of Parkinson's disease. With the competencies acquired from my work in these two research groups, it was an easy choice for me to figure out my interests for a future PhD project: *Brain drug delivery*.

EDUCATION

2015-2015	Personal license in *Laboratory Animal Science (FELASA C, EU Function ABD)* 11.7 ECTS at the Department of Experimental Medicine, University of Copenhagen
2012-2014	M.Sc. in Biomedicine from Aalborg University
2013-2014	*Project management* (5 ECTS) at Aalborg University
2012-2012	*Introduction to Using C. elegans as Model Organism in Biomedical Research* (5 ECTS) at the Department of Molecular Biology at Aarhus University
2009-2012	B.Sc. in Medicine with Industrial Specialization from Aalborg University

LIST OF PUBLICATIONS

Johnsen KB[§], Gudbergsson JM, Duroux M, Moos T, Andresen TL, Simonsen JB. On the use of liposome controls in studies investigating the clinical potential of extracellular vesicle-based drug delivery systems - A Commentary. *J Control Release. 2017 Nov. In press.*

Johnsen KB[§], Burkhart A, Andresen TL, Moos T, Thomsen LB. The use of the transferrin receptor as a target for brain drug delivery using nanomedicines. *In: Gaillard PJ, Morales J, editors. Nanomedicines for Brain Drug Delivery – Springer Nature Neuromethods Series. Springer Nature; 2017. In press.*

Johnsen KB, Burkhart A, Melander F, Kempen PJ, Vejlebo JB, Siupka P, et al. Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma. *Sci Rep. 2017 Sep 4;7(1):437.*

Siupka P, Hersom MN, Lykke-Hartmann K, **Johnsen KB**, Thomsen LB, Andresen TL, et al. Bidirectional apical-basal traffic of the cation-independent mannose-6-phosphate receptor in brain endothelial cells. *J Cereb Blood Flow Metab. 2017 Jul;37(7):2598–613.*

Andersen HH, **Johnsen KB**, Arendt-Nielsen L. On the prospect of clinical utilization of microRNAs as biomarkers or treatment of chronic pain. *Exp Neurol. 2016 Oct;284(Pt A):63–6.*

Humle N, **Johnsen KB**, Arendt GA, Nielsen RP, Moos T, Thomsen LB. Targeted Vascular Drug Delivery in Cerebral Cancer. *Curr Pharm Des. 2016;22(35):5487–504.*

Johnsen KB, Gudbergsson JM, Skov MN, Christiansen G, Gurevich L, Moos T, et al. Evaluation of electroporation-induced adverse effects on adipose-derived stem cell exosomes. *Cytotechnology. 2016 Oct;68(5):2125–38.*

Gudbergsson JM**, Johnsen KB**, Skov MN, Duroux M. Systematic review of factors influencing extracellular vesicle yield from cell cultures. *Cytotechnology. 2016 Aug;68(4):579–92.*

Johnsen KB, Moos T. Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release. 2016 Jan 28;222:32–46.*

Burkhart A, Skjørringe T, **Johnsen KB**, Siupka P, Thomsen LB, Nielsen MS, et al. Expression of Iron-Related Proteins at the Neurovascular Unit Supports Reduction and Reoxidation of Iron for Transport Through the Blood-Brain Barrier. *Mol Neurobiol. 2016 Dec;53(10):7237–53.*

Skjørringe T, Burkhart A, **Johnsen KB**, Moos T. Divalent metal transporter 1 (DMT1) in the brain: implications for a role in iron transport at the blood-brain barrier, and neuronal and glial pathology. *Front Mol Neurosci*. 2015;8:19.

Henriksen M, **Johnsen KB**, Andersen HH, Pilgaard L, Duroux M. MicroRNA expression signatures determine prognosis and survival in glioblastoma multiforme--a systematic overview. *Mol Neurobiol. 2014 Dec;50(3):896–913.*

Johnsen KB, Gudbergsson JM, Skov MN, Pilgaard L, Moos T, Duroux M. A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. *Biochim Biophys Acta. 2014 Aug;1846(1):75–87.*

Henriksen M, **Johnsen KB**, Olesen P, Pilgaard L, Duroux M. MicroRNA expression signatures and their correlation with clinicopathological features in glioblastoma multiforme. *Neuromolecular Med. 2014 Sep;16(3):565–77.*

Andersen HH*, **Johnsen KB***, Moos T. Iron deposits in the chronically inflamed central nervous system and contributes to neurodegeneration. *Cell Mol Life Sci. 2014 May;71(9):1607–22.*

Møller HG*, Rasmussen AP*, Andersen HH*, **Johnsen KB***, Henriksen M, Duroux M. A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. *Mol Neurobiol. 2013 Feb;47(1):131–44.*

* = Equal contribution

§ = Corresponding authorship

CONFERENCE ACTIVITY

Siupka P, Tóth A, Christensen SC, Bruun E, **Johnsen KB,** Hersom MNS, Abbott NJ, Moos T, Brodin B, Nielsen MS. Recycling and retrograde transported receptor trafficking in brain endothelial cells. 12th International Conference on Cerebral Vascular Biology, Nov 28 – Dec 1 2017, Melbourne, AUS.

Johnsen KB, Andresen TL & Moos T. The impact of transferrin receptor targeting on immunoliposomal cargo delivery across the blood-brain barrier. Blood Brain Barrier, 7-10 December 2016, Cold Spring Harbor Laboratory, NY, USA.

Moos T, **Johnsen KB**, Burkhart AB, Bruun J, Siupka P, Nielsen MS & Andresen TL. Targeting immunoliposomes to transferrin receptors on brain capillary endothelial cells as a mean for cargo transport across the blood-brain barrier. 46th Annual Meeting of the Society for Neuroscience, Neuroscience 2016, 12-16 November 2016, San Diego, CA, USA. Society for Neuroscience, 2016.

Johnsen KB, Burkhart AB, Melander F, Bruun J, Siupka P, Nielsen MS, Andresen TL & Moos T. Targeting immunoliposomes to transferrin receptors on brain capillary endothelial cells as a mean for cargo transport across the blood-brain barrier. 19th International Symposium on Signal Transduction at the Blood-Brain Barriers. 2016. Copenhagen.

Johnsen KB, Elbæk KJ, Gudbergsson JM, Skov MN, Gurevich L, Jørgensen M, Pilgaard L, Stensballe A, Duroux M. The potential of adipose-derived stem cell exosomes as vehicles of drug delivery. ISEV 2014. Rotterdam

Elbæk KJ, **Johnsen KB**, Skov MN, Gudbergsson JM, Gurevich L, Jørgensen M, Stensballe A, Duroux M. Characterization of protein expression on glioblastoma multiforme exosomes and evaluation of methods for their isolation. ISEV 2014. Rotterdam

Henriksen M, **Johnsen KB**, Olesen P, Pilgaard L, Duroux M. Glioblastoma miRNA signatures linked to progression-free survival. MicroRNAs Europe 2013 Meeting. Cambridge; 2013.

Johnsen KB, Andersen HH, Møller HG, Henriksen M, Pilgaard L, Duroux M. MicroRNAs in Glioblastoma Multiforme Tumors: Micro-modulators of the Mesenchymal Mode of Migration and Invasion. MicroRNAs Europe 2012 Meeting. Cambridge; 2012.

Andersen HH, **Johnsen KB**, Moos T. Migration of macrophages into the brain during neuroinflammation as a mechanism for iron accumulation in the Parkinson's disease brain. Neuroscience 2012. New Orleans; 2012.

Møller HG, **Johnsen KB**, Andersen HH, Rasmussen AP, Duroux M. Human Adipose-Derived Stem Cell Secretome Inhibits Glioblastoma Cell Migration - The Potential of microRNA Modulation? International Workshop on Small RNA in Cancer, Inflammation and Aging 2012. Copenhagen; 2012.

Johnsen KB, Møller HG, Rasmussen AP, Andersen HH, Kaa AC, Duroux M. Human adipose-derived stem cell conditioned medium causes growth arrest in an aggressive U87 malignant glioma cell culture. 22nd European Students' Conference Berlin. Berlin; 2011.

AWARDS AND FUNDING ACHIEVEMENTS

2016	Grant from Kong Christian den Tiendes Fond of 25.000 DKK
2015	Recipient of the Roblon Prize of 100.000 DKK for an innovative Master project
2014	Grant from Fonden til Lægevidenskabens Fremme of 40.000 DKK
2013	Recipient of a Novo Scholarship of 70.000 DKK
2012	First prize for Best Poster Presentation at the MicroRNAs Europe 2012 Meeting in Cambridge, UK.
2011	Co-applicant in three grant-applications with a total of 150.000 DKK from Harboefonden, Det Obelske Familiefond, and Spar Nord Fonden. The grants funded my bachelor project (Spring 2012).
2011	First prize for Best Poster Presentation at the 22nd European Students' Conference in Berlin

ENGLISH SUMMARY

Efficient drug delivery to the brain remains the largest obstacle for treatment of diseases related to the central nervous system. This obstacle is imposed by the presence of the blood-brain barrier (BBB), which constitutes the endothelial lining of the brain capillaries. The brain capillary endothelial cells of the BBB are characterized by very tight interconnections and low passive permeability, and therefore, they effectively exclude most molecules carried in the systemic circulation from entering the sensitive brain parenchyma. This also means that transport of medicines into the brain is severely impaired. One strategy to overcome the issue of the BBB has been to target the medicines as drug constructs or nanomedicines to different nutrient receptor proteins expressed on the surface of the BBB. This would in theory drag the medicines into the brain parenchyma as a blind passenger together with the nutrient molecule. One popular receptor system utilized for brain drug delivery is the transferrin receptor, which is normally responsible for transporting iron atoms into the brain. However, despite vast amounts of preclinical progress in the past three decades on the use of transferrin receptors for brain drug delivery, there is still a lack of clinical translation.

In this dissertation, transferrin receptor-mediated brain drug delivery via nanomedicines was studied to obtain knowledge about specific design aspects that could improve the current standard of transport across the BBB. Specifically, we studied the impact of ligand affinity, avidity, and valency on the subsequent uptake of gold nanoparticles and liposomes. Furthermore, we studied the fate of nanoparticles administrated directly into the brain compartment via intracerebroventricular injection. We find that between the different aspects studied, the impact imposed by decreasing the valency of a TfR-targeting antibody was superior with respect to increasing the brain parenchymal exposure of intravenously administrated nanoparticles. This amounted to a more than five-fold increase compared to current standards. We also find that a net negative surface charge is favorable for deep penetration of nanoparticles into the brain cortex after intracerebroventricular administration. In conclusion, the results presented in this dissertation provide important information about how aspects known to impact antibody-based medicines for the brain also may impact the transport of nanomedicines into the brain.

DANSK RESUME

Effektiv lægemiddeltilførsel til hjernen er fortsat den største hindring for behandling af sygdomme i centralnervesystemet. Denne hindring opstår ved tilstedeværelsen af blod-hjerne-barrieren, som udgøres af endotelcellelaget i hjernens kapillærer. Endotelcellerne i hjernens kapillærer er karakteriseret ved meget tætte intercellulære forankringer og lav passiv permeabilitet, og derved udelukker de effektivt de fleste molekyler, der findes i den blodsystemet fra at komme ind i det følsomme hjernevæv. Dette betyder også, at transport af lægemidler til hjernen i de fleste tilfælde er forhindret. En strategi til at passere blod-hjerne-barrieren har været at målrette lægemidlet som lægemiddelkonstrukter eller nanomedicin til forskellige receptorer udtrykt på overfladen af blod-hjerne-barrieren, der normalt er ansvarlig for transport af næringsstoffer ind i hjernen. Dette vil i teorien trække lægemidlet ind i hjernevævet som en blind passager sammen med næringsstofmolekylet. Et populært receptorsystem til dette formål er transferrinreceptoren, som normalt er ansvarlig for transport af jernatomer ind i hjernen. Men på trods af store mængder prækliniske fremskridt i seneste sidste tre årtier vedrørende anvendelsen af transferrinreceptorer til medicinlevering til hjernen er der dog stadig mangel på klinisk fremskridt.

I denne afhandling undersøges transferrinreceptormedieret, nanomedicinbaseret lægemiddellevering til hjernen for at opnå viden om specifikke designaspekter, der kan forbedre det nuværende niveau af transport over blod-hjerne-barrieren. Specifikt studerede vi virkningen af ligandaffinitet, aviditet og valens på den efterfølgende optagelse af guldnanopartikler og liposomer. Desuden studerede vi nanopartiklers mobilitet efter direkte administration ind i centralnervesystemet via intracerebroventrikulær injektion. Vi finder, at mellem de forskellige designaspekter, der blev studeret, var virkningen ved at nedsætte valensen af et transferrinreceptorantistof overlegen med hensyn til forøgelse af hjernens eksponering af intravenøst administrerede nanopartikler. Dette resulterede i en mere end femfolds stigning i forhold til andre undersøgte formuleringer. Vi finder også, at en negativ overfladeladning er gunstig for penetration af nanopartikler ind i hjernebarken efter intracerebroventrikulær administration. Afslutningsvis giver resultaterne i denne afhandling vigtige oplysninger om, hvordan designaspekter, der på nuværende tidspunkt vides at påvirke antistofbaserede lægemidler til hjernen, også kan påvirke transporten af nanomedicin ind i hjernen.

ACKNOWLEDGEMENTS

The execution of a PhD project is by no means a one-man achievement. Through the past three years, I have met and collaborated with many amazing people, who one way or another has made their contributions to this work:

First, I would like to give my most heartfelt recognition to Professor Torben Moos. Torben has always been a knowledgeable, caring, and trustful supervisor, who has allowed me to follow my own leads and interests to reach the most significant results. I would also like to thank him for introducing me to the field of brain research many years ago, where I was only a young (and naïve) student. Lastly, I am grateful that Torben gave me the opportunity to become a PhD student with him, even though I had no plans of staying in Aalborg. It takes a special man to supervise a PhD student 400 kilometers away!

Second, I would like to thank Professor Thomas Lars Andresen from the Technical University of Denmark. Thomas has welcomed me into his group to have as a primary working place in the Copenhagen area. I am still amazed of how lucky I have been to be introduced to the world class expertise in nanoparticle design and synthesis that Thomas and his employees have, and to be allowed to harvest from this knowledge bank to improve my own work in the field. Lastly, Thomas' spirit and opinions on specific matters have taught me invaluable lessons about how to upscale experiment and have focus on any translational aspects that the work may include.

Third, I owe great acknowledgements to my colleagues at the Laboratory of Neurobiology at Aalborg University. Especially Annette Burkhart and Maj Schneider Thomsen, who have always taken their time and put their own things aside in the short periods, where I have chosen to come to Aalborg and do experiments. Whether it have been isolation of primary brain endothelial cells or daylong sit-ins for large scale *in vivo* experiments, these Annette and Maj have always been there for me! Great thanks to our two laboratory technicians, Hanne Krone Nielsen and Merete Fredsgaard, who have been irreplaceable in my work by doing things that were impossible for me in the short periods of work time in Aalborg. Especially Hanne, from whom I have demanded almost gram-scale production of different antibodies over the years. Also, thank you to Johann Mar Gudbergsson from the Laboratory of Cancer Biology for joyful side projects related to brain cancer and extracellular vesicles, and for being a great colleague for discussing science in general.

Fourth, I wish to thank my colleagues at the Colloids and Biological Interfaces group at the Technical University of Denmark for three years with a very steep learning curve in relation to understanding nanoparticles, their design and production. Very special thanks go to: Jonas Bruun Vejlebo, who became my mentor in the group, and taught me the basic principle of liposome production and functionalization with

antibodies. Fredrik Melander, who introduced me to ICP-MS and supervised my use of the technique. Martin Bak, who became a close collaborator to me in projects regarding gold nanoparticles. Paul Joseph Kempen, who introduced me to the value of including electron microscopy in my projects, and has provided me with so many images of nanoparticles in brain capillaries (often) or inside the brain (seldom). Furthermore, thank you to Associate Professors Alireza Dolatshahi-Pirouz, Andrew Urquhart, and Jens Bæk Simonsen for hour-long office discussions on science in general.

Fifth, I am very grateful for the contributions and help, I received from the group of Morten Schallburg Nielsen at Aarhus University. Morten has allowed me to come to Aarhus to use his equipment and the expertise of him and his group. Great thanks to Piotr Siupka and Sarah Christine Christensen, who have helped me with spinning disk microscopy and biochemical techniques, which have improved several projects. Also, thanks to laboratory technician, Anne Marie Bundsgaard, for many runs of surface plasmon resonance, and for introducing me to the technique and the Biacore system.

Sixth, I would like to thank all the members of the Lundbeck Foundation Research Initiative on Brain Barriers and Drug Delivery network and our international scientific advisory board (especially N. Joan Abbott and Robert G. Thorne) for great scientific input to my projects and experiments several times each year. It has been a great privilege to have my work frequently evaluated and discussed by such skilled researchers, and to have established great friendships and collaborations through our biannual meetings and related social activities.

Still, while professional relations can get you a long way, *nothing* of this would have been possible without my dear family:

Thank you, mom and dad! You have given me a warm and welcoming home in the periods, when I worked in Aalborg away from my own family. You have allowed and facilitated my egoistic focus on work in these periods by taking care of any daily life-related tasks, and I am deeply grateful for this. I am constantly fascinated by your surplus of energy to give to people around you, which I have benefited from so much in the past three years.

Thank you, Heidi, my beautiful wife! The past three years has been the most eventful of my life. We have moved away from our families to go build our respective careers in Copenhagen. We have bought a house, sold it again, and bought a new one. We have a son now and await one more. I am so grateful for having you in my life, and because you have facilitated my wish of doing my research in several cities in Denmark. I am impressed that you so many times have allowed me to leave you and Alfred behind in Copenhagen to go to Aalborg and work. The fact that you have been able to handle both your own demanding job and all tasks related to Alfred in these periods is so fantastic, and I feel blessed to have such a strong wife that handles this

without any regrets. You remain a guiding light in my professional and personal life. However, I still will not let you read my manuscripts in preparation, because you will laugh loudly of the sample sizes!

TABLE OF CONTENTS

PREFACE

The work presented in this dissertation was carried out between 2014 and 2017 as a part of a PhD programme under the Faculty of Medicine at Aalborg University. The dissertation contains different manuscripts focusing on the use of the transferrin receptor as a target for brain drug delivery. Different aspects pertaining to the use of the transferrin receptor was studied, including the possible impact of ligand valency and affinity on the transport of nanoparticles into the brain. Furthermore, differences with respect to the transport of nanoparticles versus encapsulated cargo into the brain was studied.

The dissertation is structured such that the classical Introduction section is omitted in favor of the inclusion of two published review manuscripts (*Manuscript I – II*, one journal paper and one book chapter). The two following manuscripts will thus serve as Introduction (*Chapter 1*) for the experimental chapters presented later:

- *Manuscript I:* Johnsen KB, Moos T. Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release. 2016 Jan 28;222:32–46.*

- *Manuscript II:* Johnsen KB, Burkhart A, Andresen TL, Moos T, Thomsen LB. The use of the transferrin receptor as a target for brain drug delivery using nanomedicines. *In: Gaillard PJ, Morales J, editors. Nanomedicines for Brain Drug Delivery – Springer Nature Neuromethods Series. Springer Nature; 2017. In press.*

In addition to these introductory manuscripts, another chapter has been added to the dissertation, wherein the principles behind the main methodology used in the experimental manuscripts are described, as well as our considerations for the use of these specific methods (*Chapter 2*).

The experimental part of the dissertation is divided into four manuscripts regarding the use of the transferrin receptor as a target for brain drug delivery (*Manuscript III – V*), and the distribution of nanoparticles in the brain compartment after direct administration (*Manuscript VI*). These manuscripts (published or in preparation) are included in this dissertation as *Chapter 4*:

- *Manuscript III:* Johnsen KB, Burkhart A, Melander F, Kempen PJ, Vejlebo JB, Siupka P, et al. Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma. *Sci Rep. 2017 Sep 4;7(1):437.*

- *Manuscript IV:* Johnsen KB, Bak M, Kempen PJ, Melander F, Burkhart A, Thomsen MS, Nielsen MS, Andresen TL, Moos T. Antibody affinity and valency impact the brain uptake of transferrin receptor-targeted gold nanoparticles. *Submitted to Science Advances*.

- *Manuscript V:* Johnsen KB, Bak M, Kempen PJ, Melander F, Thomsen MS, Burkhart A, Andresen TL, Moos T. Modulating the ligand density changes the brain uptake of transferrin receptor-targeted gold nanoparticles and liposomal cargo. *In preparation*.

- *Manuscript VI:* Johnsen KB, Vejlebo JB, Andresen TL, Moos T. The surface charge depicts the brain distribution of liposomes after intracerebroventricular administration – a morphological study. *In preparation*.

The dissertation ends with a joint discussion on the features of brain drug delivery (and transferrin receptor targeting) covered in the six manuscripts, but with a focus on the on the broad aspects that were observed across multiple manuscripts (*Chapter 5*). This discussion also describes the perspectives of the studies that will be relevant to investigate in future work.

I hope that the work will be of great interest for the reader, and that the reader will enjoy the contents of the dissertation.

Kasper Bendix Johnsen, December 2017

LIST OF MANUSCRIPTS

Manuscript I: **Johnsen KB**, Moos T. Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release. 2016 Jan 28;222:32–46.*

Manuscript II: **Johnsen KB**, Burkhart A, Andresen TL, Moos T, Thomsen LB. The use of the transferrin receptor as a target for brain drug delivery using nanomedicines. *In: Gaillard PJ, Morales J, editors. Nanomedicines for Brain Drug Delivery – Springer Nature Neuromethods Series. Springer Nature; 2017. In press.*

Manuscript III: **Johnsen KB**, Burkhart A, Melander F, Kempen PJ, Vejlebo JB, Siupka P, et al. Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma. *Sci Rep. 2017 Sep 4;7(1):437.*

Manuscript IV: **Johnsen KB**, Bak M, Kempen PJ, Melander F, Burkhart A, Thomsen MS, Nielsen MS, Andresen TL, Moos T. Antibody affinity and valency impact the brain uptake of transferrin receptor-targeted gold nanoparticles. *Submitted to Science Advances*.

Manuscript V: **Johnsen KB**, Bak M, Kempen PJ, Melander F, Thomsen MS, Burkhart A, Andresen TL, Moos T. Modulating the ligand density changes the brain uptake of transferrin receptor-targeted gold nanoparticles and liposomal cargo. *In preparation*.

Manuscript VI: **Johnsen KB**, Vejlebo JB, Andresen TL, Moos T. The surface charge depicts the brain distribution of liposomes after intracerebroventricular administration – a morphological study. *In preparation*.

CHAPTER 1. INTRODUCTION

1.1. MANUSCRIPT I

Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the gate improves the fate of transferrin receptor-targeted liposomes

Kasper Bendix Johnsen & Torben Moos*

Laboratory for Neurobiology, Biomedicine, Institute of Health Science and Technology, Aalborg University, Aalborg, Denmark.

