Gene Delivery to the Blood-Brain Barrier
Gene Delivery to the Blood-Brain Barrier

Non-Viral Gene Carriers and in vitro Blood-Brain Barrier Models

PhD Thesis by

Louiza Bohn Thomsen

Laboratory for Neurobiology – Biomedicine,
Department of Health Science and Technology,
Aalborg University, Aalborg, Denmark
## Table of contents

Preface .................................................................................................................. vii
Supervisors ............................................................................................................. ix
Abbreviations ......................................................................................................... xi
Abstract ................................................................................................................ xiii
Resumé på dansk ................................................................................................... xv
Acknowledgements .............................................................................................. xvii
List of papers ........................................................................................................ xix

1 Introduction ..................................................................................................... 1

1.1 The Blood-Brain Barrier............................................................................ 2

1.2 Drug Delivery to the Brain....................................................................... 3
   1.2.1 Non-CNS-invasive approaches to enable drug delivery to the brain 3

1.3 Gene Therapy and Delivery to the BBB.................................................. 4
   1.3.1 Delivery by non-viral chemical gene vectors................................. 8
   1.3.2 Polyplexes.................................................................................... 9
   1.3.3 The novel drug carrier pullan-spermine and gene delivery.......... 9
   1.3.4 SPIOs and Blood-Brain Barrier Penetration............................... 11

1.4 In Vitro Blood-Brain Barrier Models..................................................... 12
   1.4.1 Static in vitro Blood-Brain Barrier Model.................................... 13
   1.4.2 Dynamic in vitro Blood-Brain Barrier Model............................. 13

2 Objective of the thesis .................................................................................. 17

3 Results .......................................................................................................... 19

3.1 STUDY I ................................................................................................. 19

3.2 STUDY II ............................................................................................... 27

3.3 STUDY III ............................................................................................. 45

4 Discussion ..................................................................................................... 61

4.1 Pullulan-spermine complexes as gene-carriers to BCECs................. 61
4.2 Transport of pullulan-spermine cargo into the cell nucleus .......... 62
4.3 Targeting properties of pullulan-spermine................................. 62
4.4 Pullulan-spermine and serum compatibility ........................................ 63
4.5 SPIOs and drug delivery to the brain ........................................... 64
4.6 SPIOs and possible damaging effects ............................................. 64
4.7 SPIOs and magnetic force-targeted delivery ............................... 65
4.8 Static versus dynamic in vitro BBB model .................................... 65
4.9 Immortalized BCECs and BBB integrity .................................... 65
5 Future Perspectives ........................................................................... 67
References ............................................................................................. 69
Appendix I .............................................................................................. 83
Preface

This thesis has been submitted to the Faculty of Medicine, Aalborg University, Denmark. The work in this thesis has been carried out in the Laboratory of Neurobiology, Biomedicine, Department of Health Science and Technology, Aalborg University from October 2008 until November 2011. In November 2009, I visited Dr. Damir Janigro and his research group on Cleveland Clinic, Ohio, USA and worked shortly in his laboratory to learn how to operate their dynamic in vitro blood-brain barrier system, Flocel. During my Ph.D. study I have participated in Ph.D. courses corresponding to half a year. Furthermore, I have been teaching students at the educations “Medicine with industrial specialization” and “Medicine” according to the norms of Aalborg University which also corresponds to half a year of studying.

This thesis contains the following: Introduction, Objective of the Thesis, Results, and Discussion. The results are presented in article form in three studies. Study I was published in Journal of Controlled Release in 2010. Study II has been submitted to Journal of Controlled Release and Study III are yet unpublished. The thesis also contains two review articles appendices I and II published or accepted for publication, respectively, during my Ph.D. study.

Aalborg, November 2011

Louiza Bohn Thomsen
Supervisors

**Head Supervisor:**
Professor Torben Moos  
Department of Health Science and Technology  
Aalborg University  
Aalborg, Denmark

**Co-Supervisor:**
Professor Thomas Jensen  
Department of Human Genetics  
University of Aarhus  
Aarhus, Denmark

**Co-Supervisor:**
Anker Jon Hansen  
Principal Scientist at Novo Nordisk  
Novo Nordisk A/S  
Bagsværd, Denmark
Abbreviations

ACM: astrocyte-conditioned media
ANG1: angiopoitin 1
BBB: blood-brain barrier
BCECs: brain capillary endothelial cells
BDNF: brain derived neurotropic factor
bFGF: basic fibroblast growth factor
cDNA: complementary DNA
CED: convection enhanced diffusion
CMV: cytomegalovirus
CSF: cerebrospinal fluid
EPO: Erythropoietin
GDNF: Glial cell line-derived neurotropic factor
GH: growth hormone
hGH1: human growth hormone 1
L-DOPA: L-3,4 dihydroxyphenylalanine
MRI: magnetic resonance imaging
NGF: neural growth factor
NLS: nuclear localization signal
PECAM: platelet endothelial cell adhesion molecule
PEG: polyethylene glycol
PEI: polyethylenimine
siRNA: short interfering RNA
SPIO: superparamagnetic iron oxide nanoparticle
ZO-1: zonula occludens 1
Abstract

Drug- and gene delivery to the brain is highly restricted by the vascular barriers of the brain, denoted by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barriers. Among these barriers, BBB is the main limiting factor. It is composed by the brain capillary endothelial cells (BCECs). The BCECs barrier function is supported by astrocytes, pericytes and neurons to form the blood-brain barrier. BCECs are very tightly connected to each other by tight junctions. Apart from the essential substrates needed to nourish the brain, small and/or lipophilic molecules are free to diffuse into the brain. However most pharmacologically active drugs and gene fragments are too large to enter the brain. Various kinds of drug-carriers have been constructed for delivery of large substances to the brain. Such drug-carriers have to be able to successfully carry their cargo through the BCECs and into the brain. For testing the ability of drug-carriers to deliver their cargo into the brain, investigators have constructed different in vitro BBB models, consisting of BCECs that express the main characteristics of the BBB in vivo.

In the first part of the thesis the ability of two drug-carriers, pullulan-spermine and SPIOs, to mediate transfection of BCECs or transcellular transport through BCECs in vitro was studied.

Pullulan-spermine is a polymeric complex consisting of the polysaccharide, pullulan and the polyamine, spermine. Pullulan-spermine formed a cationic complex shown to be able to bind plasmid DNA electrostatically. Pullulan-spermine was conjugated with plasmid DNA encoding a red fluorescent protein, Hc-Red-1 C1, or human growth hormone 1 (hGH1). Pullulan-spermine complexed with Hc-Red-1 C1 cDNA led to the formation of a red fluorescent signal in human brain microvascular endothelial cells (HBMECs). Furthermore, pullulan-spermine complexed with hGH1 cDNA was not only able to transflect HBMECs but also led to secretion of the hGH1 into the culture media. Pullulan-spermine-cDNA complexes could transfsect non-dividing cells although the rate of transgene cells was higher in dividing cells. This indicated that the DNA is not only entering the cell nucleus under mitosis. Unfortunately, pullulan-spermine complexes proved incapable of transflecting HBMECs in the presence of serum in the growth media and additional studies are needed to enable its use for in vivo transfection.

Another potential drug-carrier, fluorescent iron oxide nanoparticles were also shown to enter HBMECs upon incubation. These nanoparticles were also able to pass though the HBMECs forming a BBB in a static in vitro BBB model. Furthermore, their passage was increased by the aid of an external magnetic field created by placing the cell culture plates with the SPIOs on a plate magnet. Two vitality tests showed no significant change in BCEC vitality after addition of
The results of the drug-carrier studies indicate that it is possible to deliver plasmid cDNA into BCECs and transfect these cells leading to their secretion of encoded protein into the extracellular space. Moreover, SPIOs are potentially potent carriers of attachable molecules through cultured BCECs in vitro, which may have high potential for drug-delivery to the brain in vivo.

In the second part of the thesis, two in vitro BBB models, a static and a dynamic model were investigated and compared. The static model consisting of microporous membrane inserts in which immortalized BCECs is cultured. The model induces many characteristics of the BBB in vivo, but lacks the tightness induction factor of shear stress. Different experiments were performed with this static model to monitor BBB integrity. Barrier formation by the BCECs was monitored by measuring transendothelial electric resistance (TEER) and the BCEC monolayer was stained positive for zonula occludens 1 (ZO-1) a tight junction protein. It was mainly found that the tightness of the BCECs was strengthened by contact co-culture of the BCECs with astrocytes and addition of hydrocortisone to the media. The dynamic in vitro BBB model however, did not lead to any reliable results in this study and further investigation of barrier formation in this model was not pursued. In consequence a comparison between the static and dynamic in vitro models was not possible, but it could be concluded that the static model seems to be the most reliable model.
Resumé på dansk

Gen og medikament levering til hjernen har vist sig at være svært hæmmet af barriererne i hjernen, herunder hovedsageligt blod-hjerne barrieren (BBB). BBB formes af hjerne kapillær endothel celler (BCECs). BCECs er omgivet af astrocytter, pericytter og neuroner, der også menes at støtte BCECs barriere funktioner. BCECs danner tætte forbindelser mellem hinanden, kaldet “tight-junctions” og derved forhindres passage mellem cellerne. Bortset fra de essentielle næringsstoffer hjernen har brug for er det kun små og/eller fedtopløselige molekyler, der kan passere BBB. Gener og medikamenter er ofte store og vandopløselige og kan derfor ikke passere gennem BBB. Derfor er der brug for en leverings strategi af store molekyler til hjernen. Forskellige “drug carriers” er blevet udviklet til dette formål. Drug carriers bør være i stand til at levere deres last gennem BCECs og videre ind i hjernen. Til at teste en drug carriers evne bruger man ofte en *in vitro* BBB model. Disse modeller består af BCECs dyrket i kultur, der danner en barriere med de karakteristika BBB udviser *in vivo*.

I den første del af denne tese blev to potentielle drug-carriers undersøgt for deres evner til enten at passere BBB og ind i hjernen eller at levere gener ind i BCECs.

Den første carrier er et polymerisk kompleks bestående af en polysakkarid, pullulan og en polyaminosyre, spermine. Pullulan-spermine danner tilsammen et kationisk kompleks der kan binde negativt ladet plasmid DNA elektrostatisk. I dette studie blev pullulan-spermine konjugeret med plasmid DNA kodende for en rød fluorescerende markør Hc-Red C1 og humant vækst hormon 1 (hGH1). Pullulan-spermine-pHc-Red-1 C1 komplekser havde evnen til at forme transgene rød fluorescerende humane hjerne endothel celler (HBMECs) i monokultur. Endvidere kunne pullulan-spermine-pGH1 komplekser transfectere HBMECs og proteinet som det plasmide DNA kodede for, hGH1, kunne detekteres i cellerne og i celle kultur mediet, hvilket indikerede at HBMECs havde udskilt dette protein. Pullulan-spermine viste sig desværre at være uforenelig med serum, hvilket forhindrer brug af denne carrier *in vivo*. Derudover blev det fundet at celler der ikke er i det delende stadie, dvs. ikke mitotiske, kunne transfecteres, dog i en mindre grad end i delende celler. Dette indikerer at det plasmide DNA ikke kun kan diffundere ind i cellekernen, når kernembranen er midlertidigt åben, men at der også findes en mekanisme der kan hjælpe det plasmide DNA ind i cellekernen når kernembranen er intakt. Resultaterne i dette studie indikerer at det er muligt at benytte pullulan-spermine komplekser til levering af DNA til BCECs *in vitro* og at disse kan transfecteres og udskille det DNA indkodede protein.