Manuscript published in Journal of Controlled Release

Abstract

An unmet need exists for therapeutic compounds to traverse the brain capillary endothelial cells that denote the blood-brain barrier (BBB) to deliver effective treatment to the diseased brain. The use of nanoparticle technology for targeted delivery to the brain implies that targeted liposomes encapsulating a drug of interest will undergo receptor-mediated uptake and transport through the BBB with a subsequent unfolding of the liposomal content inside the brain, hence revealing drug release to adjacent drug-demanding neurons. As transferrin receptors (TfRs) are present on brain capillary endothelial, but not on endothelial cells elsewhere in the body, the use of TfR-targeted liposomes - colloidal particulates with a phospholipid bilayer membrane - remains the most relevant strategy to obtain efficient drug delivery to the brain. However, many studies have failed to provide sufficient quantitative data to proof passage of the BBB and significant appearance of drugs inside the brain parenchyma. Here, we critically evaluate the current evidence on the use of TfR-targeted liposomes for brain drug delivery based on a thorough investigation of all available studies within this research field. We focus on issues with respect to experimental design and data analysis that may provide an explanation to conflicting reports, and we discuss possible explanations for the current lack of sufficient transcytosis across the BBB for implementation in the design of TfR-targeted liposomes. We finally provide a list of suggestions for strategies to obtain substantial uptake and transport of drug carriers at the BBB with a concomitant transport of therapeutics into the brain.

1.2. MANUSCRIPT II

The use of the transferrin receptor as a target for brain drug delivery using nanomedicines

Kasper Bendix Johnsen[1,2]*, Annette Burkhart[1], Thomas Lars Andresen[2], Torben Moos[1] & Louiza Bohn Thomsen[1]

[1]Laboratory for Neurobiology, Biomedicine, Institute of Health Science and Technology, Aalborg University, Aalborg, Denmark. [2]Center for Nanomedicine and Theranostics, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark.

Manuscript published as a book chapter in *Nanomedicines for Brain Drug Delivery – Springer Nature Neuromethods Series*

Abstract

Millions worldwide suffer from neurological disorders, and even more will be affected by e.g. Alzheimer's disease and cerebrovascular diseases in the coming decades. The possibilities for treatment of neurological diseases are hampered by that drug delivery to the brain is severely impaired by the blood-brain barrier (BBB), which function is to regulate the fluxes of molecules between the circulation and the brain. Development of successful strategies to bypass the integrity of the BBB is thus pivotal to obtain tools that will allow for treatment of diseases of the central nervous system. To develop such bypassing strategies, it is also of great importance to understand the morphological and physiological features that characterize the BBB. In this chapter, the structure and function of the BBB and the current knowledge on the role of the transferrin receptor in brain iron uptake is reviewed. This is followed by a full coverage of the functions of the transferrin receptor and its ligands together with a thorough summary of studies investigating the delivery of transferrin receptor-targeted nanomedicines. The challenges of the transferrin receptor-targeting strategy are discussed with emphasis on possibilities to improve brain delivery while avoiding typical experimental caveats.

CHAPTER 2. METHODOLOGICAL CONSIDERATIONS

The purpose of this chapter is to give a broad overview of the methodology applied in the subsequent studies of TfR-mediated brain drug delivery. The individual subsections will include the main principles of each technique and the historical background regarding their development. Furthermore, the considerations we had for including the specific methods in our studies are also presented. All projects presented later in this dissertation was based on collaborations between two groups, who are experts in neurobiology and nanomedicine, respectively. Therefore, a significant amount of knowledge transfer between the groups is needed to fully comprehend the data output and the interpretations based on it. This next chapter seeks to bridge the gap.

2.1. IN VITRO MODELLING OF THE BLOOD-BRAIN BARRIER

Given the important role that the BBB plays in the regulation of the brain microenvironment by governing the entrance of most types of molecules, studies of the BBB to understand this regulation and exploit it for drug delivery is crucial (1). However, these studies are not trivial to perform *in vivo*, because the BBB exists as a complex network in the brain vasculature, which is mostly inaccessible for studies of intracellular mechanisms or transport across the BBB (2). It may also be difficult to decipher the roles of specific cellular or non-cellular components in regulating these mechanisms, because the proximity of the different components limits the resolution with which we can identify and describe these roles. Knock-out animal models exist where a specific cell type is lost, but these models only illustrate an extreme situation and do not necessarily mimic a situation that resembles any disease (3, 4). Furthermore, the bioethical aspect of the use of animals for experimental studies requires consideration about reducing the number of animals or replacing the animals with other models giving a similar answer to the scientific question (5-7).

Many researchers have tried to overcome these issues by modelling the BBB *in vitro* using advanced culture systems. These efforts were started already in the 1970s, where BCECs were cultured from microcapillaries isolated from brain homogenates via sucrose centrifugation (an early version of the brain capillary depletion technique, see below)(8, 9). These isolated BCECs were used as simple models of the BBB to study the regulation of transporters and integrity of TJ protein assemblies (Figure 2.1.1)(10-14). However, these models were limited by the fact that they did not produce a tight monolayer of BCECs, which is a main requirement for *in vitro* BBB models (1). Some also reported that the activity of the endothelial-specific enzyme γ-glutamyl transferase was lost in culture (15), hereby illustrating that in culture the BCECs were not fully differentiated and capable of producing a functional BBB.

Later, a series of studies illustrated that culturing the BCECs in the presence of astrocytes or astrocyte-like cells led to re-induction of BCEC characteristics, e.g. γ-glutamyl transferase activity and TJ protein expression (16, 17). This suggested that the presence of astrocytes was important for maintaining the integrity of the BCEC monolayer, corresponding well with the almost full coverage of the BCECs by astrocytic endfeet *in vivo* (18-24). With the introduction of the now widely popular Transwell co-culture system, *Dehouck et al.* (1990) cultured bovine BCECs and rat astrocytes in the so-called contact co-culture format (Figure 2.1.1) and showed that this raised the transendothelial electrical resistance (TEER) of the BCEC monolayer remarkably together with decreasing the passive permeability to small molecules (17). These factors are important to prove since they correlate with the BCECs obtaining their proper polarization, and thus, it is now widely accepted that co-culture of BCECs with astrocytes is a crucial requirement for obtaining a relevant *in vitro* BBB model system to perform studies in (1). The induction is even possible when using the so-called non-contact co-culture models, where the astrocytes are seeded in the bottom chamber of the Transwell co-culture system (Figure 2.1.1), illustrating how much of the inductor properties of the astrocytes that are mediated by their release of soluble factors to the microenvironment (1). Furthermore, having access to these commercially available Transwell co-culture systems also sparked the idea of including the last cellular component of the NVU, namely the pericytes (25-30). *In vivo*, the pericytes (like astrocytes) are responsible for maintaining the integrity of the BCEC monolayer, but there are also indications that they are important for regulating the capillary blood flow (3, 31-34). *In vitro*, some have found that including the pericytes into the co-culture systems (to obtain a triple co-culture, Figure 2.1.1) improved the TEER values of the resulting BBB model (25), whereas others failed to make this observation (35). Pericytes are also known to reduce the vesicular transport *in vivo*, but this role has not been described yet for *in vitro* models, although it would likely underscore the system's validity, if such intracellular mechanisms could be successfully modelled (3, 4, 36).

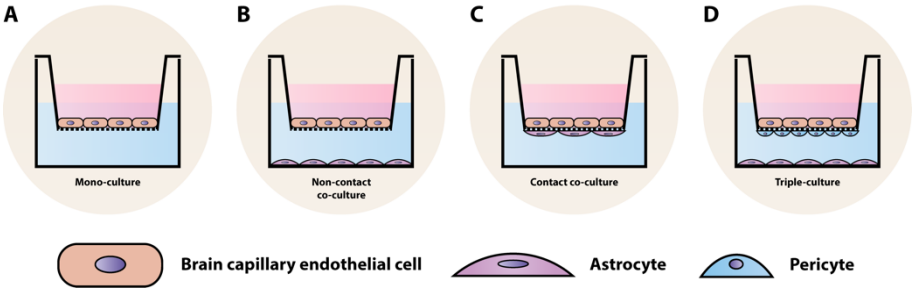


Figure 2.1.1. Schematic representation of *in vitro* models of the blood-brain barrier. BCECs can be isolated and cultured in different setups to mimic the normal blood-brain barrier, and these different setups result in very different outcomes with respect to proper differentiation and polarization. (A) The simplest model, called the mono-culture model, consists only of BCECs and is characterized by low values of TEER and

poor differentiation and polarization. (B + C) Co-culture models with astrocytes can be set up both as non-contact or contact co-cultures. These models are characterized by a marked increase in BBB integrity and mimicry. (D) Pericytes can also be introduced to create the so-called triple co-culture models. These models are not characterized by increased TEER values, but may have an impact on decreasing the vesicular transport as is evident *in vivo*.

To ease the use of *in vitro* BBB models, several groups have developed immortalized BCEC lines derived from a variety of different species, and these cell lines have gained huge popularity. For example, transfection of mouse BCECs with polyoma middle T antigen resulted in the generation of the bEnd.3 cell line, which have been a work horse in many laboratories to this day (37). Similar approaches have been used to generate the RBE4 cell line from rat cells (38), or hCMEC/D3 and HBMEC lines from human cells (39, 40). These cell lines are easy to culture and they express several important receptors and transporters, which make them useful for many purposes, especially regarding drug delivery screening processes (41). However, it is also evident that none of these cell lines can produce a tight monolayer of BCECs, as shown by the low TEER values and high passive permeability of the resulting *in vitro* BBB models (1). This can possibly be overcome for the study of larger drug molecules or carriers (e.g. antibodies and nanoparticles), if a pulse-chase strategy is employed (42). In this system, primary binding and uptake into the BCEC is allowed in one well, followed by extensive washing of the BCECs and transfer of the Transwell into another well (bottom compartment) from which the transported drug molecule is sampled (42). Still, regardless of their high-throughput qualities, the immortalized cell lines will never produce models as good as those derived from primary BCECs, and thus, to study BBB biology, the primary *in vitro* BBB models are state-of-the-art in comparison (43-45).

Primary *in vitro* models of the BBB have until now been produced from mouse (35), rat (46-49), bovine (2, 50), porcine (51), and human BCECs (52, 53), and their utility as models of the true BBB have to some extent been proven in numerous studies. However, there are still many issues to consider, when using the *in vitro* BBB models to base conclusions on, especially because only very few studies have provided correlative evidence based on parallel *in vitro* and *in vivo* studies (49). One study compared the transport profile of different antibody constructs targeting undisclosed BBB receptors and found a good correlation between the *in vitro* and *in vivo* data, which indicates that some transport is happening similarly in the two systems (45). Others have failed to reproduce the function of the TfR for transporting iron across the BBB, a function which has been known for decades to exist *in vivo* (54). This finding was interpreted be due to interaction between iron and the permeable support of the Transwell, which illustrates how transport data may be underestimated because of this interaction (54, 55). It was also shown that despite of having a high integrity *in vitro* BBB model, the barriers contain a high number of holes in the endothelial monolayer that can be impossible to detect with the resolution of normal fluorescence microscopes (55, 56). These holes represent a sink for nanoparticles administrated to

these barriers, which would result in an overestimation of the transport efficiency (55). Whether these holes are a universal phenomenon in all *in vitro* BBB models is yet to be shown.

The use of *in vitro* BBB models throughout the projects described in this dissertation has served both as tools for optimization (such as flow cytometry-based studies in immortalized cell lines) and as parts of parallel assessments together with *in vivo* experiments of how the different types of nanoparticles were taken up into BCECs and transported across the BBB. A choice was made to avoid the use of triple cultures with pericytes, since adding this cell type to the system would yield another source of error in addition to factors such as unspecific interaction between the transcytosed nanoparticles and the polycarbonate membrane of the Transwell inserts (55). However, this still produced *in vitro* BBB models with high integrity, as determined by high values of TEER, and the expression of relevant TJ proteins. Furthermore, the TEER values were measured immediately before and after a nanoparticle transport experiment to ensure that the integrity of the *in vitro* BBB model was maintained despite the presence of nanoparticles in the growth medium. Depending on the type of nanoparticle that was administrated to the models, the correlation between the *in vitro* and *in vivo* systems varied from negative to positive, but especially for experiments on AuNPs there was a good correlation. Still, in all cases, the absolute amounts of compound transported across the *in vitro* BBB models were very low. This may both be a testament to the overall low transport capacity of the nanoparticle systems, but also to the fact that while it was possible to measure transport to the 'brain' compartment of the Transwell setup, some of the transcytosed nanoparticles or cargo will inevitably still be interacting with the polycarbonate membrane (55).

2.2. BRAIN CAPILLARY DEPLETION

When studying the relevance of a new nanomedicine strategy for brain drug delivery, one must know about its uptake capacity into the brain parenchyma. The most widely used methods for this purpose are measurements of the compound of interest (nanoparticle, elements of the nanoparticle, or therapeutic cargo) in homogenates of the brain (57, 58), or labelling of the nanoparticle and therapeutic cargo to facilitate imaging of the brain accumulation using e.g. positron emission tomography (PET)(59, 60). Both methods are suitable for giving an answer to the basic question about whether the newly developed nanomedicine will preferentially accumulate in the region of the brain. Thus, many studies argue for the relevance of their nanomedicine strategies by presenting such evidence to show that brain accumulation will occur after intravenous injection, and often interpret such findings as indicative of transport into the brain parenchyma (58, 61). However, these interpretations will often be flawed by the fact that the impact of the BBB on the transport of the nanoparticle into the brain is seldom accounted for, especially if these observations are used as a standalone argument.

The experiments performed *in vivo* for this project are focused on obtaining knowledge about the transport capacity of the different strategies studied, and less about the possible therapeutic impact this would have subsequently (49). Therefore, there was a need for a methodology that would allow for answering both the broad question of whether the nanoparticles reached the brain region, together with more advanced questions on the whereabouts of the nanoparticles after this brain regional accumulation, i.e. whether the nanoparticles had the ability of being transported across the BBB into the brain parenchyma, or whether the nanoparticles simply remained confined inside the BCECs (49, 62). To answer these questions, the so-called brain capillary depletion method was employed, because this method separates the vascular compartment from the rest of the brain tissue (63). The method was originally described by *Triguero et al.* (1990), where forebrain tissue was homogenized in a buffer solution and mixed with high concentrations of dextran (Figure 2.2.1)(64). The homogenization is thought to release the microvasculature from the other cell types of the brain, which will associate with the dextran beads in the solution. The dextran bead-associated microvasculature can then be pelleted by centrifugation at high speed (Figure 2.2.1)(64). After the centrifugation, the homogenized brain samples are separated into a capillary-containing pellet and a supernatant containing the brain parenchymal fraction. The purity of these fractions can be analyzed, e.g. by measuring the activity of endothelial cell-associated enzymes (alkaline phosphatase and γ-glutamyl transferase)(64-66), or performing microscopic assessment of the different fractions to look for the morphology of the cells contained in the samples (64, 65). For the latter, immunocytochemistry can be included to illustrate high abundance of endothelial markers such as CD31 or claudin 5. Generally, studies using this method find a high purity of the two fractions after separation, which highlights the relevance of including this method in studies of drug transport across the BBB (63, 64, 67, 68).

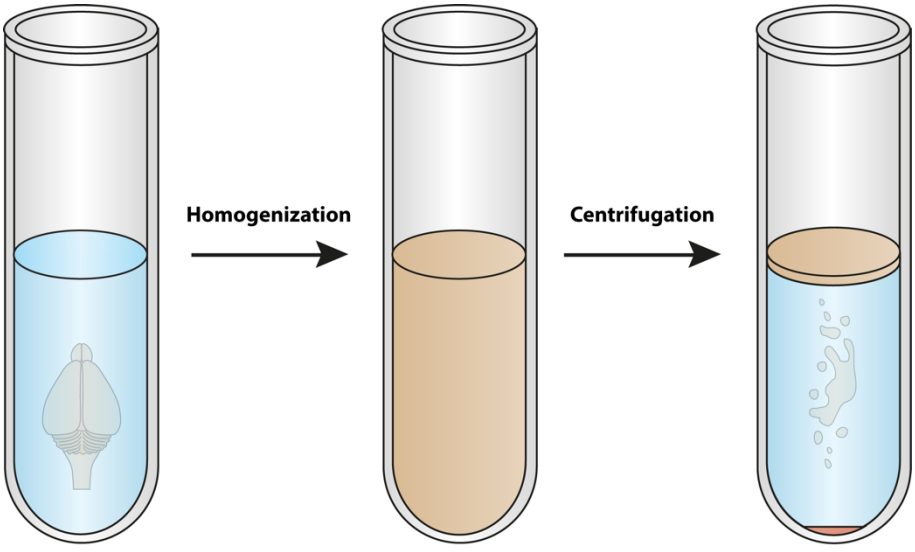


Figure 2.2.1. Schematic representation of the brain capillary depletion technique. Samples of the brains are resected and homogenized in a buffer solution, mixed with a high concentration of dextran, and centrifuged at high speed. The combined effect of this dextran addition and subsequent centrifugation is the separation of the brain capillaries (red pellet) from the brain parenchyma, which is found both as a supernatant (blue with cloud of tissue) and a so-called lipid cake (beige plug on top of the supernatant). All fractions are sampled for downstream analysis of transport across the blood-brain barrier *in vivo*.

Since the original publication of the protocol, the method has been used in a relatively steady amount of studies each year (approximately four published articles per year). Some find their compounds to accumulate mostly in the brain capillaries with low transport across the BBB (65, 69), whereas others find their compounds to be upconcentrated in the parenchymal fraction with low levels of accumulation inside the BCECs (70). In relation to studies regarding endogenous compounds (i.e. with no therapeutic purpose), the validity of the method was illustrated by showing receptor-mediated endocytosis of acetylated low-density lipoprotein (LDL) without subsequent exocytosis to the brain parenchymal compartment (64), and vascular sequestration of adenosine after carrier-mediated uptake into the BCECs (67). However, the protocol has not gained wide popularity within studies of brain drug delivery, mostly because researchers employ a functional (therapeutic) outcome measure in their studies, which indirectly proves the transport capacity. Such indirect proof may be relevant in many occasions, but given the low clinical progress of nanomedicines for brain drug delivery, there may be valuable knowledge to gain about why so little transport is happening, if more detailed studies on the nanomedicine strategy itself are performed.

Criticism has been made regarding brain capillary depletion due to possible imperfect separation of all capillaries from the parenchymal fraction, and the risk of spillover between fractions after the centrifugation procedure (63, 65, 71). This was evidenced by dissociation of compounds binding with low affinity to the plasma membrane of the BCECs, e.g. a μ-opioid peptide-dermorphin analogue (68). Purity of the fractions may be increased by subjecting the isolated brain to the currently used protocols for isolation BCECs for advanced *in vitro* culture systems (35, 46, 51), but this also undermines the quantitative aspect that is an important positive feature of the brain capillary depletion technique. Thus, it is evident that although being much more advanced than studies made on whole brain homogenates, the outcome of the brain capillary depletion technique may be used only as an approximation of the transport capacity, and should preferably be combined with other methods (e.g. morphology-based) that can underscore the quantitative findings. Another relevant combinatorial technique could be the use of microdialysis directly from the brain extracellular space (72-74).

Brain capillary depletion was employed in all quantitative *in vivo* experiments presented in this dissertation. One hemisphere from each animal was used for the technique, as was originally suggested by *Triguero et al.* (1990), whereas the other served as tissue input for the biodistribution analysis. After optimization, we found

the technique to yield a very robust and reproducible separation of the fractions, and analysis of the alkaline phosphatase and γ-glutamyl transferase activity underscored that the fractions were of high purity. Importantly, it was noted that the separation was disturbed if the deceleration of the centrifuge was too fast, and thus, it has been stressed in the protocols described in the manuscripts that slow deceleration was conditional for a good brain capillary depletion.

2.3. INDUCTIVELY-COUPLED PLASMA-MASS SPECTROMETRY

A continuous methodological aspect across the projects described in this dissertation is the use of inductively-coupled plasma mass spectrometry (ICP-MS) as the primary quantification tool. ICP-MS is known as the most powerful technique for elemental analysis in a variety of different sample types. It has been widely used for analysis of environmental samples (e.g. water) to study contamination or mineral composition, but in the recent years increasing interest has been given to the technique for use within biology and life sciences (75, 76). The principle of the technique is a classical type filtering based on the atomic mass of a given element and its isotopes using a mass spectrometer (77). However, MS is incapable of distinguishing between neutral atoms, and hence, an ICP unit is attached to the system to facilitate ionization of the sample atoms before entering the mass spectrometer. The plasma inside the ICP unit is made of the relatively inert gas, argon, which can create a highly ionized phase at extremely high temperatures (6,000 – 8,000°C)(78).

The sample is introduced into the system via a peristaltic pump attached to an auto sampler, from where it is pumped into a nebulizer that converts the fluid into aerosols (Figure 2.3.1)(76). This conversion is important since it allows for introducing only small volumes into the argon plasma, hereby increasing the plasma stability. Aerosols can also be of quite large sizes, which is the reason for nebulizing the sample into a spray chamber, where the larger aerosols are restricted from exiting and moving further into the system (78). In the ICP unit, a quartz torch inside a copper coil initiates the argon plasma by creating a magnetic field that can transfer energy to the argon gas. An alternating current (oscillating at 27 or 40 MHz) is produced within the copper coil by a radiofrequency generator operated at 1,000 – 1,500 W (76). This induces a strong magnetic field at the tip of the quartz torch in which free electrons are produced by applying a high voltage spark to the flowing argon gas. These free electrons cause collisions and ionization in the argon gas, which produces the plasma. The aerosolized sample is then introduced into the plasma with a high velocity that is sufficient to ‘punch a hole’ through the center of the plasma. Inside the plasma, the aerosols are desolvated, vaporized into a gas, and atomized. Lastly, the atoms (originating from the sample) are ionized by the plasma and extracted from the plasma chamber to continue into the MS unit (Figure 2.3.1)(76-78).

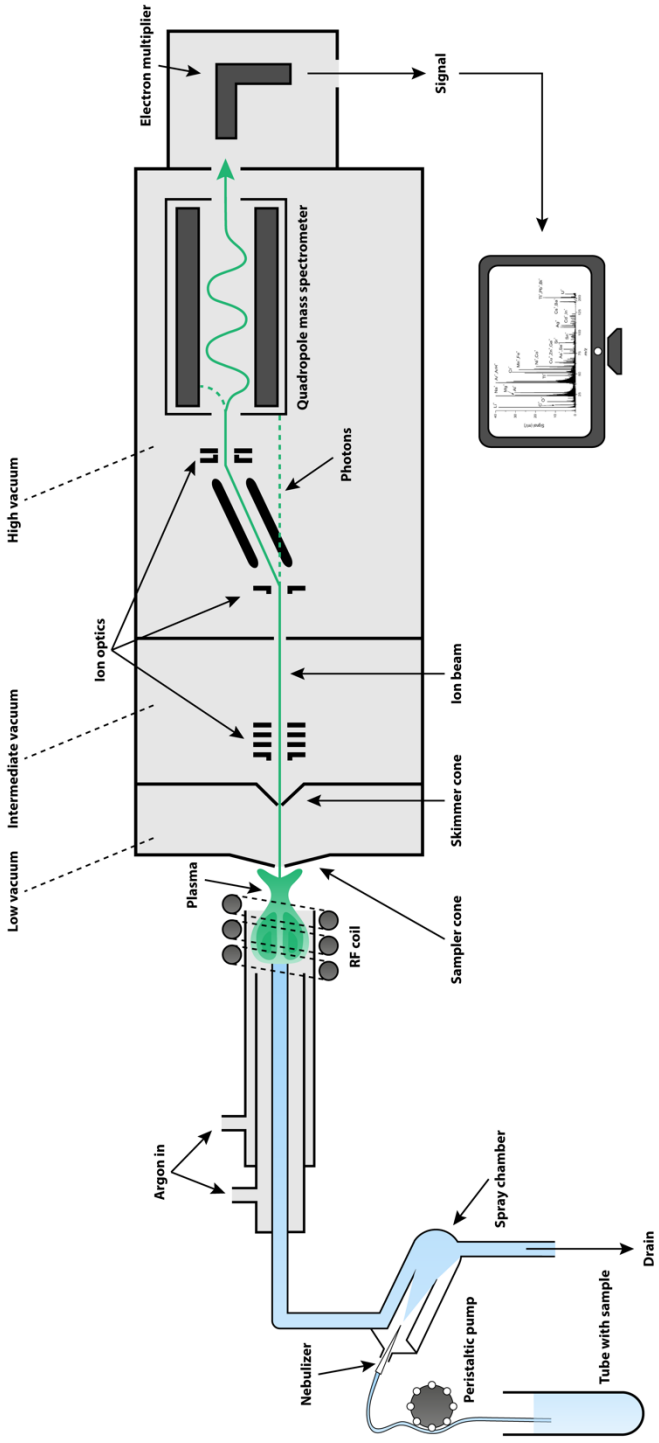

Electron multiplier
Signal
Quadropole mass spectrometer
Photons
High vacuum
Ion optics
Intermediate vacuum
Ion beam
Skimmer cone
Low vacuum
Plasma
RF coil
Sampler cone
Argon in
Spray chamber
Drain
Nebulizer
Peristaltic pump
Tube with sample

Figure 2.3.1. Schematic representation of an inductively-coupled plasma-mass spectrometry system. A liquid sample is introduced into an argon plasma after nebulization into small droplets. In the plasma, the sample is atomized and ionized. The atomized and ionized sample is extracted from the plasma into the mass spectrometry unit, where the pressure in sequentially lowered, and the sample focused using ion optics. The focus ion beam is then introduced into the quadrupole mass spectrometer, wherein the trajectory of given ions depicts its passage out onto an electron multiplier detector. From here, the signal is extracted onto a computer, where the resulting data is analyzed. The figure was re-drawn from *Linge et al.* (2009)(76).

In the interface between the high-pressure argon plasma and the ion optics inside the MS unit sit two cones responsible for ion extraction and reduction of pressure (78). At first, the ions are extracted from the plasma via a sampler cone into a low vacuum compartment, thereby reducing the pressure. The extracted ions are then passed through a second step of pressure reduction via a skimmer cone, but while this effectively reduces the pressure into an intermediate vacuum, it also diverges the ion beam substantially (76). Therefore, the ion beam passing the skimmer cone is focused via ion optics on its path into the high vacuum chamber, in which the quadrupole mass spectrometer is located. Inside the high vacuum chamber, neutral atoms and photons are filtered away from the ion beam before the ions left from the initial sample is introduced into the mass spectrometer (Figure 2.3.1)(78). In the mass spectrometer, the ions are separated based on their mass-to-charge ratio (m/z), which when a certain voltage is applied to the quadrupole will allow the ions of interest to oscillate through to the detector, whereas extreme oscillations will result in an unstable trajectory for other ions, and therefore, these ions are not passed onto the detector (Figure 2.3.2). Ions hitting the detector will be counted to yield a total amount of the ion of interest in the sample. This can be converted into a concentration of the ion of interest, if a standard curve of this ion is generated and measured together with the experimental samples (76-78).

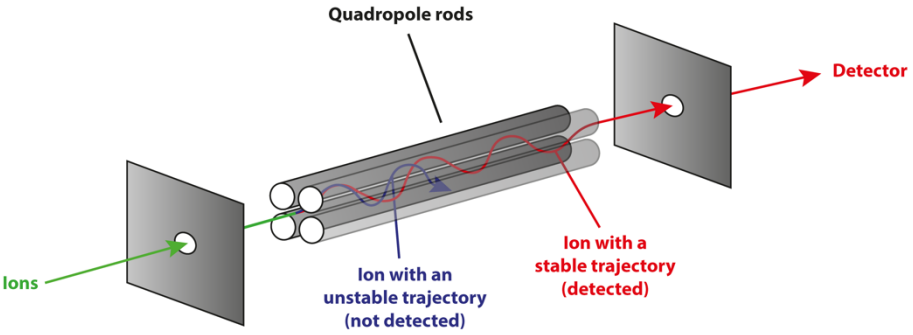


Figure 2.3.2. Schematic representation of a quadrupole mass spectrometer. The ion beam from the inductively-coupled plasma unit is directed into a quadrupole mass spectrometer, wherein the ions originating from the sample will be separated based on their trajectories in the voltage field applied in given situations. As such, ions of no relevance will be unstable in the voltage field applied to the quadrupole and

collide, whereas the ions of interest with have a stable trajectory throughout the length of the quadrupole to be detected on an electron multiplier detector immediately after the quadrupole mass spectrometer.

ICP-MS was adopted as a quantitative technique due to its very high sensitivity compared to other quantitative techniques (e.g. measurements of fluorescence intensity). Brain drug delivery is low irrespective of how many folds a given change to the drug delivery system improves the transport, and hence, the sensitivity of the ICP-MS has been favorable for our purposes. Furthermore, analyzing the presence of metals (platinum and gold) as a measure of transport across the BBB also avoids the issue of stability/photobleaching, which is an inevitable problem when quantifying based on fluorescence (79). In this project, samples taken from tissues, blood, cell cultures, and growth medium have been processed and analysed by ICP-MS (49). Regardless of the sample origin, processing included complete digestion overnight in aqua regia at 65°C followed by dilution in 2 % HCl before analysis. Iridium was chosen as an internal standard for both samples, standards, and blanks. This allowed for monitoring the stability of the measurements while analyzing 100+ samples per run (59, 80). Two points of the standard curve was measured after every ten samples to ensure stability of the concentration determination, and extra washing was performed between different types of samples (i.e. brain versus liver) to avoid spillover. Lastly, to further reduce the risk of spillover and its impact on the measurements, samples were analyzed in the order of lowest expected concentration to highest expected concentration.

2.4. SILVER ENHANCEMENT

Despite using the highly sensitive method, ICP-MS, for the quantitative parts of the project, a tool for visualizing the transport of AuNPs across the BBB was needed to substantiate the results. For this purpose, the use of electron microscopy (EM) was included in some of the projects, because this method allows for very sensitive detection of AuNPs located in tissue sections. This method cannot, however, be used to investigate larger volumes of brain tissue. Therefore, the silver enhancement technique was employed to visualize the colloidal gold in the brain tissue samples with light microscopy. The silver enhancement technique is an autometallography method, wherein silver ions derived from a silver salt in solution reacts with the surface of a nanocrystal (e.g. an AuNP) to adhere and become part of it under catalysis induced by a reducing agent (81). In this way, the size of the original colloidal gold nanocrystal is increased until reaching the resolution of the light microscope (Figure 2.4.1)(82).

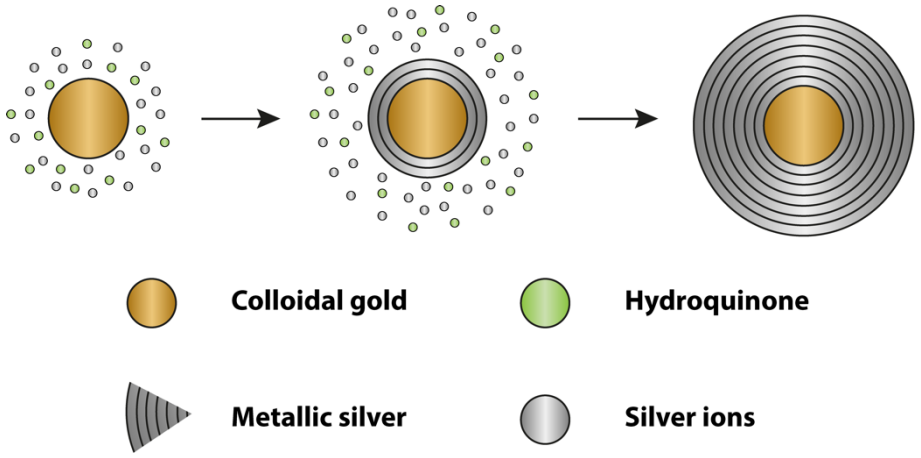


Figure 2.4.1. Schematic representation of the silver enhancement process. Silver enhancement is an autometallography technique, where colloidal gold in a tissue sample is developed using silver ions. The silver ions and the reducing agent, hydroquinone, is administrated to the sample, which initiates a silver deposition process. Colloidal gold can act as a catalyst in this process, and hence, at locations with presence of gold, the silver deposition process will be accelerated. This leads to deposition of metallic silver around the colloidal gold particles, which with increasing time will develop the original size of the colloidal gold particle to reach the resolution limit of either electron microscopy or light microscopy.

The principle behind the silver enhancement technique was introduced for histology by *Liesegang* in 1911, who took inspiration from the so-called silver-based physical developers known from early photography of the 1800s, and wanted to apply this principle on tissue specimens (83). Moreover, he wanted to perform silver stainings like those *Ramón y Cajal* used for tissue blocks directly on sections of tissue (83-86). His studies showed that this was possible, and hence, he pioneered the use of photographic developers for staining purposes on tissue sections, even to such an extent that *Ramón y Cajal* later used his techniques (83, 86).

Based on work within improvements of photographic plates using silver sulphur nanocrystals in the late 1930s (87), *Timm* (1958) developed a technique in which hydrogen sulphide and metal ions (silver) were introduced into a tissue block during the fixation process, hereby creating silver sulphur nanocrystals to visualize metals contained in the tissue block (88). Later, it was evidenced that most of the silver staining could be traced back to zinc (89), which led *Timm* to develop the method further to be able to also visualize mercury in tissue samples exposed to this (90). In 1981, a Danish group led by *Gorm Danscher* recognized that if colloidal gold was to be detected using silver staining protocols, it was a necessity that the reaction happened in a reducing environment, e.g. by exposing the sample to UV light or by adding a reducing agent to the silver enhancer solution (91, 92). This sparked the use of colloidal gold as labels on antibodies for subsequent use in immunogold labeling,

because it became possible to increase the nanocrystal size from below EM resolution up to light microscopy resolution (82, 93). Since then, the technique has been used for many purposes, which have proven its usability and robustness. For example, AuNPs (14.5 nm) were visualized in tissue sections from different organs (94), and the co-localization of gold and silver in these electron dense spot proven by energy-dispersive X-ray spectroscopy (EDS)(95). These studies have also found that injected AuNPs are cleared from the organism by macrophages in different organs (especially the liver and spleen)(96-98), and that gold blocks implanted directly into the brain are distributed as nanoparticles in neural and astrocytic processes (99). Similar findings will be presented later in this dissertation. Also, several studies employ both the ICP-MS and silver enhancement techniques to underscore their observations (98, 100).

In the field of brain drug delivery, the silver enhancement technique has been used on several occasions for visualization of AuNPs transported into the brain (100-102). *Jensen et al.* (2013) used the technique to show that injected AuNPs reached the brain microvasculature and parenchyma after intravenous injection (100). This intraparenchymal location allowed for delivery of RNAi-based medicine to prolong the survival of glioma-bearing mice. Others have used the technique more quantitatively, where light microscopy assessment and counting of silver-enhanced spots (indicative of AuNPs) showed that by modulating the ligand density, the transport of AuNPs into the brain parenchyma could be increased (101). The methodology was also used to show that a pH-sensitive linker placed between the AuNP surface coat (PEG) and the ligand (endogenous transferrin) led to more uptake of AuNPs into the brain parenchyma compared to those without this linker (102). Yet others used the method to investigate the transcytosis process of OX26 AuNPs (5 nm), although these efforts did not result in unambiguous detection of silver-enhanced AuNPs in the brain parenchyma (103). In this project, the silver enhancement technique was employed on brain tissue sections of a thickness comparable to those used by the group of *Danscher* (97, 104). No quantification was attempted with the technique (like performed in the studies presented above), but it was used in conjunction with electron microscopy to make visible the observations done using ICP-MS after brain capillary depletion.

2.5. TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) was used in studies related to AuNPs to detect the presence of them in the brain capillaries and parenchyma, in addition to the ICP-MS-based bulk quantification and silver enhancement of brain tissue sections with subsequent light microscopy assessment. The technique was included due to the possibility of studying ultrastructure of brain samples from animals having received injections with AuNPs, hereby reaching a resolution where individual AuNPs could be detected without *ex vivo*-processing (i.e. using silver enhancement).

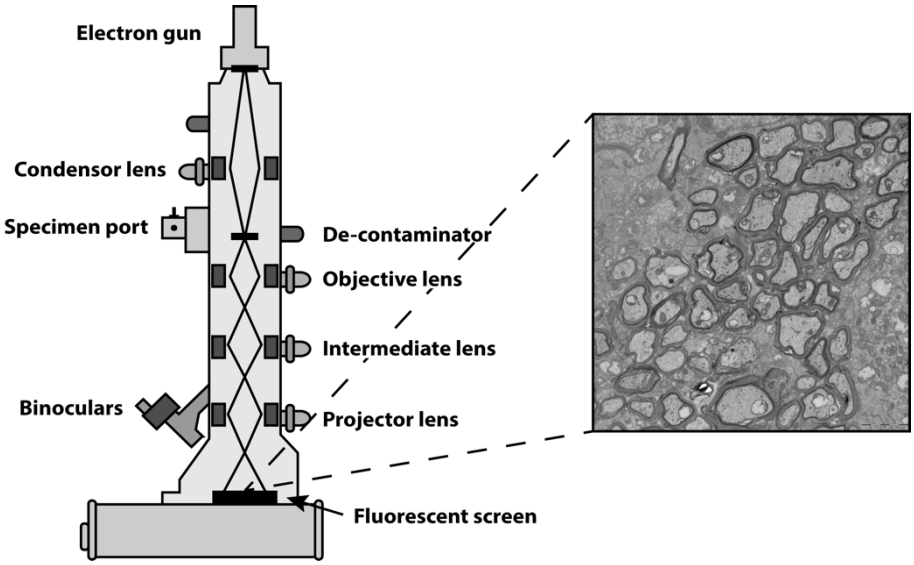


Figure 2.5.1. Schematic representation of a transmission electron microscope. In a transmission electron microscope, electrons are generated from a tungsten or LaB_6 crystal filament in the so-called electron gun. The electrons are accelerated in vacuum and focused onto the specimen by an electromagnetic condenser lens. Electron transmitted through the specimen are further focused using another lens system to generate an image on a fluorescent screen, which can be visualized through binoculars, or detected by a CCD camera to create digital images. The schematic was re-drawn from *Kuntsche et al.* (2011)(105), whereas the electron microscopy image of neuronal axons was acquired during the PhD course in Electron Microscopy at Copenhagen University in 2016.

The basic principle in TEM is based on exposing a specimen to an electron beam that transmits through and becomes detected (Figure 2.5.1)(106). Electrons are generated in the so-called electron gun, in which a filament is placed (tungsten or LaB_6 crystal) that when subjected to a high voltage source releases the electrons into the vacuum of the microscope interior. From here, the electrons of the beam are accelerated to obtain kinetic energy and focused onto the specimen using a condenser lens made of electromagnetic coils (106). When the electron beam reaches the specimen, the resulting interaction can induce the formation of several physical phenomena (see below), which allows for detection of different kinds of information from the specimen. In TEM, the information that is gathered comes from the electrons that have been transmitted through the entire thickness of the sample, and hence, it is the density of the different components of the specimen (e.g. carbon versus gold) that gives the contrast (106-108). However, electrons cannot be transmitted through specimens of a large thickness, and therefore, tissue and cell samples analyzed with TEM must be sectioned to a thickness of 80 – 150 nm for the electrons to pass through (107, 109). The electrons that pass through the sample are guided further down the TEM column via another lens system (including objective lens and projector lens) to project the

image of the sample onto a fluorescent screen, wherefrom the sample can be visualized using binoculars. The sample can also be projected to a charge-coupled device (CCD) camera to acquire images (Figure 2.5.1)(106). To enhance the contrast in the sample, the thin sections are stained using heavy metals such as lead, osmium and uranium before imaging. These compounds will scatter the electrons of the beam to yield a grey-toned appearance, e.g. of lipid rich structures such as cell membranes in the case of osmium. The resulting image can then be analyzed for its information on the ultrastructure of the sample in question, although interpretations will have to regard the fact that the sample processing procedure include both dehydration and resin embedding, which could have detrimental effects on the size of extracellular spaces, cell organelles, or the entire cell itself (110). Still, TEM provides for the best resolution compared to other microscopy techniques, and will thus be fit for many purposes.

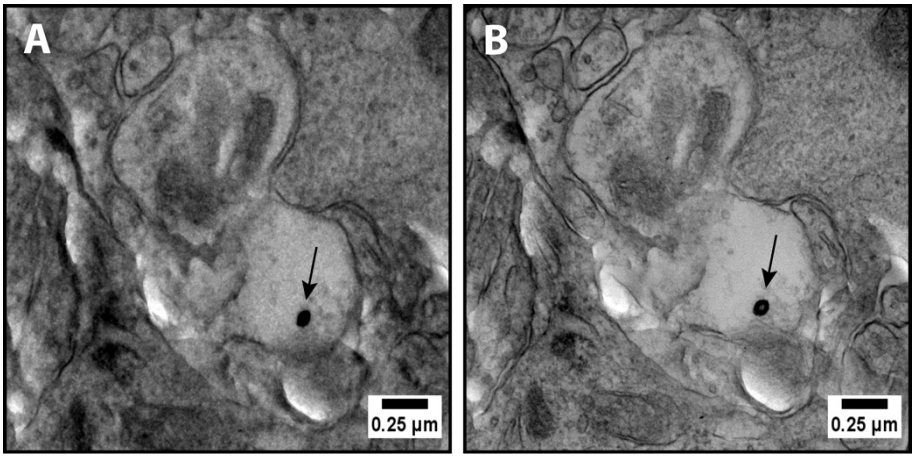


Figure 2.5.2. Electron microscopy images showing the presence of a salt artefact inside a neural process. (A) A salt crystal was observed inside a neural process closely resembling a gold nanoparticle. Therefore, without further processing, such an artefact would likely be interpreted as a gold nanoparticle having traversed the blood-brain barrier. (B) The same salt artefact could be pierced by a high intensity, focused electron beam, which proved it to be an artefact and not a gold nanoparticle, which would make the primary interpretation (without additional analysis using energy-dispersive X-ray spectroscopy) false.

TEM has served as a valuable tool because of its potential for studying ultrastructure of the nanoparticle-treated brain samples, which furthermore allowed for visualization of individual nanoparticles instead of measuring bulk quantities as with ICP-MS (75). It is well known that electron microscopy can resolve the presence of nanoparticles in tissues, but even though it is easy to see highly electron dense nanoparticles (i.e. AuNPs), processing of the tissue before the microscopic analysis can induce different types of artefacts that are likely to be interpreted as nanoparticles (*Kempen et al*., in preparation). To illustrate this issue, we analysed sections from mice that had received

no treatment with AuNPs, but had been exposed to the same kinds of tissue processing for subsequent electron microscopy (Figure 2.5.2A). Using TEM, it was evident that electron dense, punctate structures could be observed in the brain parenchyma, even though no AuNPs were injected into the animal (Figure 2.5.2A). Such structures are likely interpreted as successfully transcytosed nanoparticles, because it is very difficult to claim it to be anything else, when analyzing a sample where AuNPs were in fact injected. One way to resolve this is to boost the electron beam and focus this on the electron dense structure in question (Figure 2.5.2B). This will for AuNPs not lead to any adverse effects (although it might harm the sample), and so, the AuNP integrity will be preserved. However, for artefacts like salt precipitates induced during the processing, it will be possible to partly destroy it (Figure 2.5.2B, hole in the middle of the salt artefact). While this technique is a pragmatic solution to study the presence of true AuNPs in the brain parenchyma, it will probably result in sample destruction, which will not be applicable for routine analysis of the AuNPs in a sample.

2.6. ENERGY-DISPERSIVE X-RAY SPECTROSCOPY

We instead utilized the microanalysis technique, energy-dispersive X-ray spectroscopy (EDS), for element composition microanalysis of the tissue samples analysed using TEM. As depicted by its name, the principle of this technique is based on the early work of *Wilhelm Röntgen* on a new type of rays (Röntgen rays or X-rays), which earned him the Nobel Prize in 1901 (111, 112). Later, the work of *Charles G. Barkla* showed that the X-rays emitted from a sample is connected to the atomic weight of the elements contained in the sample (113-118), which was further expanded by *Henry G. J. Moseley*, who explained how the so-called K-line transitions (movement of electrons between different energy states) moved the same amount in the X-ray spectrum, when the atomic number increased by one (119-123). Combined, these observations led to the invention and commercialization of the first X-ray spectrometers in the late 1950s, which were the foundations for the energy-dispersive X-ray spectrometers used in connection to TEM today (124).

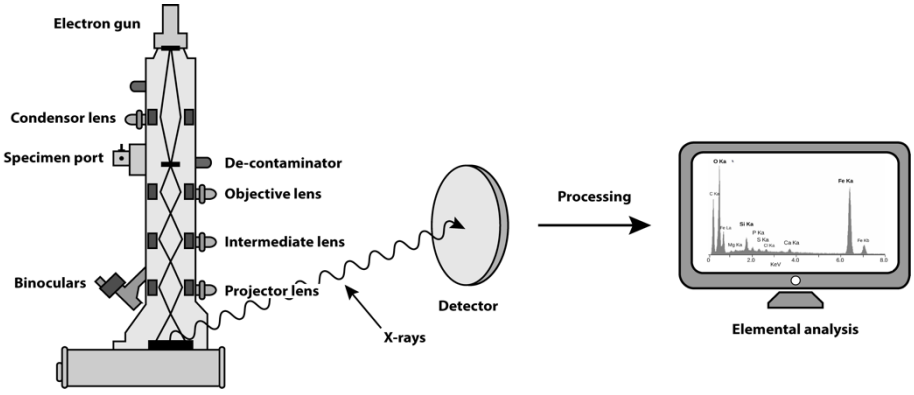


Figure 2.6.1. Schematic representation of the process in energy-dispersive X-ray spectroscopy. When an electron interacts with a specimen, it can yield a so-called characteristic X-ray, which gives information about the elemental composition in the specimen. This X-ray can be detected and processed to give an element spectrum. The schematic was re-drawn from *Kuntsche et al*. (2011)(105), and the elemental spectrum was derived from *Corbari et al.* (2008)(125).

The information acquired using EDS is based on the physics of electrons interacting with the specific atoms of a given sample (126, 127). As depicted in a simple schematic in Figure 2.6.1, electrons are accelerated inside the column of an electron microscope, hereby obtaining kinetic energy. The high-energy electrons are focused on the sample, where it interacts to generate different phenomena, which for the sake of EDS is an X-ray of a specific energy corresponding to the elemental composition of the sample. These X-rays are measured on a detector, wherefrom the information is processed and mapped to yield an element spectrum of the entire sample or a region of interest in the sample (Figure 2.6.1).