Den anden carrier type hvis evner blev undersøgt i dette studie var fluorescerende superparamagnetiske nanopartikler (SPIOs). I dette studie kunne det påvises at fluorescerende stivelses overflade behandlede jern oxid nanopartikler...
kunne optages af BCECs. Desuden kunne disse partikler passere BCECs, der dannede en BBB i en statisk \textit{in vitro} BBB model. Derudover kunne det påvises at passage af SPIOs øges markant ved hjælp af et eksternt magnetisk felt. Dette eksterne magnetiske felt blev dannet ved hjælp af en plademagnet hvorpå cellekultur pladerne med BCECs blev placeret og de magnetiske nanopartikler blev derved trukket gennem cellelaget mod magneten. To vitalitets test udført på BCECs, der havde været udsat for SPIOs alene eller suppleret med det eksterne magnetiske felt viste ingen signifikant ændring i vitaliteten. Det blev derfor konkluderet at SPIOs er potentielt potente carriere til hjernen.

I anden del af tesen var formålet at undersøge og sammenligne to forskellige former for \textit{in vitro} BBB, en statisk og en dynamisk. Den statiske model inducerer BCECs til at danne de fleste BBB karakteristika, men mangler evnen til at forme “shear stress”, der er en vigtig tætheds promoverende faktor. BCECs dannelse af barriere blev monitoreret ved hjælp af transendothel elektrisk resistans (TEER) måling og celler blev efterfølgende farvet positive for tight junction proteinet zonula occludens 1. Tætheden af barrieren viste sig at øges ved kontakt co-kultur med astrocytter og en yderligere øgning af tætheden blev observeret ved tilsættelse af binaryrebarkhormon til mediet.

Den dynamiske \textit{in vitro} BBB model kan inducere shear stress og er derfor en mere kompleks model. Desværre var det ikke muligt at indsamle pålidelige resultater fra den dynamiske model og derfor kunne dannelse af en blod hjerne barriere i denne model ikke undersøges nærmere. Desuden var det heller ikke muligt at sammenligne de to modeller, men det kunne konkluderes at den statiske \textit{in vitro} BBB model på nuværende tidspunkt virker mest stabil.
First of all I would like to thank my supervisor, Torben Moos for giving me the opportunity to start on this Ph.D. study and for support and guidance throughout the study. Torben has been very kind to give me great opportunities to evolve my skills as a scientist and teacher. I would also like to thank my co-supervisors Thomas Jensen and Anker Jon for their input to the methods and results obtained during my study.

I would like to thank everybody at the Biomedicine building for the nice times spend in the coffee room at lunch, Monday bread and social events. Especially thanks to Jacek Lichota for guidance, sharing of knowledge and for the nice talks we have had as “office mates”. A big thank to Merete Fredsgaard for being the perfect “travel mate”, for moral support and for all her help trough out the years. Also Linda Pilgaard is thanked for her input to this thesis, scientific co-operation and moral support.

“Kompetence Fonden” at the University of Aalborg is thanked for financial support.

Last but not least I would like to thank my family and friends for supporting me, not only in good times but also on a cloudy day. Furthermore I would like to thank Palle for always supporting me and believing in me.
List of papers

This Ph.D. thesis is based on the following papers:


Thomsen, L.B., Lineman, T., Lichota, J., Kim, K.S., Visser, G., Moos, T. In vitro delivery of superparamagnetic iron oxide nanoparticles through brain endothelial cells (submitted to *Journal of Controlled Release*).


1 Introduction

Drug delivery to the brain is restricted by the vascular barriers of the brain. These barriers constitute the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barriers (Fig.1). The BBB covers a 1000 times larger area of the capillary surface than that of the blood-CSF barriers, which makes the BBB the main route of interest for systemic drug delivery to the brain [1, 2]. In this thesis, drug delivery to the brain capillary endothelial cells (BCECs) forming the BBB and across the BCECs into the brain interior is the main target. Therefore, the thesis emphasizes the transfections of BCECs and transport into and through these cells.

**Figure 1** Capillaries in the brain. Cerebral capillaries of blood-brain barrier (BBB), capillaries of the circumventricular organs (CVOs) and capillaries of the blood-cerebrospinal fluid barrier (BCB) in the choroid plexus are highlighted under magnifying glasses. The endothelial cells of the capillaries of the CVO and choroid plexus are fenestrated and leaky and the barrier function is found in the surrounding cells. The capillaries of the BBB are without fenestration and the endothelial cells are interconnected by tight-junctions and they are therefore non-leaky.
1.1 THE BLOOD-BRAIN BARRIER
BCECs denote the BBB and form a major physical restraint on the transport of several molecules present in the blood plasma. Astrocytes and pericytes make intimate contacts with the BCECs and participate in the maintenance of the integrity of the BBB [3]. Astrocytes are especially important for induction and regulation of the BBB properties of the BCECs and their end-feet ensheat almost completely the abluminal surface of the BCECs [4]. Together, the BCECs and astrocytes form a basal lamina present between the BCECs and end-feet of the astrocytes. The basal lamina consists of laminin, type-IV collagen, integrins and fibronectin [5, 6]. The basal lamina is believed to act as a barrier to the passage of macromolecules [2]. The pericytes are embedded in the basal lamina. Studies indicate that pericytes have a role in regulating the paracellular permeability of the BBB by regulating the tight junctions between the endothelial cells [7, 8, 9, 10]. Pericytes have also been shown to be necessary for BBB formation, regulate BBB gene expression, and induce polarization of astrocytic end-feet [9, 10]. The BCECs are also believed to be in direct contact with neurons, suggesting that neurons also could take part in the regulation of permeability of the BBB [11].