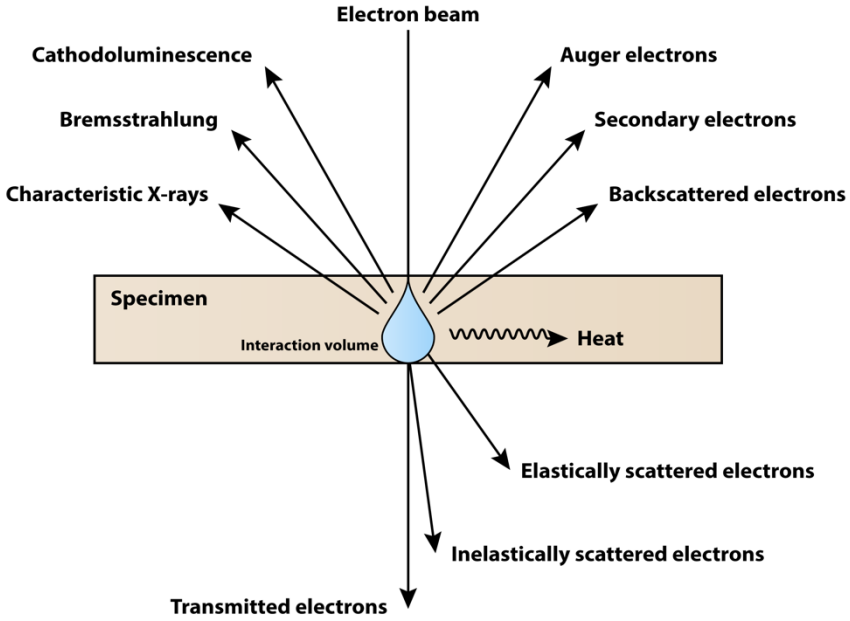


Figure 2.6.2. Schematic representation of the interaction between an electron beam and a specimen. When electrons hit a specimen, it can result in generation of many different phenomena. In TEM, it is the transmitted electrons that are detected and used from image acquisition. However, important for the energy-dispersive X-ray spectroscopy technique are the X-rays generated in the same process. By in-elastic or elastic scattering of the incoming electrons, an X-ray continuum, known as brehmsstrahlung, is generated. These X-ray does not carry any information on the elemental composition of the specimen. Conversely, generation of so-called secondary electrons can result in the generation of so-called characteristic X-rays

that are specific to the element contained in a specific region of the specimen. These can be detected in energy-dispersive X-ray spectroscopy. The schematic is re-drawn from *Vaughan* (2008)(126).

Looking at the process at an atomic level, the beam of electrons can interact with the sample in several ways (for the purpose in this section, the interactions are only summarized in Figure 2.6.2). Electrons moving close to the atomic nucleus can have their trajectory bend in the coulomb field, such that it will scatter either elastically or inelastically (Figure 2.6.3A). For the inelastically scattered electron, this process will slow the electron passing through the sample by loss of kinetic energy, and this energy will be emitted as an X-ray continuum known as bremsstrahlung (Figure 2.6.3A). This X-ray continuum cannot be traced back to determine the specific elemental composition of the sample.

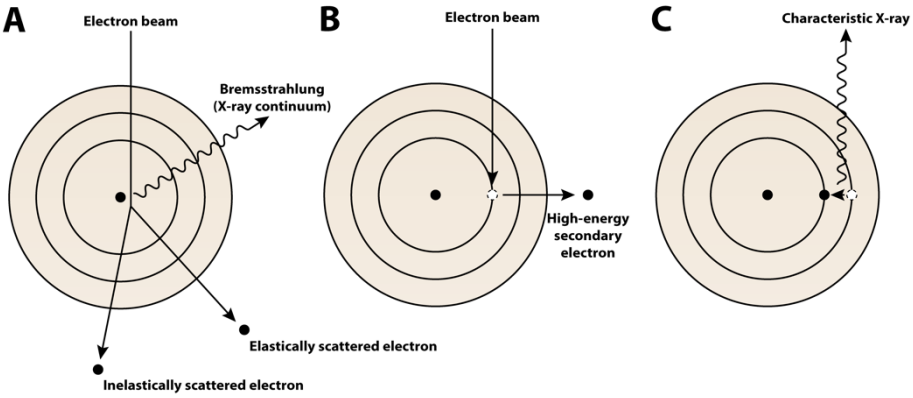


Figure 2.6.3. Schematic representation of an electron interacting with an atomic nucleus. When an electron hits a specimen inside a transmission electron microscope, different kinds of X-rays can be generated. (A) The electrons can be scattered by the atomic nucleus inelastically to generate an X-ray continuum, which is known as brehmsstrahlung. Such radiation does not carry information about the specific element. (B) In another situation, the incoming electron can eject an electron from the electron shells of the atom as a so-called secondary electron. (C) This leaves a void, wherein electrons from the outer shells can move to. In this process (which transfers the ion from an excited state to the ground state), the outer shell electrons must give up a part of its energy as X-rays of a specific energy, which can be detected as characteristic X-rays. This yields information about the elemental composition of the specimen, since the amount of energy is different for each element of the periodic table. The schematic is re-drawn from *Vaughan* (2008)(126).

In addition, the high-energy electrons of the electron beam can interact with an electron in the inner atomic shell to eject this as a so-called secondary electron (Figure 2.6.3B). This yields an ion in an excited state with a void in which another electron can move to. For the ion to return to its ground state, an electron from an outer shell must transition into the void created by the ejection of the secondary electron (128). The electron transitioning must give up some of its energy to drop into the void, and this energy is emitted as electromagnetic radiation X-rays (Figure 2.6.3C). These X-

rays (or the amount of energy/the wavelength that characterizes them) are specific for each element in the periodic table, and hence, can be processed to give information about the composition of the sample (126, 128). In EDS, the energy of the X-rays is analyzed as opposed to the analogous technique, wavelength-dispersive spectrometry, where the wavelength of the emitted X-rays is analyzed (126, 127).

EDS proved to be an important method to use in the study of AuNP transport into the brain. As described above, salt artefacts in the brain parenchyma can easily be interpreted as transcytosed AuNPs, if that is what one is looking for in the tissue. Therefore, we chose to analyze all potentially transcytosed AuNPs with EDS to ensure that they were in fact gold, and not just salt artefacts. EDS was also used to determine the presence of gold in some of the AuNPs found in brain capillaries, however, the large number of these observations (i.e. correlating with the observed upconcentration of AuNPs in the brain capillaries by ICP-MS) made it impossible to do this analysis as a standard for all of them. Nevertheless, EDS is a very advanced technique not accessible for many brain drug delivery groups, and as such, the use of this technology is an improvement with respect to TEM interpretations with high validity.

CHAPTER 3. OBJECTIVES OF THE DISSERTATION

Based on the literature review presented in *Manuscript I* and *Manuscript II*, we find that while transferrin receptor-targeting has been pursued for brain drug delivery in well over two decades, there has still not been any real progress with respect to clinical translation of the preclinical findings. For example, the phenotype has been corrected in many animal models of brain disease via delivery of drugs through transferrin receptor-targeted fusion constructs or nanomedicines, but it does not seem to hold true, when more structured preclinical or clinical testing is performed. Thus, there must be aspects of this drug delivery strategy that we still do not understand:

- Are nanoparticles being transported across the blood-brain barrier into the brain parenchyma?

- Is it only an encapsulated cargo that can be transported into the brain parenchyma after intracellular processing of a nanoparticle?

- How does ligand affinity impact the uptake of nanoparticles into the brain parenchyma?

- How does ligand valency impact the uptake of nanoparticles into the brain parenchyma?

- How does total ligand density impact the transport of solid nanoparticles into the brain parenchyma, and is this impact different from that of an encapsulated cargo?

- How do nanoparticles behave in the brain compartment after direct administration?

These questions will be sought answered in *Chapter 4* based on the methodology presented in *Chapter 2*.

CHAPTER 4. METHODOLOGY AND RESULTS

4.1. MANUSCRIPT III

Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma

Kasper Bendix Johnsen[1,2], Annette Burkhart[1], Fredrik Melander[2], Paul Joseph Kempen[2], Jonas Bruun Vejlebo[2], Piotr Siupka[3], Morten Schallburg Nielsen[3], Thomas Lars Andresen[2] & Torben Moos[1]*

[1]Laboratory for Neurobiology, Biomedicine, Institute of Health Science and Technology, Aalborg University, Aalborg, Denmark. [2]Center for Nanomedicine and Theranostics, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark. [3]Department of Biomedicine, Aarhus University, Denmark.

Manuscript published in Scientific Reports

Abstract

Drug delivery to the brain is hampered by the presence of the blood-brain barrier, which excludes most molecules from freely diffusing into the brain, and tightly regulates the active transport mechanisms that ensure sufficient delivery of nutrients to the brain parenchyma. Harnessing the possibility of delivering neuroactive drugs by way of receptors already present on the brain endothelium has been of interest for many years. The transferrin receptor is of special interest since its expression is limited to the endothelium of the brain as opposed to peripheral endothelium. Here, we investigate the possibility of delivering immunoliposomes and their encapsulated cargo to the brain via targeting of the transferrin receptor. We find that transferrin receptor-targeting increases the association between the immunoliposomes and primary endothelial cells *in vitro*, but that this does not correlate with increased cargo transcytosis. Furthermore, we show that the transferrin receptor-targeted immunoliposomes accumulate along the microvessels of the brains of rats, but find no evidence for transcytosis of the immunoliposome. Conversely, the increased accumulation correlated both with increased cargo uptake in the brain endothelium and subsequent cargo transport into the brain. These findings suggest that transferrin receptor-targeting is a relevant strategy of increasing drug exposure to the brain.

4.2. MANUSCRIPT IV

Antibody affinity and valency impact the brain uptake of transferrin receptor-targeted gold nanoparticles

Kasper Bendix Johnsen[1,2], Martin Bak[2], Paul Joseph Kempen[2], Fredrik Melander[2], Annette Burkhart[1], Maj Schneider Thomsen[1], Morten Schallburg Nielsen[3], Thomas Lars Andresen[2]* & Torben Moos[1]*

[1]Laboratory for Neurobiology, Biomedicine, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark. [2]Center for Nanomedicine and Theranostics, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark. [3]Department of Biomedicine, Aarhus University, Denmark.

Manuscript submitted to Science Advances

Abstract

The ability of treating invalidating neurological diseases is impeded by the presence of the blood-brain barrier (BBB), which inhibits the transport of most blood-borne substances into the brain parenchyma. Targeting the transferrin receptor (TfR) on the surface of brain capillaries has been a popular strategy to give a preferential accumulation of drugs or nanomedicines, but several aspects of this targeting strategy remain elusive. Here we report that TfR-targeted gold nanoparticles (AuNPs) can accumulate in the brain capillaries and transport across the BBB to enter the brain parenchyma. We find that this uptake capacity is significantly modulated by the affinity and valency of the AuNP-conjugated antibodies. Specifically, antibodies with high and low affinities mediate a low and intermediate uptake of AuNPs into the brain, respectively, whereas a monovalent (bi-specific) antibody improves the uptake capacity remarkably. We characterize this concept both in vitro using primary models of the BBB and in vivo using quantitative measurements of gold accumulation together with morphological assessments using light and transmission electron microscopy. Our findings indicate that monovalent ligands may be beneficial for obtaining transcytosis of TfR-targeted nanomedicines across the BBB, which is relevant for future design of nanomedicines for brain drug delivery.

4.3. MANUSCRIPT V

Modulating the ligand density changes the brain uptake of transferrin receptor-targeted gold nanoparticles and liposomal cargo

Kasper Bendix Johnsen[1,2], Martin Bak[2], Paul Joseph Kempen[2], Fredrik Melander[2], Maj Schneider Thomsen[1], Annette Burkhart[1], Thomas Lars Andresen[2] & Torben Moos[1]*

[1]*Laboratory for Neurobiology, Biomedicine, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark.* [2]*Center for Nanomedicine and Theranostics, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark.*

Manuscript in preparation

Abstract

Transport of therapeutic molecules to the brain is largely precluded by the presence of the blood-brain barrier (BBB), hereby rendering efficient treatment of neurological disorders impossible. The BBB may be circumvented by targeting the drug towards receptors and transport proteins expressed on the surface of the brain capillary endothelial cells (BCECs). The transferrin receptor (TfR) has remained a popular target since its original description for this purpose, although clinical progression of TfR-targeted drug constructs or nanomedicines has yet to be seen. On proposed issue pertaining to the use of TfR-targeting in nanomedicines has been the efficient tuning of the ligand density on the nanoparticle surface. In this study, we studied the impact of ligand density on the transport of nanoparticles into the brain, taking a parallel approach to investigate the impact on both antibody-functionalized gold nanoparticles (AuNPs) and cargo-loaded liposomes. We find that among three different low-range ligand densities (0.15, 0.3, and 0.6 * 10^3 antibodies/μm^2), the highest density yielded most targeting abilities towards the BCECs and subsequent transport across the BBB *in vitro* and *in vivo*. Furthermore, we find that TfR-targeting on nanoparticles may induce severe adverse effects after intravenous administration.

4.4. MANUSCRIPT VI

The surface charge depicts the brain distribution of liposomes after intracerebroventricular administration – a morphological study

Kasper Bendix Johnsen[1,2], Jonas Bruun Vejlebo[2], Thomas Lars Andresen[2] & Torben Moos[1]*

[1]Laboratory for Neurobiology, Biomedicine, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark. [2]Center for Nanomedicine and Theranostics, Department of Micro- and Nanotechnology, Technical University of Denmark, Denmark.

Manuscript in preparation

Abstract

Transport of intravenously administrated drugs into the brain is obstructed by the blood-brain barrier, which constitutes the interface between the systemic circulation and the fragile brain parenchyma. Many variants of nanomedicines have been developed for being transported into the brain, but despite employing different targeting strategies and controlled release mechanisms, none of these nanomedicines have reached the clinical stages of drug development. A solution to this issue may be to administrate the nanomedicine directly into the brain compartment, although drug delivery via this route is still poorly investigated. Here we investigated the distribution of liposomes after intracerebroventricular administration. We find that liposomes with either anionic and cationic surface charge are efficiently transported to the subarachnoid space after intracerebroventricular administration, and that much of the liposome dose is taken up by resident macrophages. Additionally, we observe a marked difference in cortical penetration, where anionic liposomes penetrate deeply into the brain cortex along the penetrating arteries, whereas the depth of penetration was much lower for cationic liposomes. These findings suggest that an anionic surface charge is favourable for obtaining a good cortical distribution of administrated liposomes.

CHAPTER 5. JOINT DISCUSSION

Based on the findings of the literature and experimental projects presented in *Manuscript I – VI*, this chapter will be devoted to a joint discussion of all projects. While there has been discussions of the different aspect of both the literature reviews and experimental results in the respective manuscripts, this joint discussion seeks to draw some broader lines and describe some interesting similarities and differences between the studies. The main focus is on the findings made in *Manuscript III – IV*, since these projects were all focusing on different aspects pertaining to the use of the TfR as a target for nanomedicine-based brain drug delivery. Furthermore, several discussion points of the literature reviews in *Manuscript I – II* are revisited and updated with the most recent literature. Further discussion of the findings in *Manuscript VI* is omitted, since this project stood out with a markedly different purpose than the other projects. The reader is thus referred to the discussion presented in this manuscript itself.

5.1. CURRENT STATUS OF BRAIN DRUG DELIVERY VIA NANOMEDICINES

Despite the large focus on brain diseases impacting the health on an aging population, there is still a lack of medicines that can combat them. However, when looking at the number of FDA approvals for neurology, there seems to be no paucity of therapies getting available for use in the clinic, as has been argued by many (129), when compared to other types of diseases within oncology and cardiology (Figure 5.1.1). The drugs that are approved are often for use against pain or against symptoms of neurodegenerative disorders rather than the disease mechanisms *per se*. Also, many of the approvals are for small molecule drugs, which means that the great promise of the biological treatments (i.e. antibody-based) has yet to come forth. This was illustrated in the case of solanezumab by Eli Lilly, a monoclonal antibody against β–amyloid, which attracted worldwide media attention in 2015 due to findings of delayed disease progression in a subgroup of patients suffering only from mild Alzheimer's disease (130-133). However, when a Phase III clinical trial was performed to study this patient group only, the original findings were not supported at primary endpoint analysis, which led to the termination of the so-called EXPEDITION3 trial (133-136). Such outcomes from clinical trials are unfortunate, but not rare. A 2014 paper calculated the failure rate of drugs for Alzheimer's disease to be 99.6 % (both antibody-based and small molecules)(137). Another striking fact is that in the current pool of clinical trials, most of the drugs studied target the β–amyloid protein present in the brain parenchyma, but provide no means of transport into the brain (138). Clinical trials using nanomedicines are rare, and they most often study brain tumors or brain metastases in very small populations (139). Thus, it seems that a burning platform exists for including BBB transport strategies for future drugs in development to improve the current success rate.

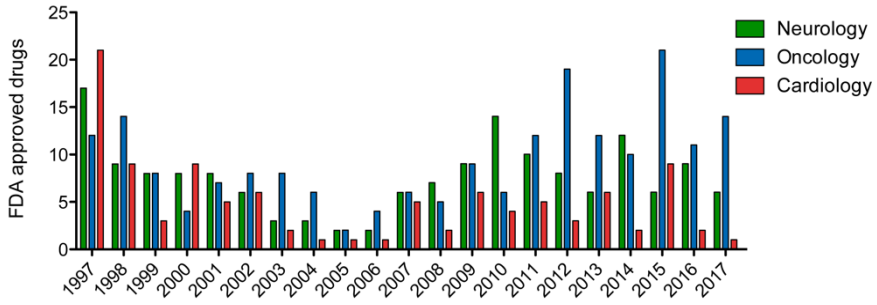


Figure 5.1.1. Overview of the number of FDA-approved drugs between 1997 and 2017.

Based on this burning platform, a vast variety of brain drug delivery strategies has been developed over the past decades, especially nanomedicines strategies that have a theoretical potential of delivering large payloads of drugs to the brain parenchyma, if the nanoparticles are designed to cross the BBB (140, 141). The development of these BBB-passing nanomedicines has not yet amounted into true clinical progress, and as such, the fate of most new nanomedicine designs resembles that of small molecule drugs or biologicals, namely that most of them will fail (140). In principle, this is not problematic as this is the normal path in drug development (142). However, for brain drug delivery, the use of nanomedicines has shown to be particularly poor with respect to clinical translation, leaving no true examples of its clinical utility until now (141). Since no critical assessments of the current status on nanomedicines for the treatment of brain diseases are available in the literature, one can turn towards the nanomedicine field as a whole.

In the recent years there have been an increased recognition in the field of nanomedicine that much of the potential of these strategies for the treatment of diseases (cancer in particular) rely on several unvalidated assumptions, and that the downstream experimentation is flawed by confirmation bias related to these assumptions (143, 144). One prominent example of such an assumption is the universal presence of the so-called enhanced permeation and retention (EPR) effect as a positive driver of tumor accumulation of nanoparticles, leading to increased drug exposure and therapeutic efficacy (145). While there are numerous cases of evidence to suggest a role for the EPR effect in preclinical models of malignant diseases, the impact of the effect in clinical trials seems limited at most (146). This was especially evident in a recent meta-analysis of available preclinical and clinical studies on the use of liposomal doxorubicin, which showed a markedly improved efficacy of this chemotherapeutic drug in solid tumors compared to the free drug in preclinical models, whereas meta-analysis of the clinical evidence showed no effect at all (147). Leading researchers of the nanomedicine field even state that there has been *no* clinical progression at all despite clinical approval of a few formulations, because most of these do not present with a better therapeutic efficacy (143, 148). This lack of clinical progression is discussed to be due to poor disease modelling in preclinical

studies, and relying too much on the EPR effect for the therapeutic efficacy, when increasing amounts of evidence suggest that it is not a universal phenomenon (145, 149). Poor understanding of the drug release kinetics, uptake into tumor cells, and distribution within the tumor tissue compartment is another problematic aspect in the current nanomedicine strategies. Also, there have been no limitations of the peripheral accumulation in the liver, spleen, and lungs, which upholds an unfavorable adverse effect profile of the developed nanomedicines (143). In general, it seems that many of the problems related to the development of efficient nanomedicines (for cancer treatment) are due to a poor understanding of or lack of interest in considering the complex disease biology and the fact that all tumors are unique (143, 145, 150). Thus, if all preclinical development focuses on the limiting the variability (in homogenous animal disease models) to increase the chance of success, then the downstream clinical potential may in fact be reduced substantially. For clinical progression of nanomedicines, there must be considerations of many other aspects than what is currently included in experiments (142).

Returning to the use of nanomedicines for brain drug delivery, several of the problematic features described above seem to apply here as well. First, many studies of nanomedicine-based brain drug delivery disregard the potential diminishing aspects of the strategy, such as unfavorable accumulation in peripheral organs or hemolysis when in circulation. This likely follows the logic that if the improvement is large enough, e.g. with respect to cognitive performance in Alzheimer's disease patients, then the potential adverse effects can be tolerated (140). However, at this point, nothing suggests that such large improvements in disease-related parameters are to be expected. Second, in studies of brain drug delivery (clinical or preclinical), there is a general lack of understanding of the amount of drug that reaches the brain parenchyma, and what fraction of this that will be unbound and available for eliciting its therapeutic effect (151). Furthermore, the extent of distribution inside the brain parenchyma after effective BBB transport is also not understood. This means that even though we are able to transport therapeutic compounds across the BBB, we cannot yet correlate this with the amount of therapeutic efficacy that is to be expected, because the aspect of dosing and drug distribution is so poorly studied. Interestingly, though, there are now indications that encapsulation of a drug in a liposome is enough to increase brain exposure, because this encapsulation will improve the circulatory half-life of the drug and avoid unspecific binding with components of the blood (74, 152-154). Follow-up studies on this concept will likely improve our understanding of the extent of which BBB transport of nanomedicines can be viable for effective treatment of disease processes in the brain parenchyma, or whether the same effects can be achieved by better circulation properties (73, 74, 152, 154). Third, the field of nanomedicine-based drug delivery also build its potential of largely unvalidated assumptions, including that the BBB will be open for passive accumulation of nanoparticles as a result of neurodegenerative or neuromalignant disease (see below)(155-157), or that specific target receptors are said to be transcytosing (often stated by brain drug delivery researchers), while this does not follow the

normophysiological sorting of these receptors (as described by researchers in BBB cell biology and biochemistry)(158). Fourth, the brain drug delivery field is (like many other research fields) severely impacted by poor animal models used to describe the downstream clinical potential (140, 159, 160). This is especially problematic for a range of neurodegenerative diseases, which can be modelled to some extent in rodents, e.g. to observe overexpression of disease-related proteins or ongoing neuroinflammation. However, the human-associated symptomatology is rarely observed, because rodents do not have analogous neurodegenerative diseases because of their short lifespan (140, 161, 162).

How to progress from this state of poor translational value of currently available nanomedicine strategies for brain drug delivery is difficult to resolve at this point. It will likely have to encompass much deeper considerations of the end-point that is targeted, e.g. a relevant drug concentration needed to be obtained within a certain time-frame to induce therapeutic effects inside the brain parenchyma. The same is true for the choice of target, where just choosing a relevant one is now the strategy of the past, in favor of more detailed studies of how the nanomedicine (and targeting ligands) can interact with the receptor of choice, and what parameters that can be modified to control both the interaction and the sorting/transport processes (49, 141, 163).

5.2. IS THERE A NEED FOR TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER?

There is still great dispute regarding the possible loss of BBB integrity in neurodegenerative and neuromalignant disorders, and the possibility that an intravenously administrated drug or nanomedicine will reach the brain parenchyma due to this loss of integrity (156, 157, 164, 165). In *Manuscript I* and *Manuscript II*, this issue was discussed in depth in relation to the impact it may have on nanoparticle accumulation in the brain parenchyma, and how this disruption may leave strategies to overcome the BBB with nanoparticles or drug constructs irrelevant (141, 163). Here, a few points of this discussion are revisited and updated with newer references to highlight the main arguments for continuing the work on targeted nanoparticle strategies for transport across the BBB.

Many neurodegenerative disorders have been characterized with an impacted BBB, including Alzheimer's disease (166, 167), Parkinson's disease (168-170), Huntington's disease (171), multiple sclerosis (155, 156, 172), cerebral malaria (173), and glioblastoma multiforme (157). Investigations into the impact of such a loss of integrity have shown passive accumulation of drugs or blood constituents in the diseased area (167, 168, 170), which indicates that the brain parenchyma is readily accessible from the systemic circulation, but it also raises the opportunity of getting preferable drug accumulation at the site, where it is needed (174). This was especially evident in a series of paper showing passive accumulation of drugs encapsulated into stealth-like (non-targeted) liposomes in multiple models of human neurodegenerative

disease, including the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (155, 173, 175-177). However, the EAE model is known to have a very impaired BBB, which does not readily mimic the level seen in human disease or other neurodegenerative diseases (156), which decreases the translational value of these findings.

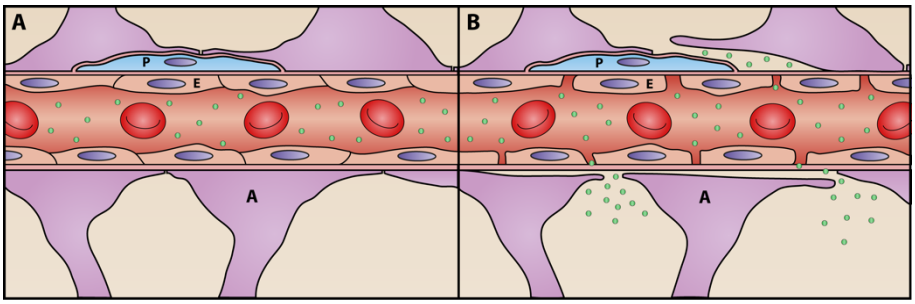


Figure 5.2.1. The theoretical impact of BBB disruption for brain delivery of nanomedicines. The figure describes the potential for transport of nanoparticles (green) into the brain parenchyma during neurodegenerative disease suggested by either (A) *Bien-Ly et al.* (2015)(156) or (B) *Turjeman et al.* (2015)(155).

The BBB disruption (including the increased permeability of newly formed vessels observed in several diseases) and its impact on disease progression was also highlighted in many studies based on both preclinical (168, 169, 171, 178-185) and clinical data (164-167, 186), although data on its impact on drug delivery is still scarce (for a detailed review, please refer to *Obermeier et al.* (2013)(164) or *Zhao et al.* (2015)(165)). BBB disruption seems to correlate with increasing age in humans, and not only with progressing neurodegenerative disease (166). Interestingly, such breakdown of the BBB was correlated with increasing amounts of extravasated IgG in the hippocampal area of the brain during Alzheimer's disease, suggesting that the opening is rather prominent, and that the disruption may aggravate with progressing disease (164, 165, 167). Still, in the case of solanezumab explained above, those patients with late-stage Alzheimer's disease had a very poor therapeutic response to the treatment. If we assume that target of solanezumab was indeed relevant for these patients, this indicates that regardless of the presence and magnitude of BBB disruption in these patients, it was not enough to obtain a therapeutically relevant dosage of IgG in the brain parenchyma (i.e. hippocampus). This was underscored in another series of studies in multiple preclinical models of Alzheimer's disease, where BBB opening even to small molecule drugs (266 – 720 Da) or dextrans (3 kDa) was insignificant (156, 187-191), and where increased intraparenchymal IgG concentrations could be obtained only if they were endowed with capacity to target and transport with the TfR (156). Together, these findings, both preclinical and clinical, indicate that disruption of the BBB may indeed be a factor in most brain-related disorders, but the magnitude of this disruption is still difficult to decipher.

Even though clinical data exist on significant opening of the BBB, e.g. in Alzheimer's disease, its utility with respect to increasing drug exposure seems largely unresolved, if existing at all. This was demonstrated in a recent review of BBB disruption in glioblastoma multiforme (157). Here, it was argued that even though BBB disruption is evident in this disease (like the general increased vascular permeability in many cancer types), it is non-uniformly distributed with large areas of maintained or increased integrity. This correlates with poor uptake of even small chemotherapeutics in many regions of the tumor and subsequent poor therapeutic outcome for the patient (157). Moreover, even in cancer, where there is a high chance of passive accumulation due to increased leakiness of the tumor vasculature, it can be difficult to obtain drug accumulation at a level more than 0.7 %ID (148). There is thus still a clear reason to study drug delivery strategies that can overcome the BBB and gain access to the brain parenchyma.

In this dissertation, a choice was made to study only healthy animals when assessing the transport capacity of the different nanoparticle strategies developed. This had several advantages, including the ease and lower cost of performing large-scale studies and performing studies in models where the receptor systems were expected to behave 'normally'. In *Manuscript III*, we chose to study the uptake of OX26 immunoliposomes in young rats. The choice of using young rats instead of adults was based on the evidence that the expression of TfRs is high during development (65), which could increase the likelihood of interaction between the TfR and OX26 immunoliposomes. In *Manuscript IV* and *Manuscript V*, the female mice used for the assessment of AuNP and liposome transport into the brain were also considered to be young of age (approximately 8 weeks by the time of experiments)(192). The choice of age was made to mimic that of the published papers that our work was inspired by, namely that of *Yu et al.* (2011)(193) and *Wiley et al.* (2013)(107). Especially, we figured that to study the aspects of antibody affinity and valency on the transport of AuNPs into the brain (*Manuscript IV*), the most proper choice of experimental setup was one like *Yu et al.* (2011)(193), since we used the antibodies developed in this study. Thus, in all studies of this dissertation concerning the use of targeted nanoparticles for brain drug delivery, we used 'prime' animals that were healthy and young, and were expected to have no changes in their BBB integrity or receptor transport pathways.

It is indeed a limitation in our studies that we do not consider modelling of the brain disease, for which our nanomedicine strategy would be relevant to use. This means that the possibility of BBB disruption as an additive factor in the accumulation on nanoparticles was not tested in our setup, and thus, the studies presented in this dissertation can only provide knowledge on the transport across the properly maintained BBB, but not the full scale of nanoparticle accumulation in a state of neurodegenerative or neuromalignant disease. It would, however, be highly relevant to test this hypothesis in preclinical models of brain disease, to gain knowledge about how a disruption would impact the nanoparticle accumulation.

5.3. BIODISTRIBUTION ANALYSES

Accumulation of nanoparticles in peripheral tissues are expected after intravenous administration (148), especially in cases of brain drug delivery where only a very small fraction of the injected dose reaches the area of interest (< 1 %ID)(141, 163). This process of unspecific accumulation, despite targeting qualities etc., has been studied greatly in the last two decades. The efforts have shown that both the liver and spleen are preferable sites of accumulation of nanoparticles, mostly because the injected nanoparticles are opsonized by serum proteins in the systemic circulation and subsequently cleared by resident macrophages (194, 195). This happens instantly after injection, hereby leaving only little left of the dose to interact with the target (196-198). These findings have paved the way of designing nanoparticles that are able to avoid this clearance, e.g. by mimicking the surface of a red blood cells or surface coating the nanoparticles with polymers such as PEG (199). The creation of a so-called protein corona has also been described for such surface-coated nanoparticles, which could decrease the targeting qualities of conjugated ligands, and in general reduce the circulatory half-life of the nanoparticles (200-204). The impact of this protein corona is, however, still heavily debated, because newer findings suggest that the total number of proteins that can interact with and bind to a PEGylated nanoparticle surface is low, and thus, only impacts the targeting qualities of the nanoparticles very little (205). Regardless of the magnitude of the impact, there is little doubt that most of an injected dose of nanoparticles will accumulate in other tissues than the brain.

Biodistribution analysis was performed in all studies that utilized the intravenous administration route presented in this dissertation (summary presented in Figure 5.3.1). The choice of including the analysis was the logical consequence of the issue presented above, namely that most of the injected liposomes and AuNPs were expected to accumulate other places than the brain (163). Across these studies of TfR-targeted liposomes or AuNPs, there seemed to be a clear tendency that most of the injected dose would end up in either the liver or the spleen. Still, we observed that while the accumulation of nanoparticles in the liver did not correlate with the TfR-targeting, the splenic accumulation of nanoparticles did. In *Manuscript III*, isotype IgG and OX26 immunoliposomes were compared in the rat, showing that there was no difference in the uptake in the liver after 1 hour of circulation. In the spleen, isotype IgG immunoliposomes accumulated at a level of approximately 10 %ID/g, whereas this accumulation was three-fold higher for the TfR-targeting, OX26 immunoliposomes (approximately 30 %ID/g). In *Manuscript IV*, mPEG AuNP accumulation in the liver was lower than that of protein (i.e. antibody)-functionalized AuNPs, including isotype IgG AuNPs, indicating no effect of TfR-targeting on liver accumulation. Conversely, the two high-affinity-binding variants of TfR-antibodies (anti-TfR^A and anti-TfR^A/BACE1) increased the splenic accumulation to 25 – 40 %ID/g, much higher than the accumulation observed for the mPEG and isotype IgG control AuNPs (10 – 13 %ID/g). In *Manuscript V*, there was no difference between

mPEG and TfR-targeted AuNPs with respect to liver accumulation, but in the spleen, the accumulation seems to correlate with increasing amounts of anti-TfR antibodies. For the liposomes, a somewhat low liver accumulation was observed with no differences between the individual groups, whereas increased splenic accumulation was observed for all TfR-targeted variants. Importantly, in this study, there were no isotype IgG control nanoparticles included, which make clear-cut interpretations of a TfR-mediated accumulation difficult. Nevertheless, in combination, the observations made in these three studies clearly indicate that splenic accumulation of nanoparticles are reinforced by targeting the TfR.

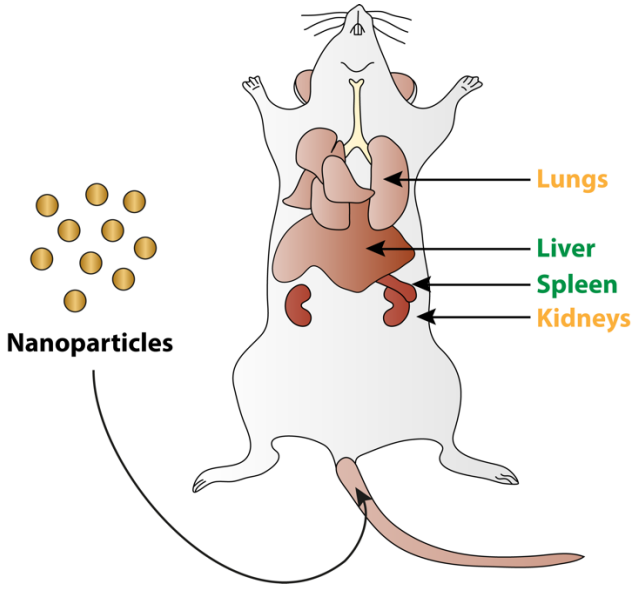


Figure 5.3.1. Overview of the sites of peripheral accumulation after intravenous administration of nanoparticles. High levels of accumulation (green) were observed in both the liver and the spleen. In the spleen, there was also a tendency of improved accumulation as a function of TfR-targeting. Intermediate levels (yellow) of accumulation were observed in the lungs and the kidneys.

TfR expression is present both in the liver and spleen (Figure 5.3.2). In the liver, the TfR expression has been described both for the hepatocytes and Kupffer cells (both TfR1 and TfR2) with subcellular localization both in the cytoplasm and cell membrane (206-209). Thus, in principle, the nanoparticles injected in the studies presented in this dissertation could accumulate in the liver via the TfR. However, as presented above, we find no evidence of any TfR-mediated accumulation in this organ. Instead, we find that the accumulation may be increased by the conjugation of proteins on the nanoparticle surface (e.g. antibodies). This could leave the targeted nanoparticles more susceptible for opsonization, and hence, uptake by macrophages (Kupffer cells) residing in the liver. This corresponds well with our EM-based

observation that AuNPs could only be detected in Kupffer cells in the sinusoids (*Manuscript IV*), whereas no AuNPs were detected inside or even binding to a hepatocyte. Such results have also been presented in other detailed studies of nanoparticle biodistribution (97, 210-212). In the spleen, the TfR expression has been observed throughout the tissue, although with an overweight in the red pulp and residing macrophages (206-208, 213). As for the liver, we performed EM on spleen sections to study the AuNP uptake (*Manuscript IV*), however, with no clear answer as to the type of cell, in which the AuNPs located after splenic accumulation. We speculate that the macrophages also in this organ are the primary cells taking up nanoparticles in the spleen, maybe with a TfR-mediated potential increase in accumulation. Experiments on anti-TfR antibodies showed that these accumulated to a high degree in the spleen (214), thus, also suggesting an impact of the splenic TfR expression on accumulation in this organ in addition to the size effect, which could be another explanation in the case of nanoparticles. Others have shown no additional spleen accumulation of TfR-targeted AuNPs after intravenous administration (210). However, this study was very small (n = 3) and did the assessment 24 hours after administration, which is not comparable to most of our time points. Still, in *Manuscript III*, we observed that the difference between isotype IgG and OX26 immunoliposomes, with respect to splenic accumulation after 1 hour, was lost after 24 hours, suggesting that the impact of TfR-targeting on splenic accumulation may be an acute phenomenon that levels out days after the administration.

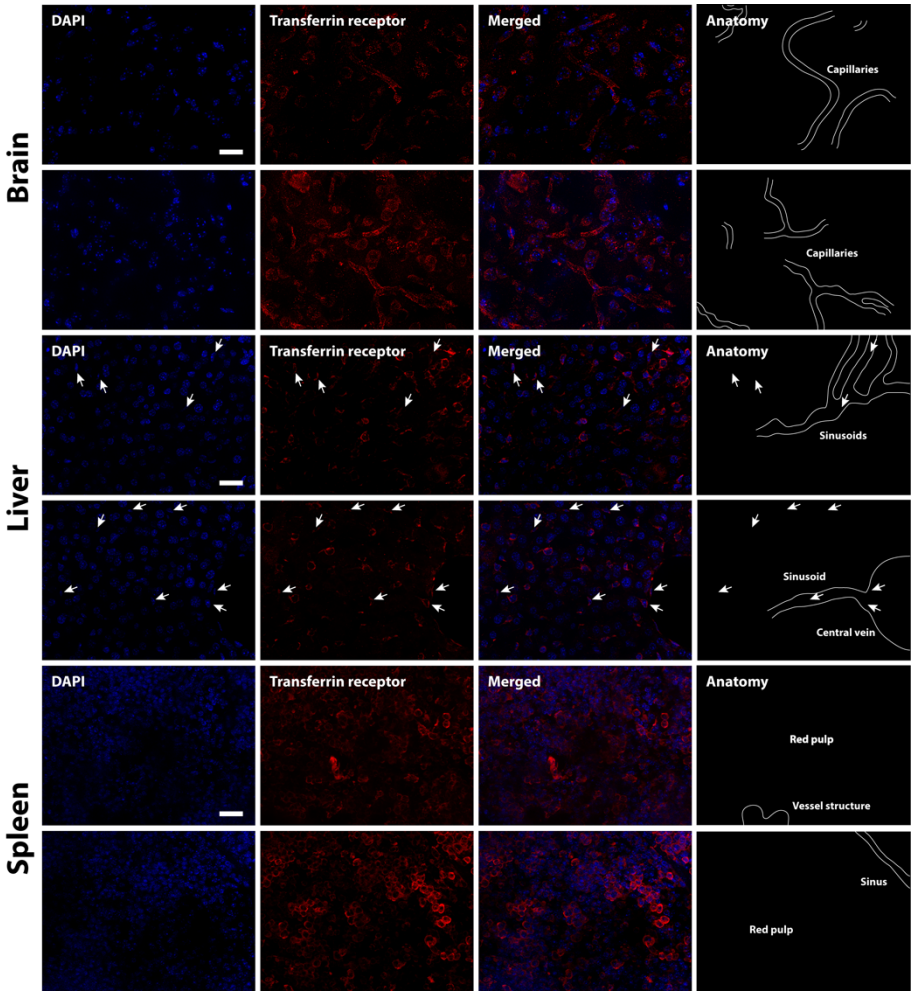


Figure 5.3.2. Immunohistochemistry staining of TfR in healthy mouse brain, liver, and spleen. In the brain, the expression of TfRs are distributed evenly across the tissue, hereby staining both the brain endothelial cells lining the capillaries, and the surrounding neuronal cell bodies. In the liver, the expression is observed both on the hepatocytes and on Kupffer cells (resident macrophages, arrow). In the spleen, the expression is mostly observed in the red pulp, where the tissue-specific macrophages are residing. Several microanatomical features are illustrated in the far-right column. Scale bars depict 20 µm.

Overcoming the issue of passive (or active) accumulation of nanoparticles in peripheral organs are a great interest for the field of nanomedicine. Particularly, the big reduction in clearance seen for PEGylated nanoparticles was a big stepping stone (199), although new findings indicate that the immune system will respond to the polymer molecules (215-221). Others have presented evidence that the molecule CD47 is crucial for omitting phagocytosis of nanoparticles (222, 223). For example,

by coating targeted nanoparticles (against ICAM-1, PECAM-1, and TfR) with CD47, the subsequent clearance was significantly reduced, although with an increase in lung targeting (222). This corresponded well with a recent finding in the field of extracellular vesicles (here: exosomes), where CD47 was found to be the reason why liposomes were cleared much faster than exosomes, and how CD47 expression on the exosome surface ensured that the exosome was not phagocytosed by macrophages (223).

In *Manuscript V*, we observed a severe adverse reaction after injecting the RI7 antibody-functionalized liposomes into the systemic circulation of mice. The reaction happened immediately after the dose had been given, and continued for approximately two hours before the mice showed complete recovery. There were no observable reactions in the mice treated with only mPEG oxaliplatin-loaded liposomes, which indicates that the cause of the adverse reaction converges to the RI7 antibodies. This is further underscored by the fact that there seemed to be a dose-dependency of the adverse effects with increasing numbers of deaths per group with increasing RI7 antibody density. The mice treated with RI7 antibody-functionalized AuNPs did not present with any adverse effects, which may be due to the overall low protein concentration in these formulations, and the possible inaccessibility of the conjugated RI7 antibodies for interacting with immune cells, as has been observed by others (224).

We interpreted the symptoms observed in the mice as a clear indication of hemolysis due to TfR-targeting on circulating reticulocytes, which constitutes the majority of TfRs residing in the systemic circulation (225, 226). This amounts to approximately 100,000 receptor molecules/cell with some interspecies differences (227, 228), which is comparable to the expression level in BCECs (229, 230). The expression will, however, be lost in the process yielding mature red blood cells (225). Nevertheless, the fact that such severe reactions can be induced by TfR-targeting should raise some concern when designing TfR-targeted drug constructs or nanomedicines for any disease indication (225, 231-233). With regards to the use of full anti-TfR antibodies as drugs for brain diseases, it was shown that by reducing the effector function of the Fc domain, the level of adverse effects (i.e. hemolysis) decreased substantially (225, 233), whereas complete removal of the Fc domain reduced peripheral toxicity of anti-DLL4 antibodies (234). Correlating with these observations, for many purposes of nanomedicine development, the use of full antibodies is often omitted in favor of single chain variable fragment or Fab fragments to reduce the potential of a severe peripheral toxicity profile (235, 236). No additional investigations into the possible adverse effects imposed on the BCECs were attempted, but studies on TfR antibodies show receptor degradation as a result of high-affinity targeting of the TfR with no signs of detrimental effects of the BBB integrity (193, 237-239).

5.4. RELEVANCE OF TRANSFERRIN RECEPTOR-TARGETING

The popularity of TfR as a target for drug delivery has not declined since the concept was originally proposed in the mid-1980s (240). In *Manuscript I* and *Manuscript II*, detailed literature reviews were presented regarding the use of TfR as a target for liposome-based brain drug delivery, or for brain drug delivery in general regardless of the choice of drug design. In this joint discussion, only a few major points will be summarized (141, 163).

Despite the discrepancy between the widely-accepted model for TfR sorting inside the BCECs, there is little doubt that the TfR has proven its relevance as a good target for getting drugs near the brain parenchyma (141, 151, 158, 163, 241, 242). This is evidenced in numerous reports, where the strategy has been tested in many diseases using many carriers. Common to many of these reports is the lack of investigation into the drug/nanoparticle transport itself in favor of more clinically relevant experimental outcomes such as improvements in preclinical models of neurodegenerative or neuromalignant diseases (141, 242). Some reports provide indirect evidence of transport, e.g. where PET tracers binding to targets inside the brain parenchyma indicate that successful transport did indeed happen (60, 243-245). The choice of leaving out any assessment of the transport qualities of the construct is not wrong, but it adds to the number of missed opportunities to gain knowledge about the transport process that we greatly need (242). The reason for this complaint is that despite loads of preclinical progress, we have yet to see this potential translated into clinically relevant ‘leads’ that can progress through the drug development process and become effective therapeutics for the patients (141, 242). Hence, the large number of positive papers regarding this targeting concept cannot hide the underlying disappointment of so few examples of clinical progression (e.g. TransMID (Tf-DT-CRM107) from Celtic Pharma, which was discontinued in early Phase III due to lack of improved therapeutic efficacy compared to standard care that could defend the very difficult handling and administration of the construct (246-249)). The picture is even worse for TfR-targeted nanomedicines, which illustrates how new innovations are crucial for the strategy to survive and prove its potential further than the preclinical stages (141, 163).