The BCECs are thin and non-fenestrated cells [12, 2]. BCECs are rich in mitochondria, hence high metabolic activity, but low in vesicles involved in endocytotic and transcytotic activity [2, 4]. Furthermore BCEC have a higher concentration of drug and nutrient metabolizing enzymes, such as gamma-glutamyl transpeptidase and alkaline phosphatase compared with non-neural endothelial cells [4]. The BCECs are closely interconnected with tight and adherence junctions, which highly impair paracellular trafficking of even small molecules [13, 14]. The tight junctions are considered to be the main structures responsible for the strict barrier properties. The tight junctions are composed of the integral transmembrane proteins occludins, claudins (predominantly claudin 3 and 5), junctional adhesion molecules (JAMs) and endothelial selective adhesion molecule (ESAM). The transmembrane proteins are anchored to the cytoskeleton by zonula occludens 1, 2 and 3 (ZO-1, ZO-2, ZO-3) [3, 4, 6]. The adherence junctions are formed by vascular endothelial cadherins and these are linked to the cytoskeleton by catenins. The platelet endothelial cell adhesion molecule (PECAM) is also a part of the adherence junctions [3, 6].

Transcellular transport across the BCECs takes place by mechanisms like passive diffusion of small lipid soluble, nonpolar compounds; carrier mediated transport of essential nutrients like glucose and amino acids; receptor mediated transport of e.g. insulin and transferrin; adsorptive mediated transport of e.g. albumin; and carrier mediated efflux transporters of amphilic lipid soluble substrates [15, 6]. Furthermore leukocytes can penetrate the BBB transcellularly by diapedesis, giving raise to transvascular transport in the brain [6].

Several lipophilic and cationic drugs which enter BCECs are returned to the plasma by efflux transporters expressed by the BCECs [2]. The entry of large molecules like most drugs into the brain is therefore limited which is additionally supported by the fact that the number of endocytotic and transcytotic vesicles in BCECs are significantly smaller compared with those of capillaries of many other
Non-viral delivery strategies into/across the brain capillary endothelial cells

About 98% of all small lipophilic drugs and all large drug molecules with a molecular weight above ~400 Dalton are unable to penetrate the BBB without an enhancing transport strategy [12].

1.2 DRUG DELIVERY TO THE BRAIN

Several strategies have been taken to enable drug transport into the brain via bypass of the BBB impermeability.

Transiently disruption of the BBB integrity can be employed to facilitate entry of drugs to the brain. Disruption is achieved by either opening of tight junctions, by enhancing pinocytosis or by creating lesions in the cell membrane [19, 20]. Disruption can e.g. be mediated by osmotic substances, vasoactive agents, chemicals, and ultrasonic waves. Hyperosmotic substances, such as mannitol cause shrinkage of BCECs and opening of tight-junctions due to an elevation of osmotic pressure [21]. Vasoactive molecules such as bradykinin and histamine are also known to disrupt the BBB [22, 23]. Chemicals like dimethylsulfoxide (DMSO) and ethanol enhance permeation of the BBB by solubilizing the BCECs membrane [19]. Furthermore ultrasonic waves can be employed to create micro-bubbles bursting in the BCECs membrane leading to a higher permeability of the BBB [20]. Administering a drug together with one of these approaches will lead to entry of the drug into the brain through the disrupted areas of the BBB.

Unfortunately, not only the drug has access to the brain. The brain is also exposed to e.g. infection, toxins in circulation and plasma proteins. Therefore these procedures can lead to severe damage e.g. serum albumin have damaging effects on astrocytes [19].

Invasive strategies for drug delivery directly to the CNS can also be employed. These delivery methods have the advantage of delivering high drug concentrations directly to the CSF or parenchymal space of the brain and low drug distribution outside CNS [24]. Drugs can be injected by intrathecal catheters in a bolus or continuous infusions [25]. Delivery is high at the site of administration but limited in success due to the poor diffusion of drugs into the brain tissue. Convection-enhanced diffusion (CED) by implanted osmotic pumps increases the distribution of the drug. The diffusion rate is though still not high enough for the drug to reach into the entire brain parenchyma [12]. Intracerebral implants have also shown to lead to controlled release of drugs in the brain. Implants are made of polymeric materials which encapsulate the drug [26]. This strategy is also based on diffusion of the drug from the implant into the brain parenchyma and has the same diffusion limitations as CED [19]. With the invasive delivery strategies follows a risk of increased intracranial pressure due to the increased fluid volume. There is furthermore a higher risk of infection in the brain, because of the need of repeated craniotomy to allow continuous drug infusion [19].

1.2.1 Non-CNS-invasive approaches to enable drug delivery to the brain

Systemic delivery of drugs into the blood-stream for transvascular delivery to the CNS is non-invasive strategies for drug delivery. Drugs are administered through
intravenous, intra-arterial or intra-nasal delivery [26]. The delivery bypasses the first-pass metabolism allowing fast access to the brain vasculature [24].

Intra-nasal delivery bypasses the BBB. Due to the highly permeable nasal epithelium, drugs can diffuse across the nasal mucosa, through the arachnoid membrane and into the olfactory CSF compartments [12, 27]. Frequent intra-nasal administration of drugs damages the nasal mucosa and only some drugs, mostly lipid soluble reached into the CSF by this strategy [19].

Intravenous delivery is limited by the non-brain-specific delivery as the drug is circulated throughout the entire vascular system of the body [26].

Intra-arterial delivery is local delivery to the brain as the blood is supplied directly to the brain before entering peripheral tissue. The intra-arterial delivery ensures a higher concentration of drugs delivered to the brain compared with intravenous delivery [28]. Drugs delivered intravenously or intra-arterially for the purpose of entering into the brain are limited by the BBB. If a drug in circulation is to cross the BBB there are great restrictions. To overcome the blood-brain barrier a drug should meet one of the following criteria:

1) Affinity for nutrient transporters or membrane receptors. An example of a substrate that is able to penetrate the BBB by this criterion is a precursor of dopamine, L-3,4 dihydroxyphenylalanine (L-DOPA). L-DOPA is a substrate for the BCEC receptor, large amino acid transporter 1 (LAT1) and is therefore able to cross the BBB without modification. L-DOPA is considered a pro-drug administered to patients with Parkinson’s disease to increase dopamine concentration [12].

2) Capability to undergo adsorptive transport e.g. by means of positive charge. Cationic albumin is able to be internalized by BCECs by electrostatic interaction with BCEC membrane proteins [19].

3) Small in size and high lipophilicity. Diazepam, a benzodiazepine, is small (284.7 Da) and highly lipophilic. Diazepam is able to diffuse passively through the BBB [29]. Diazepam is administered e.g. to patients with epileptic seizures or anxiety disorders.

If a drug does not have affinity for BCEC membrane transporters, receptors, or are small and lipophilic, it can be transported by a substance that fulfills these criteria. Drug and gene carriers are such transport vectors that enable or improve delivery of large molecules such as drugs and genetic material to a target organ.

1.3 GENE THERAPY AND DELIVERY TO THE BBB

Gene therapy was first proposed as a treatment of human diseases in 1972 by Fiedmann and Roblin [30]. Expression of disease causing genes can be corrected by gene therapy by the transfer of genetic material into target cells in order to enhance or inhibit production of a protein [31]. Gene inhibitors, such as oligonucleotides and short interfering RNA (siRNA), silence defective genes on the mRNA level in the cell cytosol. Gene enhancers such as complementary DNA (cDNA) compensates for a deficiency in the production of a specific protein [32]. Ideally cDNA is transported into the target cells by a carrier and further into the nucleus where it is integrated into the host cell genome (Fig. 2). If the integration
of the cDNA is successful, it will be transcribed and the encoded protein synthesized by the transfected cell [32, 33].

![Graphical Representation of Gene Delivery](image)

**Figure 2** Schematic drawing showing delivery of genetic material to an endothelial cell. A drug-carrier loaded with plasmid cDNA binds to the cell surface, is transported into an endosome from where it escapes and releases its plasmid cDNA into the cytosol. The plasmid DNA may enter the nucleus and incorporate into the host genome.

Table 1 displays a list of proteins which have been shown to have therapeutic effects on CNS disorders. The proteins in Table 1 are not able to cross the BBB but gene delivery enables delivery of cDNA coding for proteins across the BBB. Introducing cDNA coding for one of the proteins listed in Table 1 to cells of a patient with a CNS disease would lead to production of the protein in the target cells. Genetic material coding for glial cell line-derived neurotrophic factor (GDNF) was introduced into mice before dopaminergic nerve damages were induced. GDNF was shown to act as a neuroprotective agent on the dopaminergic neurons [34].

For the introduction of cDNA to a cell it is important to develop an efficient delivery agent, a gene carrier. Naked plasmid cDNA can be delivered systemically without a gene carrier but is rapidly broken down by nucleases and cleared by the mononuclear phagocytic system [35]. Naked genetic material are therefore typically conjugated to the surface or encapsulated inside the core of a gene carrier [33]. Gene carriers can be of viral or non-viral origin. Viral delivery is administered by viral vectors which are based on a natural virus [36]. Non-viral delivery can grossly be divided into physical and chemical approaches [32]. Table 2 displays common viral and non-viral drug-carriers used for delivery of genetic material to the CNS. Physical non-viral gene delivery methods (see table 2) are directly delivered to the cell cytosol whereas the chemical non-viral vectors needs to escape the endosome/lysosome after cellular uptake [32]. Physical non-viral delivery is invasive, difficult to apply and inappropriate for large scale transfection [32]. Gene delivery by viral vectors exploits the natural abilities of viral gene transfer to host cells. Delivery can be successful in both dividing and non-dividing
cells as they are able to escape the endosomes and deliver DNA into the cell nucleus [37].

**Table 1:** Proteins with therapeutic effect in CNS disorders

<table>
<thead>
<tr>
<th>Protein</th>
<th>Therapeutic effect</th>
<th>CNS disorder</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain-derived neurotropic factor (BDNF)</td>
<td>Neuroprotection and neuroregeneration</td>
<td>Multiple Sclerosis</td>
<td>Makar et al 2009 [112]</td>
</tr>
<tr>
<td></td>
<td>Differentiation of oligodendrocytes</td>
<td>Cerebral Ischemia</td>
<td>Yong 2009 [124]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Depression</td>
<td>Zhu et al 2011 [125]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parkinson’s disease</td>
<td>Yu and Chen 2011 [126]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sun et al 2005 [127]</td>
</tr>
<tr>
<td>Glial cell line-derived neurotropic factor (GDNF)</td>
<td>Neuroprotection and neuroregeneration</td>
<td>Parkinson’s disease</td>
<td>Biju et al 2010 [34]</td>
</tr>
<tr>
<td>Neural growth factor (NGF)</td>
<td>Promote neuronal growth</td>
<td>Alzheimer’s disease</td>
<td>Li et al 2008 [128]</td>
</tr>
<tr>
<td>Growth hormone (GH)</td>
<td>Neuroregeneration and neuroprotection</td>
<td>Mild cognitive impairment and</td>
<td>Zhang et al 2010 [129]</td>
</tr>
<tr>
<td></td>
<td>Proliferation of astrocytes, neurons and</td>
<td>Alzheimer’s disease</td>
<td>Isgaard et al 2007 [114]</td>
</tr>
<tr>
<td></td>
<td>oligodendrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic fibroblast growth factor (bFGF)</td>
<td>Neuroprotective</td>
<td>Brain Ischemia</td>
<td>Song et al 2002 [130]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ma et al 2008 [121]</td>
</tr>
<tr>
<td>Erythropoetin (EPO)</td>
<td>Neuroprotective and neuroregenerative oligodendrogenesis</td>
<td>Parkinson’s Disease</td>
<td>Boado et al 2010 [132]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain Ischemia/hypoxia</td>
<td>Xue et al 2007 [133]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Iwai et al 2010 [134]</td>
</tr>
</tbody>
</table>