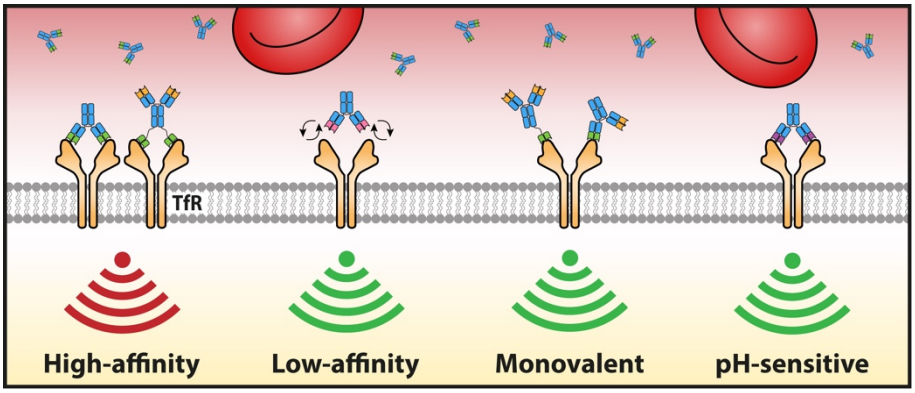


Figure 5.4.1. Overview of the literature regarding transport of anti-TfR antibodies into the brain as a function of affinity, valency, and pH-sensitivity. The transport of different binding strategies is illustrated with high-affinity (bivalent) antibodies (green variable domains) are characterized by low transport (red) into the brain parenchyma. Conversely, low-affinity (pink variable domains), monovalency (high-affinity, green variable fragments), and pH-sensitivity (purple variable fragments) improve the transport into the brain markedly (green), hereby yielding therapeutic effects of other antibody fragments targeting intraparenchymal targets such as β-amyloid (yellow variable fragments). The setup of the figure was inspired by *Freskgård et al.* (2017)(151). TfR: Transferrin receptor.

Fortunately, the recent years has seen some new design innovations to the targeting strategy (i.e. to the antibodies) that has proven much better than the previous designs both in rodent and non-human primates (Figure 5.4.1)(42, 193, 225, 238, 239, 243, 244, 250-253). This development has been driven largely by the biotechnological and pharmaceutical industries that had the required resources to study concepts in depth that had been proposed or indicated in studies 15 – 20 years ago (65, 193, 239, 254). What has come out of these efforts are indeed some fascinating results showing that it is not enough to choose the right target, one must also have detailed knowledge about the interaction (i.e. binding modus) between the ligand and the TfR, since this seems to depict the intracellular sorting route and subsequent potential for transport through the BCECs (151, 241, 255). Whether it is the modulation of the overall antibody affinity towards the TfR (or other targets like CD98hc)(193, 225, 238, 250-252), monovalent binding to the TfR (42, 193, 225, 238, 239, 243, 244, 250, 251), or even the pH-sensitivity of the binding (42, 253) will presumably be proven within the coming years, where it must be expected that these concepts will be tested in clinical trials.

In relation to nanomedicines, very little of the abovementioned new innovations for the TfR-targeting ligands has been tested on nanoparticles for brain drug delivery. The concept of affinity was tested to some degree in one study, but here, antibodies known to have a very high affinity served as the low-affinity variant on liposomes (256). The aspects of avidity and valency was also tested in a few studies (107, 257, 258), but

several questions still need to be answered. The next section is devoted to a discussion of the findings presented in this dissertation, and how these findings may improve our knowledge on relevant TfR-targeting ligands for brain drug delivery.

5.5. AFFINITY, AVIDITY, OR VALENCY – WHICH ONE IS THE HOLY GRAIL FOR BRAIN TARGETED NANOMEDICINES?

An underlying question in most of the projects described in this dissertation was whether the recently presented evidence on antibody binding modus and subsequent transport into the brain could be applied in a nanomedicine setting (193, 239, 252). We therefore took different approaches to study the affinity, avidity, and valency phenomena in relation to transport of nanoparticles into the brain. An overview of the findings is presented in Figure 5.5.1.

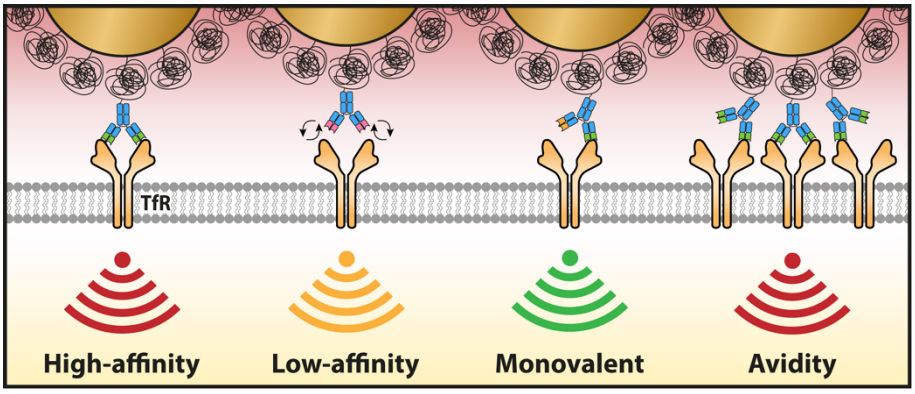


Figure 5.5.1. Overview of the findings related to nanoparticle transport into the brain as a function of affinity, avidity, and valency. The different strategies are illustrated as antibodies attached to AuNPs together with color coding representing their respective efficiencies in the studies presented in this dissertation. The use of high-affinity antibodies had a general low uptake (red) into the brain parenchyma, irrespective of the overall avidity of the antibodies on both AuNPs and liposomes. Low-affinity antibodies were found to produce modest increases (yellow) in the brain uptake of AuNPs, although the absolute amounts transported was still below 0.1 %ID/g. A monovalent binding antibody proved to be the best ligand for obtaining higher AuNP transport (green) into the brain parenchyma. The setup of the figure was inspired by *Freskgård et al.* (2017)(151). TfR: Transferrin receptor.

In Manuscript III (49), we sought to recapitulate previous findings of liposome-based transport into the brain, which is an area of great dispute (163). It was evident that targeting the TfR on the surface of BCECs was efficient for obtaining high accumulation of liposomes at the BBB, but transport of the liposomes into the brain parenchyma could not be evidenced by our data, even though we utilized the same fluorescence microscopy-based approach as presented in Reimold et al. (2008)(259). While fluorescent signals indicative of liposomes could be readily observed, these

signals could largely be explained by paraformaldehyde fixation artefacts, and as such, it was not possible to prove transcytosis of the TfR-targeted liposomes. These observations were in line with what was previously shown by our group using both OX26 immunoliposomes or the OX26 antibody itself. OX26 is a high-affinity antibody directed against the rat TfR, and according to the new knowledge on antibody transport, it should not be able to mediate transport across the BBB. This is mainly due to the bivalent binding of many TfRs on the BCEC surface, which will induce a multimerization of the receptors, not favoring subsequent intracellular sorting leading to trans-BBB transport (151). Still, the antibody density used in this study ranged between 0.7 and 1.0 * 10^3 antibodies/μm^2, which was shown to be favorable for nanoparticle delivery to the brain compared to higher densities (107, 257).

Instead, the cargo encapsulated in the OX26 immunoliposomes was found in the brain parenchyma. This finding suggests that even though the nanoparticle may not transport through the BBB, the cargo may be released to do so. The findings in Manuscript III on cargo transport showed a relatively high absolute concentration of cargo in the brain parenchyma, compared to what was shown for other types of cargo in TfR-targeted liposomes (49, 163). The concentration was also much higher than that measured in the brains of mice receiving RI7-functionalized, oxaliplatin-loaded immunoliposomes in Manuscript V. The discrepancy between these findings may be multifaceted. First, the experiments in Manuscript III were performed with n = 5 (49), whereas those in Manuscript V were performed with n = 8, thus allowing for more of the experimental variation to become visible. As a result, it is possible that the approximations of brain parenchymal transport of oxaliplatin after capillary depletion in Manuscript V are more valid. Second, the antibody reaction observed in animals receiving the RI7-functionalized immunoliposomes in Manuscript V, may have resulted in an increased clearance of the liposomes, which could result in an underestimation of the transport of oxaliplatin into the brain. Third, the species difference, and the difference in antibody may also have impacted the results of the two studies. If the difference in antibodies should have impacted the results, it suggests that the antibody is playing a role in the transport of the cargo into the brain parenchyma. However, it was especially evident in the data of Manuscript III (and Manuscript IV for AuNPs) that the pattern of transport into the brain seemed to scale with that of the initial uptake of the carrier into the BCECs (49). Therefore, we cannot rule out the interpretation that while the BCEC uptake may be correlated to the targeting qualities of the nanoparticle, the subsequent transport into the brain parenchyma may be the result of a passive process following the intracellular processing and sorting of the endocytosed nanoparticles.

In Manuscript IV, we had the opportunity to study the aspect of both affinity and valency on the transport of TfR-targeted nanoparticles into the brain. For this purpose, AuNPs was chosen as the model nanoparticle, because it allowed for several parallel analyses that would not have been possible when using liposomes. Most significant

was the adoption of TEM and EDS as techniques for validating the transport of AuNPs into the brain both by visualizing the individual AuNPs and prove their content of gold. The results of the study suggested that there may be some impact of reducing the affinity of the antibody attached to the AuNP surface, but that the most prominent increase in AuNP transport into the brain parenchyma was observed when using an antibody binding to the TfR monovalently. This is an important finding, because it underscores the theory put forward by others that multivalent binding does not favor transport into the brain of antibodies or nanomedicines (151). Hence, for an antibody with a monovalent binding modus (bi-specific antibody or brain shuttle construct), the transport into the brain parenchyma is increased, and it seems (based on our data) that in situations of low antibody density, it holds true for nanomedicines as well. These findings are, however, based on work on the TfR only, and other reports suggest bivalent targeting modes to be beneficial for other BBB targets (260).

In the seminal papers by the group of Mark E. Davis, it was shown that while an optimized density of endogenous Tf on AuNPs (analogous to a monovalent binding antibody) led to significantly increased brain transport, these findings could not be recapitulated using the same density of anti-TfR antibodies (clone RI7). The results of Manuscript IV support this notion, although here in a setting where the IgG protein structure is present in all the ligands compared. While whole nanoparticle transport could not be evidenced in Manuscript III (49), the experimental setup in Manuscript IV allowed for studying this aspect, since the AuNPs were not expected to be degraded. Importantly, it was possible measure increased concentrations of gold in the parenchymal fraction of mouse brains subjected to brain capillary depletion, and the AuNPs could be visualized using TEM to be present in the brain parenchyma (e.g. in neural processes). This underscores the findings of several papers showing that transport of the AuNPs across the BBB is possible, albeit often at very low levels, and with a clear impact of the AuNP hydrodynamic diameter on the absolute efficacy of transport (107, 258, 261-268). This does, however, oppose older findings showing that the endothelial basement membrane of the BBB will function as another barrier beyond the first barrier, hereby inhibiting the movement of nanoparticles into the brain parenchyma (269). In addition to the impact of the basement membrane, the AuNPs could also be bound to the TfR when exposed to the abluminal side of the BBB, which would inhibit its entrance into the brain parenchyma (267, 268). Nevertheless, we and others observe AuNPs to be present in to brain parenchyma (albeit in low numbers), which illustrates that some nanoparticle transport across both the BCECs and the underlying basement membrane is possible (107, 258, 263). Further studies should elucidate this transport process further, e.g. by injecting cationic AuNPs into mice and evaluate whether these AuNPs are sequestered in the basement membrane and inhibited from entering the brain parenchyma.

As described above, the brain uptake data in Manuscript IV seems to fall into the same pattern as the initial uptake (i.e. gold concentration) into the BCECs, and the interpretation of this could be that the release of the AuNPs from the BCECs is an

unspecific process that it not impacted by the attached ligand. Acknowledging this possibility, an alternative interpretation is that what is observed is largely mediated by the monovalent binding modus of the anti-TfR^A/BACE1 antibody. Resolving this is difficult, because some of the results in Manuscript IV does not follow the theory of high-affinity ligands. It is striking that we were unable to prove a higher level of capillary uptake of the anti-TfR^A AuNPs (at least compared to anti-TfR^D AuNPs), since such an observation would support the notion of high-affinity antibody (and nanoparticle) confinement to the brain capillaries. However, basing the expected outcome on findings made for single antibodies may not be fully reasonable because of possible nanoparticle-related uptake and sorting processes. For example, one could speculate that a part of the sorting between nanoparticles that are able or unable to transport in the BCEC happened already at the level of BCEC uptake, where the TfR multimerization and distorted intracellular signaling induced by high-affinity and multivalent binding would halt the endocytosis process. This seems not to be the case for single antibodies, where uptake was evidenced in many studies (correlating with TfR degradation)(65, 66, 69, 238, 239, 270), but whether it impacts the uptake of nanoparticle should be studied further. An interesting observation can be made by comparing the data obtained in Manuscript IV and Manuscript V. In Manuscript V, the group functionalized with the highest density ($0.6 * 10^3$ antibodies/μm^2) had an uptake of 0.15 and 0.05 %ID/g in the brain capillaries and parenchyma, respectively, whereas in Manuscript IV, these numbers were 0.2 and 0.04 %ID/g for the anti-TfR^A AuNP group. Both antibodies presented with a high-affinity against the mouse TfR when analyzed using surface plasmon resonance (anti-TfR^A: K_D = 21 nM, RI7: K_D = 6 nM), which is meaningful taking their equal behavior in vivo into account. Thus, the level of uptake of anti-TfR^A AuNPs may be what can be expected from these high-affinity antibodies on AuNPs. This was not quantified directly in other studies using RI7 antibodies for AuNP functionalization, making direct comparisons impossible (258). Based on our observations, we suggest that monovalently binding TfR-ligands are relevant to pursue in future studies of brain drug delivery via nanomedicines.

In Manuscript V, the aspect of avidity/ligand density was studied in parallel on oxaliplatin-loaded immunoliposomes and AuNPs. This allowed for assessing both the transport of whole nanoparticles (AuNPs) and an encapsulated cargo (oxaliplatin). The choice of ligand density was based on a thorough literature review of published studies regarding either liposomes or AuNPs. This review illustrated that ligand density in many studies centered around 10^3 antibodies/μm^2 with indications that this ligand density was more appropriate for trans-BBB transport of nanoparticles compared to higher densities. This study was therefore dedicated to analyzing densities lower than 10^3 antibodies/μm^2 to see if the transport could be increased even further, or whether there existed a lower limit for binding, and hence, transport into the brain. The choice of antibody for the study was clone RI7 (full clone code: RI7217)(271, 272), which had been studied in our laboratory before, and shown to bind the mouse TfR with high-affinity. This antibody (like several other high-affinity anti-TfR antibodies (65)) was shown to be confined inside the BCECs after

endocytosis (66, 270), and thus, we did not expect it to mediate high levels of transport into the brain parenchyma.

The findings of the study indicated that a lower limit of antibody density does exist, where no additional transport capacity is obtained compared to mPEG versions of the nanoparticles (AuNPs or liposomes). This was most evident for the AuNPs, whereas more variation was observed in the data relating to uptake of the oxaliplatin-loaded liposomes. Importantly, in vivo (and partly in vitro), the highest density of antibodies mediated the highest level of transport across the BBB (albeit in disappearingly small concentrations for both AuNPs and oxaliplatin-loaded liposomes), thus serving as validation for this observation. The inefficient transport of AuNPs and oxaliplatin supports the findings of several other reports using both the free RI7 antibodies or conjugated to AuNPs (66, 258, 270).

Surprisingly, the two model nanoparticles studied in Manuscript V (AuNPs vs. oxaliplatin encapsulated in liposomes) behaved very similarly with respect to the fraction of the dose that reached the brain side of the BBB. This was also true when comparing the in vitro and in vivo data. The logical interpretation of this would be that both the AuNPs and liposomes are transported as whole nanoparticles across the BBB. However, due to time constraints, no TEM analysis was performed for this study before submission of this dissertation, and so, this interpretation will only rely on the validity and robustness of the brain capillary depletion method (49, 64). Furthermore, the AuNPs have a hydrodynamic diameter of approximately 70 nm, whereas the liposome diameter is twice as big, which raises the possibility that AuNPs fall within a size range, where full transcytosis is possible, whereas the liposomes are too big for this to happen. This is underscored in many studies, where an impact of size has been described for many types of nanoparticles (107, 273-275). An additional factor to this discussion is the observation that the most realistic outcome in an interaction between a liposome (lipid bilayer) and a BCEC surface is fusion between the two (74). Still, as described above, we observed a severe reaction to the antibodies on the liposome surface (presumably a reaction against the rat Fc domain or the TfR-targeting itself), which may have caused an increase in the clearance of the liposomes from the circulation, wherefore the uptake of oxaliplatin into the brain parenchyma should be expected to be higher, and hence, approaching that of Manuscript III (49).

5.6. CAN TFR-TARGETED NANOMEDICINES BE MODIFIED TO IMPROVE BRAIN UPTAKE FURTHER?

Based on the results described throughout *Manuscript III – V*, TfR-mediated targeting seems to be a viable way to ensure preferential accumulation of nanoparticles at the BBB. This was evidence by varying degree of upconcentration of nanoparticles in BCECs compared to the amounts found in the brain parenchyma or whole brain homogenate. The transport across the BBB and into the brain parenchyma is, however, still very low regardless of the improvements presented in this dissertation.

The studies presented in this dissertation are all characterized by being acute with respect to the time points of analysis in the experimental setup, and therefore, we do not know how the nanomedicine strategies will behave as a continuously administrated drug. Also, we do not know if the low level of transport that we observe will be enough to mediate a therapeutic effect of the encapsulated cargo. Therefore, we can ask the following questions to be answered by future research:

- Will a continuous dosing regimen lead to intraparenchymal accumulation of nanoparticles larger than observed for single dosing?

- What is the impact of continuous dosing on peripheral organs?

- What is a relevant drug concentration in the brain parenchyma, and can this be achieved via nanomedicine-based drug delivery?

- Is the distribution of drugs entering the brain parenchyma uniform, or will specific regions be more fitted for nanomedicine-based drug delivery?

- Can the pathophysiological process of brain diseases be exploited for stimuli-sensitive drug release or nanoparticle charge reversal?

- Can dual-targeting approach be employed to transport through the BBB using two receptor transport systems?

CHAPTER 6. CONCLUSION

In conclusion, the projects described in this dissertation have provided new insight into the utility of TfR-targeting for brain drug delivery. The results presented throughout the different projects have shown that TfR-targeting can facilitate acute accumulation in the brain capillaries following intravenous administration, which illustrates the exclusive endothelial expression of this protein in the brain capillaries. Transport of nanoparticles and encapsulated cargo was in general increased with TfR-targeting, with pronounced effects of antibody binding modus on the absolute amounts accumulating inside the brain parenchyma. The observed increases in brain transport of nanoparticles or cargo may suffice a viable way of increasing drug exposure to diseased regions inside the brain parenchyma, although further studies should investigate how much accumulation that can be achieved with multiple dosing over time. Furthermore, the projects have provided large amounts of biodistribution data to locate the nanoparticles not entering the brain parenchyma. These peripheral tissues with high accumulation of nanoparticles may be sites of adverse effects upon continuous treatment using such a drug delivery strategy. Importantly, the results of the different biodistribution analyses clearly indicate that TfR-targeting will increase the nanoparticle exposure in the spleen. While this likely illustrates a high level of macrophage uptake, there should be a focus on this increased accumulation and potential adverse effects that this would facilitate. Lastly, efforts were made to provide knowledge about another administration route, namely intracerebroventricular administration. Using this administration route, nanoparticle penetration was seen in the brain cortex alongside the penetrating arteries, especially if the nanoparticles had a net negative surface charge. This suggests that deep areas of the brain can be reached by direct administration, although the value of this technique will have to be validated to show that this nanoparticle distribution will lead to efficient drug exposure inside the brain parenchyma. Together, these findings may serve as an inspiration for the design of nanoparticle-based drug delivery strategies of the future. If the TfR-mediated transport into the brain parenchyma is deemed too small, the future strategies could possibly include a controlled release mechanism into the nanoparticle design, e.g. to mediate a surface charge-based uptake across the BBB after initial TfR-mediated accumulation of nanoparticles in the brain capillaries. The way forward is thus to develop strategies to efficiently exit the BCECs towards the brain parenchyma.

LITERATURE LIST

1. Helms HC, et al. (2016) In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use. *J Cereb Blood Flow Metab* 36(5):862–890.

2. Siupka P, et al. (2017) Bidirectional apical-basal traffic of the cation-independent mannose-6-phosphate receptor in brain endothelial cells. *J Cereb Blood Flow Metab* 37(7):2598–2613.

3. Armulik A, et al. (2010) Pericytes regulate the blood-brain barrier. *Nature* 468(7323):557–561.

4. Villaseñor R, et al. (2016) Trafficking of Endogenous Immunoglobulins by Endothelial Cells at the Blood-Brain Barrier. *Sci Rep* 6(1):25658.

5. Percie du Sert N, et al. (2017) The Experimental Design Assistant. *PLoS Biol* 15(9):e2003779.

6. Percie du Sert N, et al. (2017) The Experimental Design Assistant. *Nat Methods*. doi:10.1038/nmeth.4462.

7. Holman C, et al. (2016) Where Have All the Rodents Gone? The Effects of Attrition in Experimental Research on Cancer and Stroke. *PLoS Biol* 14(1):e1002331.

8. Joó F, Karnushina I (1973) A procedure for the isolation of capillaries from rat brain. *Cytobios* 8(29):41–48.

9. Mrsulja BB, Mrsulja BJ, Fujimoto T, Klatzo I, Spatz M (1976) Isolation of brain capillaries: a simplified technique. *Brain Research* 110(2):361–365.

10. Miller DS, et al. (2000) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol Pharmacol* 58(6):1357–1367.

11. Hartz AMS, Miller DS, Bauer B (2010) Restoring blood-brain barrier P-glycoprotein reduces brain amyloid-beta in a mouse model of Alzheimer's disease. *Mol Pharmacol* 77(5):715–723.

12. Erdlenbruch B, et al. (2003) Alkylglycerol opening of the blood-brain barrier to small and large fluorescence markers in normal and C6 glioma-bearing rats and isolated rat brain capillaries. *Br J Pharmacol* 140(7):1201–1210.

13. Fricker G, Nobmann S, Miller DS (2002) Permeability of porcine blood brain barrier to somatostatin analogues. *Br J Pharmacol* 135(5):1308–1314.

14. Hartz AMS, Bauer B, Fricker G, Miller DS (2006) Rapid modulation of P-glycoprotein-mediated transport at the blood-brain barrier by tumor necrosis factor-alpha and lipopolysaccharide. *Mol Pharmacol* 69(2):462–470.

15. DeBault LE, Cancilla PA (1980) gamma-Glutamyl transpeptidase in isolated brain endothelial cells: induction by glial cells in vitro. *Science* 207(4431):653–655.

16. Tao-Cheng JH, Nagy Z, Brightman MW (1987) Tight junctions of brain endothelium in vitro are enhanced by astroglia. *J Neurosci* 7(10):3293–3299.

17. Dehouck MP, Méresse S, Delorme P, Fruchart JC, Cecchelli R (1990) An easier, reproducible, and mass-production method to study the blood-brain barrier in vitro. *J Neurochem* 54(5):1798–1801.

18. Cecchelli R, et al. (1999) In vitro model for evaluating drug transport across the blood-brain barrier. *Adv Drug Deliv Rev* 36(2-3):165–178.

19. Gaillard PJ, et al. (2001) Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* 12(3):215–222.

20. Cohen-Kashi Malina K, Cooper I, Teichberg VI (2009) Closing the gap between the in-vivo and in-vitro blood-brain barrier tightness. *Brain Research* 1284:12–21.