Although most viral genetic material is removed from the viral vectors, leaving only sequences for delivery of the exogenous genetic material, there are still concerns about the use of viral vectors [36]. Integration into the host genome has been shown to come with a high risk of insertional mutagenesis. Furthermore the innate immune system are of risk of recognizing surface antigens on the viral vectors which can lead to destruction of all the virally transfected cells [36, 37].
Table 2: Common viral and non-viral gene delivery vectors

<table>
<thead>
<tr>
<th>Delivery vector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral vectors:</strong></td>
<td></td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Lundberg et al 2008 [135]</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Thaci et al 2011 [37]</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>Gray et al 2010 [136]</td>
</tr>
<tr>
<td>Herpes virus</td>
<td>Sun et al 2005 [127]</td>
</tr>
<tr>
<td><strong>Non-viral vectors:</strong></td>
<td></td>
</tr>
<tr>
<td>Physical techniques:</td>
<td></td>
</tr>
<tr>
<td>Microinjection</td>
<td>Zang and Yu 2008 [137]</td>
</tr>
<tr>
<td>Gene gun</td>
<td>Benedicksson et al 2005 [138]</td>
</tr>
<tr>
<td>Electroporation</td>
<td>De Vry et al 2010 [139]</td>
</tr>
<tr>
<td>Magnetofection</td>
<td>Scherer et al 2002 [77]</td>
</tr>
<tr>
<td>Chemical techniques:</td>
<td></td>
</tr>
<tr>
<td>Lipid based:</td>
<td></td>
</tr>
<tr>
<td>Cationic liposomes (Lipoplexes):</td>
<td>Tros de Ilarduya et al 2010 (Review) [140]</td>
</tr>
<tr>
<td>e.g.</td>
<td></td>
</tr>
<tr>
<td>Anionic PEGylated immunoliposomes (PILs)</td>
<td>Caveletti et al 2009 [141]</td>
</tr>
<tr>
<td>Lipid coated DNA complexes (LCDC)</td>
<td>Skjørringe et al 2009 [123]</td>
</tr>
<tr>
<td><strong>Polymer-based:</strong></td>
<td></td>
</tr>
<tr>
<td>Cationic polymers (Polyplexes)</td>
<td>Lehtinin et al 2008 [142]</td>
</tr>
<tr>
<td>e.g.: Polyethylenimine</td>
<td>Tros de Ilarduya et al 2010 (Review) [140]</td>
</tr>
<tr>
<td>Son et al 2011 [143]</td>
<td></td>
</tr>
<tr>
<td>Dendrimers</td>
<td></td>
</tr>
<tr>
<td>Polymeric micelles</td>
<td>Svenson 2009 [144]</td>
</tr>
<tr>
<td>Shao et al 2010 [145]</td>
<td></td>
</tr>
</tbody>
</table>

Non-viral chemical gene vectors are less efficient than the viral vectors as they often lack natural strategies for endosomal/lysosomal escape and nuclear delivery, but the non-viral chemical gene vectors are less immunogenic and easy to prepare in large scale [33, 38].
1.3.1 Delivery by non-viral chemical gene vectors

Non-viral chemical gene vectors can be conjugated with various targeting molecules to increase BCEC internalization [2, 38]. Targeting the BBB can be achieved by conjugating the non-viral gene vector with a ligand that has affinity for a membrane receptor on the luminal side of BCECs [2]. Transferrin is such a targeting molecule which has affinity for the BCEC transferrin receptor. Unfortunately exogenous transferrin is in direct competition with endogenous transferrin and this limits the possibility of its use for delivery. Anti-receptor antibodies are also widely used to target BCEC receptors and are not in competition with the endogenous proteins. OX26 is a monoclonal anti-rat-transferrin receptor antibody that has been shown to be taken up by BCECs [39, 40]. Unlike transferrin which undergoes transcytosis after receptor binding the monoclonal antibody OX26 has been shown to mainly stay within the BCECs after receptor binding and internalization [41, 42]. OX26 is therefore appropriate for targeting BCECs. The transferrin receptor is not only expressed by BCECs but also by other cell types such as epithelial cells of the intestinal crypts, orthochromatic normoblasts, reticulocytes, trophoblasts cells of the hemochorial type of placenta, Sertoli cells of blood-testis barrier, immature erythroid cells, and hepatocytes [43, 44]. Therefore, targeting the transferrin receptor will possibly not lead to exclusive uptake by BCECs. The uptake by BCECs could though be heightened if the conjugates were administered into the carotid artery and thereby pass the capillaries of the brain early in circulation.

When internalized the non-viral gene vectors are enclosed inside an early endosome which matures to a late endosome and fuses with a lysosome. To avoid degradation in the lysosome the carrier has to escape into the cytosol. Some non-viral carriers for example polyethylenimine (PEI) are capable of escaping the lysosomes by a process called the proton sponge effect: In the acidic lysosome, PEI will bind protons which are pumped in and these are followed by chloride ions and water. Eventually this makes the lysosome swell and burst [45]. After escape from the endosome/lysosome the gene vector needs a rapid trafficking to the nucleus, because DNA is degraded as quickly as within 50-90 min in the cytosol due to nucleases [46]. In dividing cells the nuclear envelope is momentarily open during mitosis, hence allowing transport of DNA into the nucleus. In non-dividing cells DNA reside in the cytosol in between cell divisions and is therefore prone to degradation.

Transport through the nuclear membrane pores is restricted because of a pore diameter of only approximately ~25nm [47]. Most DNA fragments are therefore not able to cross the pores without nuclear trafficking. For gene therapy plasmid DNA can be coupled to a nuclear localization signal (NLS) that enables docking to the nuclear membrane pores and subsequent transport to the nucleus [48, 49]. Coupling NLS to plasmid DNA has been shown to enhance nuclear uptake by 10 to 1000 fold [50, 51]. Overall the optimal characteristics of a non-viral carrier would be that it is biodegradable, non-toxic and have a high delivery
rate. Furthermore it should protect its cargo from degradation and be able to deliver its cargo to the cell nucleus.

In this thesis the focus is on two different non-viral carriers, the polyplex: Pullulan-Spermine and superparamagnetic iron oxide nanoparticles (SPIOs) and will be described in further detail in the next sections.

1.3.2 Polyplexes
Polyplexes are complexes consisting of cationic polymers and DNA. Cationic polymers consist of large organic molecules; they include polypeptides, polysaccharides, polyamines and dendrimers.

Many different cationic polymers have been developed for the purpose of polyplex formation e.g. PEI [52], poly-L-lysine (PLL) [53], polysaccharides like chitosan [54], and polyamidoamine dendrimers (PAMAM) [55].

Due to the electrostatic bindings between cationic polymers and anionic DNA, the polymers are excellent carriers of DNA and able to condense DNA to a small size of importance for BBB penetration. An important criterion for the strength of the polymer binding to the DNA is that it has to be sufficiently strong to carry the DNA into the target cell, but at the same time weak enough to allow the separation from the DNA in the cytosol.

The ratio of cationic polymer and DNA in a polyplex is determined by its N/P ratio in where the N refers to the number of nitrogen atoms in the amine groups of the polymer and P to the phosphor content in the DNA. If the polymer contains many branches of amine groups the transfection rate is increased and the toxicity is lowered, e.g. as seen in branched PEI [56].

In circulation, cationic complexes are in risk of being bound to negatively charged albumin, which hinders them from entering the cells. This phenomenon may occur both in vitro and in vivo [33, 57]. Moreover, when polyplexes are administered intravenously they are often recognized by the immune system as exogenous material and scavenged [33]. Coating the polyplexes with polyethylene glycol (PEG) known as PEGylation shields the polyplexes from this clearance [58, 59].

The positive charge of the polymers enables interaction with anionic glycoproteins and proteoglycans residing on the surface of the cells [60]. Concerning their cellular entry, polyplexes are believed to undergo unspecific cellular uptake by endocytosis [61, 62]. Thereafter, they need to escape the endosome/lysosomal system to avoid degradation, which can occur by the so-called proton sponge effect (see above). The proton sponge effect can be created by introducing histidine residues to the polymers [63]. Surface modifications of the polyplexes may also facilitate their escape into the cytosol from the endosome/lysosomal system. Hence, PEGylation of the cationic polymers is known to enhance this escape [53].

1.3.3 The novel drug carrier pullan-spermine and gene delivery
Pullulan-spermine is a novel natural cationic complex suitable for forming polyplexes (Fig. 3). Pullulan is a water-soluble extracellular polysaccharide with
repeated units of maltotriose condensed through α-1,6 linkage [64] (Fig. 3). Pullulan is produced by the polymorphic fungus *Aureobasidium pullulans* [65]. Spermine is a polyamine present in all eukaryotic cells and is involved in basic cellular metabolism (Fig. 3). Coupling spermine to a non-viral carrier increases the transfection efficiency [66]. Pullulan is not a cationic molecule but can be cationized by introducing spermine into its hydroxyl groups [64]. Negatively charged plasmid DNA interacts with cationized spermine branches and the more spermine the more DNA is complexed with pullulan-spermine [64].

![Figure 3](image)

**Figure 3** The chemical structures of pullulan, spermine and the pullulan-spermine complex.

Pullulan has affinity for asialoglycoprotein receptors which is highly expressed by hepatocytes in the liver [65]. However, the pullulan-spermine complex is also internalized in cells that do not express asialoglycoprotein receptors [65]. Pullulan-spermine is thought to undergo cellular endocytosis both with clathrin or raft/caveolae-dependent endocytosis [65]. It is believed that pullulan-spermine complexes larger than 200nm enters the cells via calveolae-dependent endocytosis and complexes smaller than 200nm are internalized by clathrin-dependent endocytosis [65]. Following internalization of plasmid DNA conjugated with pullulan-spermine, plasmid DNA enters into the nucleus while pullulan-spermine complexes only gets into the cytosol [65]. This polyplex does
not have any NLSs suggesting nuclear entrance may occur mainly during mitosis [65].

Pullulan-Spermine has shown good potential as a non-viral carrier of DNA for transfection of various cell types in vitro i.e. human bladder cancer cells (T24) [65], human hepatoma cells (HepG2) [64, 67] and mesenchymal stem cells [68, 69].