21. Abbott NJ, Dolman DEM, Drndarski S, Fredriksson SM (2012) An improved in vitro blood-brain barrier model: rat brain endothelial cells co-cultured with astrocytes. *Methods Mol Biol* 814(Chapter 28):415–430.

22. Boveri M, et al. (2005) Induction of blood-brain barrier properties in cultured brain capillary endothelial cells: comparison between primary glial cells and C6 cell line. *Glia* 51(3):187–198.

23. Culot M, et al. (2008) An in vitro blood-brain barrier model for high throughput (HTS) toxicological screening. *Toxicol In Vitro* 22(3):799–811.

24. Perrière N, et al. (2007) A functional in vitro model of rat blood-brain

barrier for molecular analysis of efflux transporters. *Brain Research* 1150:1–13.

25. Nakagawa S, et al. (2007) Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. *Cell Mol Neurobiol* 27(6):687–694.

26. Dohgu S, et al. (2005) Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-beta production. *Brain Research* 1038(2):208–215.

27. Hayashi K, et al. (2004) Effects of hypoxia on endothelial/pericytic co-culture model of the blood-brain barrier. *Regul Pept* 123(1-3):77–83.

28. Vandenhaute E, et al. (2011) Modelling the neurovascular unit and the blood-brain barrier with the unique function of pericytes. *Curr Neurovasc Res* 8(4):258–269.

29. Wilhelm I, Fazakas C, Krizbai IA (2011) In vitro models of the blood-brain barrier. *Acta Neurobiol Exp (Wars)* 71(1):113–128.

30. Hatherell K, Couraud P-O, Romero IA, Weksler B, Pilkington GJ (2011) Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models. *J Neurosci Methods* 199(2):223–229.

31. Daneman R, Zhou L, Kebede AA, Barres BA (2010) Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468(7323):562–566.

32. Bell RD, et al. (2010) Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron* 68(3):409–427.

33. Hall CN, et al. (2014) Capillary pericytes regulate cerebral blood flow in health and disease. *Nature*. doi:10.1038/nature13165.

34. Hill RA, et al. (2015) Regional Blood Flow in the Normal and Ischemic Brain Is Controlled by Arteriolar Smooth Muscle Cell Contractility and Not by Capillary Pericytes. *Neuron* 87(1):95–110.

35. Thomsen MS, Birkelund S, Burkhart A, Stensballe A, Moos T (2017) Synthesis and deposition of basement membrane proteins by primary brain capillary endothelial cells in a murine model of the blood-brain barrier. *J Neurochem* 140(5):741–754.

36. Armulik A, Genové G, Betsholtz C (2011) Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell* 21(2):193–215.

37. Wagner EF, Risau W (1994) Oncogenes in the study of endothelial cell growth and differentiation. *Semin Cancer Biol* 5(2):137–145.

38. Roux F, et al. (1994) Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. *J Cell Physiol* 159(1):101–113.

39. Weksler BB, et al. (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J* 19(13):1872–1874.

40. Greiffenberg L, et al. (1998) Interaction of Listeria monocytogenes with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. *Infect Immun* 66(11):5260–5267.

41. Vandenhaute E, Sevin E, Hallier-Vanuxeem D, Dehouck M-P, Cecchelli R (2012) Case study: adapting in vitro blood-brain barrier models for use in early-stage drug discovery. *Drug Discov Today* 17(7-8):285–290.

42. Sade H, et al. (2014) A human blood-brain barrier transcytosis assay reveals antibody transcytosis influenced by pH-dependent receptor binding. *PLoS ONE* 9(4):e96340.

43. Ogunshola OO (2011) In vitro modeling of the blood-brain barrier: simplicity versus complexity. *Curr Pharm Des* 17(26):2755–2761.

44. Ahmad Al A, Taboada CB, Gassmann M, Ogunshola OO (2011) Astrocytes and pericytes differentially modulate blood-brain barrier characteristics during development and hypoxic insult. *J Cereb Blood Flow Metab* 31(2):693–705.

45. Stanimirovic DB, Bani-Yaghoub M, Perkins M, Haqqani AS (2015) Blood-brain barrier models: in vitro to in vivo translation in preclinical development of CNS-targeting biotherapeutics. *Expert Opin Drug Discov* 10(2):141–155.

46. Burkhart A, et al. (2015) Transfection of brain capillary endothelial cells in primary culture with defined blood-brain barrier properties. *Fluids Barriers CNS* 12(1):19.

47. Burkhart A, et al. (2016) Expression of Iron-Related Proteins at the Neurovascular Unit Supports Reduction and Reoxidation of Iron for

Transport Through the Blood-Brain Barrier. *Mol Neurobiol* 53(10):7237–7253.

48. Burkhart A, Andresen TL, Aigner A, Thomsen LB, Moos T (2017) Transfection of primary brain capillary endothelial cells for protein synthesis and secretion of recombinant erythropoietin: a strategy to enable protein delivery to the brain. *Cell Mol Life Sci*. doi:10.1007/s00018-017-2501-5.

49. Johnsen KB, et al. (2017) Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma. *Sci Rep* 7(1):437.

50. Helms HC, et al. (2014) An Electrically Tight In Vitro Blood-Brain Barrier Model Displays Net Brain-to-Blood Efflux of Substrates for the ABC Transporters, P-gp, Bcrp and Mrp-1. *AAPS J*:1–10.

51. Thomsen LB, Burkhart A, Moos T (2015) A Triple Culture Model of the Blood-Brain Barrier Using Porcine Brain Endothelial cells, Astrocytes and Pericytes. *PLoS ONE* 10(8):e0134765.

52. Lippmann ES, et al. (2012) Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. *Nat Biotechnol* 30(8):783–791.

53. Lippmann ES, Ahmad Al A, Azarin SM, Palecek SP, Shusta EV (2014) A retinoic acid-enhanced, multicellular human blood-brain barrier model derived from stem cell sources. *Sci Rep* 4(1):178.

54. Hersom M, et al. (2016) Transferrin receptor expression and role in transendothelial transport of transferrin in cultured brain endothelial monolayers. *Mol Cell Neurosci* 76:59–67.

55. Berg C (2016) Quantitative analysis of nanoparticle transport through in vitro blood-brain barrier models. *Tissue Barriers* 4(1):e1143545.

56. Bramini M, et al. (2014) Imaging approach to mechanistic study of nanoparticle interactions with the blood-brain barrier. *ACS Nano* 8(5):4304–4312.

57. Sharma G, Modgil A, Zhong T, Sun C, Singh J (2014) Influence of short-chain cell-penetrating peptides on transport of Doxorubicin encapsulating receptor-targeted liposomes across brain endothelial barrier. *Pharm Res* 31(5):1194–1209.

58. Papadia K, et al. (2017) Multifunctional LUV liposomes decorated for BBB and amyloid targeting - B. In vivo brain targeting potential in wild-

type and APP/PS1 mice. *Eur J Pharm Sci* 102:180–187.

59. Henriksen JR, et al. (2015) Remote Loading of (64)Cu(2+) into Liposomes without the Use of Ion Transport Enhancers. *ACS Appl Mater Interfaces* 7(41):22796–22806.

60. Sehlin D, et al. (2016) Antibody-based PET imaging of amyloid beta in mouse models of Alzheimer's disease. *Nat Commun* 7:10759.

61. Markoutsa E, et al. (2014) Mono and dually decorated nanoliposomes for brain targeting, in vitro and in vivo studies. *Pharm Res* 31(5):1275–1289.

62. Johnsen KB, Moos T (2016) Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release* 222:32–46.

63. Bickel U (2005) How to measure drug transport across the blood-brain barrier. *NeuroRx* 2(1):15–26.

64. Triguero D, Buciak J, Pardridge WM (1990) Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins. *J Neurochem* 54(6):1882–1888.

65. Moos T, Morgan EH (2001) Restricted transport of anti-transferrin receptor antibody (OX26) through the blood-brain barrier in the rat. *J Neurochem* 79(1):119–129.

66. Paris-Robidas S, Brouard D, Emond V, Parent M, Calon F (2016) Internalization of targeted quantum dots by brain capillary endothelial cells in vivo. *J Cereb Blood Flow Metab* 36(4):731–742.

67. Pardridge WM, Yoshikawa T, Kang YS, Miller LP (1994) Blood-brain barrier transport and brain metabolism of adenosine and adenosine analogs. *J Pharmacol Exp Ther* 268(1):14–18.

68. Samii A, Bickel U, Stroth U, Pardridge WM (1994) Blood-brain barrier transport of neuropeptides: analysis with a metabolically stable dermorphin analogue. *Am J Physiol* 267(1 Pt 1):E124–31.

69. Gosk S, Vermehren C, Storm G, Moos T (2004) Targeting anti-transferrin receptor antibody (OX26) and OX26-conjugated liposomes to brain capillary endothelial cells using in situ perfusion. *J Cereb Blood Flow Metab* 24(11):1193–1204.

70. van Rooy I, Mastrobattista E, Storm G, Hennink WE, Schiffelers RM (2011) Comparison of five different targeting ligands to enhance

accumulation of liposomes into the brain. *J Control Release* 150(1):30–36.

71. Freskgård PO, Niewoehner J, Urich E (2014) *Time to open the blood-brain barrier gate for biologics?* (Future Neurology) doi:10.2217/fnl.14.15.

72. Hammarlund-Udenaes M (2017) Microdialysis as an Important Technique in Systems Pharmacology-a Historical and Methodological Review. *AAPS J*:1–10.

73. Hammarlund-Udenaes M, Fridén M, Syvänen S, Gupta A (2008) On the rate and extent of drug delivery to the brain. *Pharm Res* 25(8):1737–1750.

74. Lindqvist A, Fridén M, Hammarlund-Udenaes M (2016) Pharmacokinetic considerations of nanodelivery to the brain: Using modeling and simulations to predict the outcome of liposomal formulations. *Eur J Pharm Sci* 92:173–182.

75. Male D, Gromnicova R, McQuaid C (2016) Gold Nanoparticles for Imaging and Drug Transport to the CNS. *Int Rev Neurobiol* 130:155–198.

76. Linge KL, Jarvis KE (2009) Quadrupole ICP-MS: Introduction to Instrumentation, Measurement Techniques and Analytical Capabilities. *Geostandards and Geoanalytical Research* 33(4):445–467.

77. Pröfrock D, Prange A (2012) Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) for Quantitative Analysis in Environmental and Life Sciences: A Review of Challenges, Solutions, and Trends. *Appl Spectrosc* 66(8):843–868.

78. Delafiori J, Ring G, Furey A (2016) Clinical applications of HPLC–ICP-MS element speciation: A review. *Talanta* 153:306–331.

79. Sewell SL, Higgins MM, Bell CS, Giorgio TD (2011) Quantification of quantum dot concentration using inductively coupled plasma-mass spectrometry (ICP-MS). *J Biomed Nanotechnol* 7(5):685–690.

80. Frellsen AF, et al. (2016) Mouse Positron Emission Tomography Study of the Biodistribution of Gold Nanoparticles with Different Surface Coatings Using Embedded Copper-64. *ACS Nano* 10(11):9887–9898.

81. Danscher G (1984) Autometallography. A new technique for light and electron microscopic visualization of metals in biological tissues (gold, silver, metal sulphides and metal selenides). *Histochemistry* 81(4):331–335.

82. Danscher G, Stoltenberg M (2006) Silver enhancement of quantum dots resulting from (1) metabolism of toxic metals in animals and humans, (2) in vivo, in vitro and immersion created zinc-sulphur/zinc-selenium nanocrystals, (3) metal ions liberated from metal implants and particles. *Prog Histochem Cytochem* 41(2):57–139.

83. Liesegang RE (1911) Die Kolloidchemie der histologischen Silberfarbungen. *Kolloidchemische Beihefte*.

84. DeFelipe J, Jones EG (1992) Santiago Ramón y Cajal and methods in neurohistology. *Trends Neurosci* 15(7):237–246.

85. Delgado-García JM (2015) Cajal and the Conceptual Weakness of Neural Sciences. *Front Neuroanat* 9:128.

86. Liesegang RE, Rieder W (1921) Versuche mit einer "'Keimmethode'" zum Nachweis von Silber in Gewebsschnitten. *Z Wiss Mikrosk*.

87. Zeiger K (1938) Physikochemische Grundlagen der histologischen Methodik. *Wiss Forschungsber*.

88. Timm F (1958) Zur Histochemie der Schwermetalle - Das Sulfid-Silberverfahren. *Dtsch Z Gerichtl Med*.

89. Timm F (1961) Der histochemische nachweis des kupfers im gehirn. *Histochemie*.

90. Timm F (1962) Histochemische Lokalisation und Nachweis der Schwermetalle. *Acta Histochem*.

91. Danscher G (1981) Localization of gold in biological tissue. A photochemical method for light and electronmicroscopy. *Histochemistry* 71(1):81–88.

92. Danscher G (1981) Light and electron microscopic localization of silver in biological tissue. *Histochemistry* 71(2):177–186.

93. Hacker G, et al. (1996) Electron Microscopical Autometallography: Immunogold-Silver Staining (IGSS) and Heavy-Metal Histochemistry. *Methods* 10(2):257–269.

94. Danscher G, Nörgaard JO (1983) Light microscopic visualization of colloidal gold on resin-embedded tissue. *J Histochem Cytochem* 31(12):1394–1398.

95. Danscher G, Hansen HJ, Møller-Madsen B (1984) Energy dispersive X-

ray analysis of tissue gold after silver amplification by physical development. *Histochemistry* 81(3):283–285.

96. Christensen MM, Danscher G, Ellermann-Eriksen S, Schiønning JD, Rungby J (1992) Autometallographic silver-enhancement of colloidal gold particles used to label phagocytic cells. *Histochemistry* 97(3):207–211.

97. Sadauskas E, et al. (2007) Kupffer cells are central in the removal of nanoparticles from the organism. *Part Fibre Toxicol* 4:10.

98. Sadauskas E, et al. (2009) Biodistribution of gold nanoparticles in mouse lung following intratracheal instillation. *Chem Cent J* 3(1):16.

99. Danscher G (2002) In vivo liberation of gold ions from gold implants. Autometallographic tracing of gold in cells adjacent to metallic gold. *Histochem Cell Biol* 117(5):447–452.

100. Jensen SA, et al. (2013) Spherical nucleic acid nanoparticle conjugates as an RNAi-based therapy for glioblastoma. *Sci Transl Med* 5(209):209ra152–209ra152.

101. Wiley DT, Webster P, Gale A, Davis ME (2013) Transcytosis and brain uptake of transferrin-containing nanoparticles by tuning avidity to transferrin receptor. *Proc Natl Acad Sci USA* 110(21):8662–8667.

102. Clark AJ, Davis ME (2015) Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core. *Proc Natl Acad Sci USA* 112(40):12486–12491.

103. Bickel U, Kang YS, Yoshikawa T, Pardridge WM (1994) In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. *J Histochem Cytochem* 42(11):1493–1497.

104. Danscher G, Locht LJ (2010) In vivo liberation of silver ions from metallic silver surfaces. *Histochem Cell Biol* 133(3):359–366.

105. Kuntsche J, Horst JC, Bunjes H (2011) Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems. *Int J Pharm* 417(1-2):120–137.

106. Goodhew P (2011) General Introduction to Transmission Electron Microscopy (TEM). *Aberration-Corrected Analytical Transmission Electron Microscopy*, Brydson/Aberration-Corrected Analytical Transmission Electron Microscopy. (John Wiley & Sons, Ltd, Chichester, UK), pp 1–19.

107. Wiley DT, Webster P, Gale A, Davis ME (2013) Transcytosis and brain uptake of transferrin-containing nanoparticles by tuning avidity to transferrin receptor. *Proc Natl Acad Sci USA* 110(21):8662–8667.

108. Gromnicova R, et al. (2016) Localization and mobility of glucose-coated gold nanoparticles within the brain. *Nanomedicine (Lond)* 11(6):617–625.

109. Kempen PJ, et al. (2013) Locating and Characterizing Self-Assembled Gadolinium Chelate Nanoparticles in Stem Cells Using TEM. *Microsc Microanal* 19(S2):178–179.

110. Pallotto M, Watkins PV, Fubara B, Singer JH, Briggman KL (2015) Extracellular space preservation aids the connectomic analysis of neural circuits. *eLife* 4:108.

111. Röntgen WC (1896) On a new kind of rays. *Science* 3(59):227–231.

112. Röntgen WC (1896) A new form of radiation. *Science* 3(72):726–729.

113. Barkla CG (1906) Secondary Röntgen Rays and Atomic Weight. *Nature* 73(1894):365–365.

114. Barkla CG (1907) The Atomic Weight of Nickel. *Nature* 75(1946):368–368.

115. Barkla CG, Sadler CA (1907) Secondary x-rays and the atomic weight of nickel. *Philosophical Magazine Series 6* 14(81):408–422.

116. Barkla CG, Sadler CA (1908) Homogeneous secondary Röntgen radiations. *Philosophical Magazine Series 6* 16(94):550–584.

117. Barkla CG (1909) Ionisation by Röntgen Rays. *Nature* 80(2059):187–187.

118. Barkla CG (1911) The spectra of the fluorescent Röntgen radiations. *Philosophical Magazine Series 6* 22(129):396–412.

119. Moseley HGJ, Darwin CG (1913) The reflexion of the X-rays. *Philosophical Magazine Series 6* 26(151):210–232.

120. Moseley HGJ (1913) The high-frequency spectra of the elements. *Philosophical Magazine Series 6* 26(156):1024–1034.

121. Moseley HGJ (1913) The Attainment of High Potentials by the Use of Radium. *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences* 88(605):471–476.

122. Moseley HGJ (1914) The high-frequency spectra of the elements. Part II. *Philosophical Magazine Series 6* 27(160):703–713.

123. Moseley HGJ, Robinson H (1914) The number of ions produced by the β and γ radiations from radium. *Philosophical Magazine Series 6* 28(165):327–337.

124. Sinclair R, Kempen PJ, Chin R, Koh AL (2014) The Stanford Nanocharacterization Laboratory (SNL) and Recent Applications of an Aberration-Corrected Environmental Transmission Electron Microscope. *Adv Eng Mater* 16(5):476–481.

125. Corbari L, et al. (2008) Iron oxide deposits associated with the ectosymbiotic bacteria in the hydrothermal vent shrimp *Rimicaris exoculata*. *Biogeosciences* 5(5):1295–1310.

126. Vaughan D (2008) Energy Dispersive X-ray Microanalysis - An Introduction. *Thermo Scientific - Brief Primer*.

127. Goldstein JI, et al. (1981) *Scanning Electron Microscopy and X-Ray Microanalysis* (Springer) doi:10.1007/978-1-4939-6676-9.

128. Brydson R, Hondow N (2011) Electron Energy Loss Spectrometry and Energy Dispersive X-ray Analysis. *Aberration-Corrected Analytical Transmission Electron Microscopy*, Brydson/Aberration-Corrected Analytical Transmission Electron Microscopy. (John Wiley & Sons, Ltd, Chichester, UK), pp 211–240.

129. Neuwelt E, et al. (2008) Strategies to advance translational research into brain barriers. *The Lancet Neurology* 7(1):84–96.

130. Doody RS, et al. (2014) Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med* 370(4):311–321.

131. Liu-Seifert H, et al. (2015) Delayed-start analysis: Mild Alzheimer's disease patients in solanezumab trials, 3.5 years. *Alzheimer's & Dementia: Translational Research & Clinical Interventions* 1(2):111–121.

132. Siemers ER, et al. (2016) Phase 3 solanezumab trials: Secondary outcomes in mild Alzheimer's disease patients. *Alzheimers Dement* 12(2):110–120.

133. McCartney M (2015) The "breakthrough" drug that“s not been shown to help in Alzheimer”s disease. *BMJ* 351:h4064.

134. Lilly E (2016) Lilly Announces Top-Line Results of Solanezumab Phase 3 Clinical Trial. *httpsinvestorlillycomreleasedetailcfmReleaseID*. Available

at: https://investor.lilly.com/releasedetail.cfm?ReleaseID=1000871 [Accessed October 18, 2017].

135. Lilly E (2017) Progress of Mild Alzheimer's Disease in Participants on Solanezumab Versus Placebo (EXPEDITION 3). *httpsclinicaltrialsgovctshowrecordNCT*. Available at: https://clinicaltrials.gov/ct2/show/record/NCT01900665 [Accessed October 18, 2017].

136. The Lancet Neurology (2017) Solanezumab: too late in mild Alzheimer's disease? *The Lancet Neurology* 16(2):97.

137. Cummings JL, Morstorf T, Zhong K (2014) Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimers Res Ther* 6(4):37.

138. Cummings J, Morstorf T, Lee G (2016) Alzheimer's drug-development pipeline: 2016. *Alzheimer's & Dementia: Translational Research & Clinical Interventions* 2(4):222–232.

139. ClinicalTrialsgov (2017) Search of: (nanoparticle OR liposome) AND (brain) - List Results - ClinicalTrials.gov. Available at: https://clinicaltrials.gov/ct2/results?cond=%28nanoparticle+OR+liposome%29+AND+%28brain%29&term=&cntry1=&state1=&Search=Search.

140. Saraiva C, et al. (2016) Nanoparticle-mediated brain drug delivery: Overcoming blood-brain barrier to treat neurodegenerative diseases. *J Control Release* 235:34–47.

141. Johnsen KB, Burkhart A, Andresen TL, Moos T, Thomsen LB (2017) The use of the transferrin receptor as a target for brain drug delivery using nanomedicines. *Nanomedicines for Brain Drug Delivery - Springer Nature Neuromethods Series*, eds Gaillard PJ, Morales J (Springer).

142. Hare JI, et al. (2017) Challenges and strategies in anti-cancer nanomedicine development: An industry perspective. *Adv Drug Deliv Rev* 108:25–38.

143. Park K (2017) The drug delivery field at the inflection point: Time to fight its way out of the egg. *J Control Release*. doi:10.1016/j.jconrel.2017.07.030.

144. Björnmalm M, Thurecht KJ, Michael M, Scott AM, Caruso F (2017) Bridging Bio–Nano Science and Cancer Nanomedicine. *ACS Nano* 11(10):9594–9613.

145. Nichols JW, Bae YH (2014) EPR: Evidence and fallacy. *J Control Release* 190:451–464.

146. Natfji AA, Ravishankar D, Osborn HMI, Greco F (2017) Parameters Affecting the Enhanced Permeability and Retention Effect: The Need for Patient Selection. *J Pharm Sci* 106(11):3179–3187.

147. Petersen GH, Alzghari SK, Chee W, Sankari SS, La-Beck NM (2016) Meta-analysis of clinical and preclinical studies comparing the anticancer efficacy of liposomal versus conventional non-liposomal doxorubicin. *J Control Release* 232:255–264.

148. Wilhelm S, et al. (2016) Analysis of nanoparticle delivery to tumours. *Nature Reviews Materials* 1(5):16014.

149. Nichols JW, Sakurai Y, Harashima H, Bae YH (2017) Nano-sized drug carriers: Extravasation, intratumoral distribution, and their modeling. *J Control Release*. doi:10.1016/j.jconrel.2017.08.003.

150. Chen H, Zhang W, Zhu G, Xie J, Chen X (2017) Rethinking cancer nanotheranostics. *Nature Reviews Materials* 2. doi:10.1038/natrevmats.2017.24.

151. Freskgård P-O, Urich E (2017) Antibody therapies in CNS diseases. *Neuropharmacology* 120:38–55.

152. Lindqvist A, Rip J, van Kregten J, Gaillard PJ, Hammarlund-Udenaes M (2015) In vivo Functional Evaluation of Increased Brain Delivery of the Opioid Peptide DAMGO by Glutathione-PEGylated Liposomes. *Pharm Res*. doi:10.1007/s11095-015-1774-3.

153. Lindqvist A, Jönsson S, Hammarlund-Udenaes M (2016) Exploring Factors Causing Low Brain Penetration of the Opioid Peptide DAMGO through Experimental Methods and Modeling. *Mol Pharm* 13(4):1258–1266.

154. Hu Y, Rip J, Gaillard PJ, de Lange ECM, Hammarlund-Udenaes M (2017) The Impact of Liposomal Formulations on the Release and Brain Delivery of Methotrexate: An In Vivo Microdialysis Study. *J Pharm Sci* 106(9):2606–2613.

155. Turjeman K, et al. (2015) Nano-Drugs Based on Nano Sterically Stabilized Liposomes for the Treatment of Inflammatory Neurodegenerative Diseases. *PLoS ONE* 10(7):e0130442.

156. Bien-Ly N, et al. (2015) Lack of Widespread BBB Disruption in

Alzheimer's Disease Models: Focus on Therapeutic Antibodies. *Neuron* 88(2):289–297.

157. Sarkaria JN, et al. (2017) Is the blood-brain barrier really disrupted in all glioblastomas? - A critical assessment of existing clinical data. *Neuro-oncology*. doi:10.1093/neuonc/nox175.

158. Pardridge WM (2014) Blood-brain barrier drug delivery of IgG fusion proteins with a transferrin receptor monoclonal antibody. *Expert Opin Drug Deliv*:1–16.

159. Chesselet M-F, Carmichael ST (2012) Animal Models of Neurological Disorders. *Neurotherapeutics* 9(2):241–244.

160. Editorial (2015) Neurological disease models made clear. *Nat Med* 21(9):964–964.

161. Yartsev MM (2017) The emperor's new wardrobe: Rebalancing diversity of animal models in neuroscience research. *Science* 358(6362):466–469.

162. Jagmag SA, Tripathi N, Shukla SD, Maiti S, Khurana S (2016) Evaluation of Models of Parkinson's Disease. *Front Neurosci* 9(92):6428.

163. Johnsen KB, Moos T (2016) Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J Control Release* 222:32–46.

164. Obermeier B, Daneman R, Ransohoff RM (2013) Development, maintenance and disruption of the blood-brain barrier. *Nat Med* 19(12):1584–1596.

165. Zhao Z, Nelson AR, Betsholtz C, Zlokovic BV (2015) Establishment and Dysfunction of the Blood-Brain Barrier. *Cell* 163(5):1064–1078.

166. Montagne A, et al. (2015) Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* 85(2):296–302.

167. Sengillo JD, et al. (2013) Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease. *Brain Pathol* 23(3):303–310.

168. Carvey PM, et al. (2005) 6-Hydroxydopamine-induced alterations in blood-brain barrier permeability. *Eur J Neurosci* 22(5):1158–1168.

169. Desai Bradaric B, Patel A, Schneider JA, Carvey PM, Hendey B (2012) Evidence for angiogenesis in Parkinson's disease, incidental Lewy body

disease, and progressive supranuclear palsy. *J Neural Transm* 119(1):59–71.

170. Gray MT, Woulfe JM (2015) Striatal blood-brain barrier permeability in Parkinson's disease. *J Cereb Blood Flow Metab* 35(5):747–750.

171. Duran-Vilaregut J, et al. (2009) Blood-brain barrier disruption in the striatum of rats treated with 3-nitropropionic acid. *Neurotoxicology* 30(1):136–143.

172. Graesser D, et al. (2002) Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice. *J Clin Invest* 109(3):383–392.

173. Waknine-Grinberg JH, et al. (2013) Glucocorticosteroids in nano-sterically stabilized liposomes are efficacious for elimination of the acute symptoms of experimental cerebral malaria. *PLoS ONE* 8(8):e72722.

174. Lee H, Pienaar IS (2014) Disruption of the blood-brain barrier in Parkinson's disease: curse or route to a cure? *Front Biosci* 19:272–280.

175. Schmidt J, et al. (2003) Drug targeting by long-circulating liposomal glucocorticosteroids increases therapeutic efficacy in a model of multiple sclerosis. *Brain* 126(Pt 8):1895–1904.

176. Kizelsztein P, Ovadia H, Garbuzenko O, Sigal A, Barenholz Y (2009) Pegylated nanoliposomes remote-loaded with the antioxidant tempamine ameliorate experimental autoimmune encephalomyelitis. *J Neuroimmunol* 213(1-2):20–25.

177. Avnir Y, et al. (2011) Fabrication principles and their contribution to the superior in vivo therapeutic efficacy of nano-liposomes remote loaded with glucocorticoids. *PLoS ONE* 6(10):e25721.

178. Duran-Vilaregut J, et al. (2011) Role of matrix metalloproteinase-9 (MMP-9) in striatal blood-brain barrier disruption in a 3-nitropropionic acid model of Huntington's disease. *Neuropathol Appl Neurobiol* 37(5):525–537.

179. Zhao C, Ling Z, Newman MB, Bhatia A, Carvey PM (2007) TNF-alpha knockout and minocycline treatment attenuates blood-brain barrier leakage in MPTP-treated mice. *Neurobiol Dis* 26(1):36–46.

180. Monahan AJ, Warren M, Carvey PM (2008) Neuroinflammation and peripheral immune infiltration in Parkinson's disease: an autoimmune hypothesis. *Cell Transplant* 17(4):363–372.

181. Carvey PM, Hendey B, Monahan AJ (2009) The blood-brain barrier in neurodegenerative disease: a rhetorical perspective. *J Neurochem* 111(2):291–314.

182. Desai BS, Schneider JA, Li J-L, Carvey PM, Hendey B (2009) Evidence of angiogenic vessels in Alzheimer's disease. *J Neural Transm* 116(5):587–597.

183. Patel A, et al. (2011) An angiogenic inhibitor, cyclic RGDfV, attenuates MPTP-induced dopamine neuron toxicity. *Exp Neurol* 231(1):160–170.

184. Biron KE, Dickstein DL, Gopaul R, Jefferies WA (2011) Amyloid triggers extensive cerebral angiogenesis causing blood brain barrier permeability and hypervascularity in Alzheimer's disease. *PLoS ONE* 6(8):e23789.

185. Biron KE, Dickstein DL, Gopaul R, Fenninger F, Jefferies WA (2013) Cessation of neoangiogenesis in Alzheimer's disease follows amyloid-beta immunization. *Sci Rep* 3:1354.

186. Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57(2):178–201.

187. Cheng Z, et al. (2010) Central nervous system penetration for small molecule therapeutic agents does not increase in multiple sclerosis- and Alzheimer's disease-related animal models despite reported blood-brain barrier disruption. *Drug Metab Dispos* 38(8):1355–1361.

188. St-Amour I, et al. (2013) Brain bioavailability of human intravenous immunoglobulin and its transport through the murine blood-brain barrier. *J Cereb Blood Flow Metab* 33(12):1983–1992.

189. Do TM, et al. (2014) Altered cerebral vascular volumes and solute transport at the blood-brain barriers of two transgenic mouse models of Alzheimer's disease. *Neuropharmacology* 81:311–317.

190. Minh Do T, et al. (2015) Age-Dependent Regulation of the Blood-Brain Barrier Influx/Efflux Equilibrium of Amyloid-β Peptide in a Mouse Model of Alzheimer's Disease (3xTg-AD). *J Alzheimers Dis*. doi:10.3233/JAD-150350.

191. Alata W, Ye Y, St-Amour I, Vandal M, Calon F (2015) Human apolipoprotein E ε4 expression impairs cerebral vascularization and blood-brain barrier function in mice. *J Cereb Blood Flow Metab* 35(1):86–94.

192. Laboratory RBJM, Green EL (1966) *Biology of the laboratory mouse*.

193. Yu YJ, et al. (2011) Boosting brain uptake of a therapeutic antibody by reducing its affinity for a transcytosis target. *Sci Transl Med* 3(84):84ra44–84ra44.

194. Blanco E, Shen H, Ferrari M (2015) Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol* 33(9):941–951.

195. Nie S (2010) Understanding and overcoming major barriers in cancer nanomedicine. *Nanomedicine* 5(4):523–528.

196. Moghimi SM, Patel HM (1989) Differential properties of organ-specific serum opsonins for liver and spleen macrophages. *Biochim Biophys Acta* 984(3):379–383.

197. Moghimi SM, Patel HM (1989) Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes. *Biochim Biophys Acta* 984(3):384–387.

198. Patel H, Moghimi S (1998) Serum-mediated recognition of liposomes by phagocytic cells of the reticuloendothelial system - The concept of tissue specificity. *Adv Drug Deliv Rev* 32(1-2):45–60.

199. Allen TM, Cullis PR (2013) Liposomal drug delivery systems: from concept to clinical applications. *Adv Drug Deliv Rev* 65(1):36–48.

200. Salvati A, et al. (2013) Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface. *Nat Nanotechnol* 8(2):137–143.

201. Hadjidemetriou M, et al. (2015) In Vivo Biomolecule Corona around Blood-Circulating, Clinically Used and Antibody-Targeted Lipid Bilayer Nanoscale Vesicles. *ACS Nano* 9(8):8142–8156.

202. Bertoli F, Garry D, Monopoli MP, Salvati A, Dawson KA (2016) The Intracellular Destiny of the Protein Corona: A Study on its Cellular Internalization and Evolution. *ACS Nano* 10(11):10471–10479.

203. Vilanova O, et al. (2016) Understanding the Kinetics of Protein-Nanoparticle Corona Formation. *ACS Nano* 10(12):10842–10850.

204. Lara S, et al. (2017) Identification of Receptor Binding to the Biomolecular Corona of Nanoparticles. *ACS Nano* 11(2):1884–1893.

205. Kristensen K, Urquhart AJ, Thormann E, Andresen TL (2016) Binding of human serum albumin to PEGylated liposomes: insights into binding

numbers and dynamics by fluorescence correlation spectroscopy. *Nanoscale* 8(47):19726–19736.

206. Singh R, et al. (2016) Dose-Dependent Therapeutic Distinction between Active and Passive Targeting Revealed Using Transferrin-Coated PGMA Nanoparticles. *Small* 12(3):351–359.

207. Naz N, et al. (2012) Ferroportin-1 is a "nuclear-"negative acute-phase protein in rat liver: a comparison with other iron-transport proteins. *Lab Invest* 92(6):842–856.

208. Lu JP, Hayashi K, Awai M (1989) Transferrin receptor expression in normal, iron-deficient and iron-overloaded rats. *Acta Pathol Jpn* 39(12):759–764.

209. Malik IA, et al. (2011) Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. *Cell Tissue Res* 344(2):299–312.

210. Choi CHJ, Alabi CA, Webster P, Davis ME (2010) Mechanism of active targeting in solid tumors with transferrin-containing gold nanoparticles. *Proc Natl Acad Sci USA* 107(3):1235–1240.

211. Zhang Y-N, Poon W, Tavares AJ, McGilvray ID, Chan WCW (2016) Nanoparticle–liver interactions: Cellular uptake and hepatobiliary elimination. *Journal of Controlled Release* 240:332–348.

212. Tsoi KM, et al. (2016) Mechanism of hard-nanomaterial clearance by the liver. *Nat Mater* 15(11):1212–1221.

213. Hunt DW, Jiang H, Levy JG (1998) Photofrin increases murine spleen cell transferrin receptor expression and responsiveness to recombinant myeloid and erythroid growth factors. *Immunopharmacology* 38(3):267–278.

214. Sugyo A, et al. (2017) Uptake of 111In-labeled fully human monoclonal antibody TSP-A18 reflects transferrin receptor expression in normal organs and tissues of mice. *Oncol Rep* 37(3):1529–1536.

215. Hamad I, Hunter AC, Szebeni J, Moghimi SM (2008) Poly(ethylene glycol)s generate complement activation products in human serum through increased alternative pathway turnover and a MASP-2-dependent process. *Mol Immunol* 46(2):225–232.

216. Moghimi SM, et al. (2010) Complement activation cascade triggered by PEG-PL engineered nanomedicines and carbon nanotubes: the challenges ahead. *J Control Release* 146(2):175–181.

217. Andersen AJ, et al. (2013) Complement activation by PEG-functionalized multi-walled carbon nanotubes is independent of PEG molecular mass and surface density. *Nanomedicine* 9(4):469–473.

218. Wang X, Ishida T, Kiwada H (2007) Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *J Control Release* 119(2):236–244.

219. Ishida T, Wang X, Shimizu T, Nawata K, Kiwada H (2007) PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *J Control Release* 122(3):349–355.

220. Shiraishi K, et al. (2016) Exploring the relationship between anti-PEG IgM behaviors and PEGylated nanoparticles and its significance for accelerated blood clearance. *J Control Release* 234:59–67.

221. Sroda K, et al. (2005) Repeated injections of PEG-PE liposomes generate anti-PEG antibodies. *Cell Mol Biol Lett* 10(1):37–47.

222. Kim J, et al. (2017) Co-coating of receptor-targeted drug nanocarriers with anti-phagocytic moieties enhances specific tissue uptake versus non-specific phagocytic clearance. *Biomaterials* 147:14–25.

223. Kamerkar S, et al. (2017) Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 18:4249.

224. Ahmed M, Pan DW, Davis ME (2015) Lack of in vivo antibody dependent cellular cytotoxicity with antibody containing gold nanoparticles. *Bioconjug Chem* 26(5):812–816.

225. Couch JA, et al. (2013) Addressing safety liabilities of TfR bispecific antibodies that cross the blood-brain barrier. *Sci Transl Med* 5(183):183ra57–1–12.

226. R'zik S, Loo M, Beguin Y (2001) Reticulocyte transferrin receptor (TfR) expression and contribution to soluble TfR levels. *Haematologica* 86(3):244–251.

227. Iacopetta BJ, Morgan EH, Yeoh GC (1982) Transferrin receptors and iron uptake during erythroid cell development. *Biochim Biophys Acta* 687(2):204–210.

228. Frazier JL, Caskey JH, Yoffe M, Seligman PA (1982) Studies of the Transferrin Receptor on both Human Reticulocytes and Nucleated Human Cells in Culture. *J Clin Invest* 69(4):853–865.

229. Raub TJ, Newton CR (1991) Recycling kinetics and transcytosis of transferrin in primary cultures of bovine brain microvessel endothelial cells. *J Cell Physiol* 149(1):141–151.

230. Visser CC, Voorwinden LH, Crommelin DJA, Danhof M, de Boer AG (2004) Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. *Pharm Res* 21(5):761–769.

231. Gadkar K, et al. (2016) Mathematical PKPD and safety model of bispecific TfR/BACE1 antibodies for the optimization of antibody uptake in brain. *Eur J Pharm Biopharm* 101:53–61.

232. Kanodia JS, et al. (2016) Prospective Design of Anti-Transferrin Receptor Bispecific Antibodies for Optimal Delivery into the Human Brain. *CPT Pharmacometrics Syst Pharmacol* 5(5):283–291.

233. Lo M, et al. (2017) Effector-attenuating Substitutions That Maintain Antibody Stability and Reduce Toxicity in Mice. *J Biol Chem* 292(9):3900–3908.

234. Couch JA, et al. (2016) Balancing Efficacy and Safety of an Anti-DLL4 Antibody through Pharmacokinetic Modulation. *Clin Cancer Res* 22(6):1469–1479.

235. Cheng WW, Allen TM (2010) The use of single chain Fv as targeting agents for immunoliposomes: an update on immunoliposomal drugs for cancer treatment. *Expert Opin Drug Deliv* 7(4):461–478.

236. Alibakhshi A, et al. (2017) Targeted cancer therapy through antibody fragments-decorated nanomedicines. *J Control Release* 268:323–334.

237. Lesley J, Schulte R, Woods J (1989) Modulation of transferrin receptor expression and function by anti-transferrin receptor antibodies and antibody fragments. *Exp Cell Res* 182(1):215–233.

238. Bien-Ly N, et al. (2014) Transferrin receptor (TfR) trafficking determines brain uptake of TfR antibody affinity variants. *J Exp Med* 211(2):233–244.

239. Niewoehner J, et al. (2014) Increased brain penetration and potency of a therapeutic antibody using a monovalent molecular shuttle. *Neuron* 81(1):49–60.

240. Jefferies WA, et al. (1984) Transferrin receptor on endothelium of brain capillaries. *Nature* 312(5990):162–163.

241. Watts RJ, Dennis MS (2013) Bispecific antibodies for delivery into the

brain. *Curr Opin Chem Biol* 17(3):393–399.

242. Paterson J, Webster CI (2016) Exploiting transferrin receptor for delivering drugs across the blood-brain barrier. *Drug Discov Today Technol* 20:49–52.

243. Syvänen S, et al. (2017) A bispecific Tribody PET radioligand for visualization of amyloid-beta protofibrils - a new concept for neuroimaging. *Neuroimage* 148:55–63.

244. Hultqvist G, Syvänen S, Fang XT, Lannfelt L, Sehlin D (2017) Bivalent Brain Shuttle Increases Antibody Uptake by Monovalent Binding to the Transferrin Receptor. *Theranostics* 7(2):308–318.

245. Sehlin D, Fang XT, Meier SR, Jansson M, Syvänen S (2017) Pharmacokinetics, biodistribution and brain retention of a bispecific antibody-based PET radioligand for imaging of amyloid-β. *Sci Rep* 7(1):306.

246. Laske DW, Youle RJ, Oldfield EH (1997) Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nat Med* 3(12):1362–1368.

247. Weaver M, Laske DW (2003) Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. *J Neurooncol* 65(1):3–13.

248. Debinski W (2008) Molecular targeting with recombinant cytotoxins for the treatment of brain tumors. *Drug Dev Res* 69(7):407–414.

249. Yoon DJ, et al. (2010) Intratumoral Therapy of Glioblastoma Multiforme Using Genetically Engineered Transferrin for Drug Delivery. *Cancer Research* 70(11):4520–4527.

250. Yu YJ, et al. (2014) Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. *Sci Transl Med* 6(261):261ra154–261ra154.

251. Zuchero YJY, et al. (2016) Discovery of Novel Blood-Brain Barrier Targets to Enhance Brain Uptake of Therapeutic Antibodies. *Neuron* 89(1):70–82.

252. Webster CI, et al. (2017) Enhanced delivery of IL-1 receptor antagonist to the central nervous system as a novel anti-transferrin receptor-IL-1RA fusion reverses neuropathic mechanical hypersensitivity. *Pain* 158(4):660–668.

253. Tillotson BJ, Goulatis LI, Parenti I, Duxbury E, Shusta EV (2015) Engineering an Anti-Transferrin Receptor ScFv for pH-Sensitive Binding Leads to Increased Intracellular Accumulation. *PLoS ONE* 10(12):e0145820.

254. Lichota J, Skjørringe T, Thomsen LB, Moos T (2010) Macromolecular drug transport into the brain using targeted therapy. *J Neurochem* 113(1):1–13.

255. Dennis MS, Watts RJ (2012) Transferrin antibodies into the brain. *Neuropsychopharmacology* 37(1):302–303.

256. Gregori M, et al. (2016) Novel Antitransferrin Receptor Antibodies Improve the Blood-Brain Barrier Crossing Efficacy of Immunoliposomes. *J Pharm Sci* 105(1):276–283.

257. Huwyler J, Wu D, Pardridge WM (1996) Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci USA* 93(24):14164–14169.

258. Clark AJ, Davis ME (2015) Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core. *Proc Natl Acad Sci USA*:201517048.

259. Reimold I, et al. (2008) Delivery of nanoparticles to the brain detected by fluorescence microscopy. *Eur J Pharm Biopharm* 70(2):627–632.

260. Haqqani AS, et al. (2017) Endosomal trafficking regulates receptor-mediated transcytosis of antibodies across the blood brain barrier. *J Cereb Blood Flow Metab*:271678X17740031.

261. Dixit S, et al. (2015) Dual Receptor-Targeted Theranostic Nanoparticles for Localized Delivery and Activation of Photodynamic Therapy Drug in Glioblastomas. *Mol Pharm* 12(9):3250–3260.

262. Dixit S, et al. (2015) Transferrin receptor-targeted theranostic gold nanoparticles for photosensitizer delivery in brain tumors. *Nanoscale* 7(5):1782–1790.

263. Prades R, et al. (2012) Delivery of gold nanoparticles to the brain by conjugation with a peptide that recognizes the transferrin receptor. *Biomaterials* 33(29):7194–7205.

264. Gromnicova R, et al. (2013) Glucose-coated gold nanoparticles transfer across human brain endothelium and enter astrocytes in vitro. *PLoS ONE* 8(12):e81043.

265. Gromnicova R, et al. (2016) Localization and mobility of glucose-coated gold nanoparticles within the brain. *Nanomedicine (Lond)*:nnm.15.215.

266. Gromnicova R, et al. (2016) Transport of Gold Nanoparticles by Vascular Endothelium from Different Human Tissues. *PLoS ONE* 11(8):e0161610.

267. Cabezón I, et al. (2017) Serial block-face scanning electron microscopy applied to study the trafficking of 8D3-coated gold nanoparticles at the blood-brain barrier. *Histochem Cell Biol* 36:437.

268. Cabezón I, et al. (2015) Trafficking of gold nanoparticles coated with the 8D3 anti-transferrin receptor antibody at the mouse blood-brain barrier. *Mol Pharm*:151005220742000.

269. Muldoon LL, et al. (1999) A physiological barrier distal to the anatomic blood-brain barrier in a model of transvascular delivery. *AJNR Am J Neuroradiol* 20(2):217–222.

270. Paris-Robidas S, Emond V, Tremblay C, Soulet D, Calon F (2011) In vivo labeling of brain capillary endothelial cells after intravenous injection of monoclonal antibodies targeting the transferrin receptor. *Mol Pharmacol* 80(1):32–39.

271. Lesley J, Hyman R, Schulte R, Trotter J (1984) Expression of transferrin receptor on murine hematopoietic progenitors. *Cell Immunol* 83(1):14–25.

272. Lesley JF, Schulte RJ (1985) Inhibition of cell growth by monoclonal anti-transferrin receptor antibodies. *Molecular and Cellular Biology* 5(8):1814–1821.

273. Betzer O, et al. (2017) The effect of nanoparticle size on the ability to cross the blood-brain barrier: an in vivo study. *Nanomedicine (Lond)* 12(13):1533–1546.

274. Hoshyar N, Gray S, Han H, Bao G (2016) The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine (Lond)* 11(6):673–692.

275. Bharadwaj VN, Lifshitz J, Adelson PD, Kodibagkar VD, Stabenfeldt SE (2016) Temporal assessment of nanoparticle accumulation after experimental brain injury: Effect of particle size. *Sci Rep* 6:29988.

ISSN (online): 2246-1302
ISBN (online): 978-87-7210-111-8

AALBORG UNIVERSITY PRESS