1.3.4 SPIOs and Blood-Brain Barrier Penetration
A relatively new approach within the field of drug delivery to the brain is the use of magnetic nanoparticles as drug carriers. Magnetic nanoparticles have been applied for diagnostic purposes for about 40 years, but in the last decade their applications have been intensified [70]. They are currently used for many purposes both in basic research and clinical medicine e.g. as a contrast agent for magnetic resonance imaging (MRI) [71], induction of hyperthermia for tumor therapy [72], cell labeling and separation [73, 74], drug delivery [75, 76], and magnetofection [77].

SPIOs are a subtype of SPIOs that is highly magnetizable and have a core of iron-oxide like magnetite (Fe₃O₄) or maghemite (γ-Fe₂O₃) that both are half-metallic. SPIOs have a mean diameter of around 50-100nm [78]. The iron oxide particles show low toxicity and will in time be broken down in the organism to Fe²⁺ and Fe³⁺ that gets incorporated in hemoglobin [78]. SPIOs have been shown to induce oxidative stress in murine macrophage (J774) cells, but only in doses higher than 100µg/ml [79]. For improved visual detection, the magnetic core can be coated with a fluorescent dye. The surface of the SPIOs can furthermore be coated with organic or inorganic substrates e.g. dextran, chitosan, starch, phospholipids or PEG [80, 81, 82, 83]. A coat of PEG can prolong the time in systemic circulation, just as with the polyplexes described above, because they are made less prone to clearance by the mononuclear phagocytic system [76, 83]. Uncoated SPIOs tend to aggregate because of a strong dipole-dipole attraction between the particles. This can be avoided by coating the particles with monomers, inorganic materials or polymers e.g. starch or dextran [80, 84]. A coat of polymeric materials has also been shown to protect the particles from oxidation and thereby making the particles more biocompatible [80, 84]. Furthermore a surface coat of e.g. chitosan or phospholipids enables conjugation of e.g. antibodies, DNA and/or drugs to SPIOs [80, 82, 84, 85].

The SPIOs are also very potent for targeted drug delivery. With the aid of a magnetic force, they are able to very precisely deliver their cargo to a target organ. A magnetic field is supplied by an external magnet or an implanted magnet. When applied, the SPIOs are drawn towards the magnet and concentrated in the area where the magnet is located. The delivery can therefore be very locally and in consequence, fewer particles will be directed towards other non-target areas enabling reduced dosage. The lower dose of nanoparticles will presumably also lead to a reduced risk of unwanted side-effect [79, 80, 85].
1.4 IN VITRO BLOOD-BRAIN BARRIER MODELS

Modeling the morphology and permeability of the BBB has been an important issue for decades. The experimental conditions in vitro are often more controllable than those in vivo and they are overall also more ethically acceptable as the usage of cell lines results in lower use of laboratory animals. Although the BBB formed in vitro models lacks the full complexity of the in vivo BBB many parameters of the in vivo conditions can be assayed in vitro e.g. tight junction expression, luminal to abluminal transport of large molecules, and gene expression experiments of the BBB.

A valid real-time monitor of the integrity of the BBB in vitro is made by measurements of the trans endothelial electrical resistance (TEER). Unfortunately, BBB in in vitro models does not express as high TEER values as can be measured on the BBB in vivo [86]. In vivo BBB TEER values are in the range of 1200-1900 $\Omega \cdot cm^2$ and have even been measured as high as 8000 $\Omega \cdot cm^2$ [86, 87]. In vitro models using cultured endothelial cells generally have a TEER value around 6-10 times lower as those recorded in vivo [86].

For in vitro studies of the BBB both primary and immortalized cells are being used. BCECs of an in vitro BBB model should express as many endothelial markers e.g. ZO-1 and PECAM-1 as possible. Primary BCECs have been isolated and cultured from most mammals with the foremost coming from rat, human and bovine brains (e.g.[88, 89, 90]). The major advantage of primary cells is that they express most of the in vivo BBB properties to a higher extent than those of immortalized cells. Most of the immortalized cell lines have been derived from the same species as those of the primary cells and subsequently immortalized e.g. by introducing simian virus 40 (SV40) T antigen. Examples of immortalized cell lines are rat brain endothelial cells, RBE4 and human brain endothelial cells, hCMEC/D3 [91, 92]. Immortalized endothelial cells form less tight BBB properties, which can be seen as a lower TEER values than in vivo or in primary culture, and they do not consistently express endothelial cell markers [93]. Many immortalized cell lines also tend to lose their BBB properties after having been passaged many times in culture [87].

In a model of a well formed BBB BCECs obtain the same polarized properties as can be found in BCECs in vivo. The polarized BCECs will form a barrier with an apical membrane facing the lumen of the vessel, a basal membrane facing the abluminal brain side, and a lateral membrane containing tight junction proteins facing the lateral membrane of adjacent BCECs. The various domains of the BCEC membrane have distinctive characteristics determining their function. The mechanisms that induce polarization are not fully understood, but astrocytes are known to secrete a number of substances that participates in the induction of the BBB e.g. basic fibroblast growth factor (bFGF) and angiopoitin 1 (ANG1) [3].

Astrocyte conditioned medium (ACM) have been shown to increase the barrier properties of the endothelial cells [94]. The ACM is obtained from astrocytes in culture and is believed to contain some of these BBB inducible factors like bFGF and ANG1. The in vitro BBB model is improved by addition of ACM to the culture media or even better by co-culturing astrocytes with BCECs.
Pericytes are also known to induce a tighter BBB and therefore they may be included in a triple co-culture model for in vitro BBB studies together with BCECs and astrocytes [95]. Furthermore, elevation of cAMP in the growth media by addition of hydrocortisone strengthens the BBB properties of BCECs [88, 94, 96].

Optimal properties of an in vitro BBB model are reflected in high expression of tight junction proteins that lead to an accordingly high TEER value, expression of BBB transporters, and in low tracer permeability of e.g. sodium fluorescein or sucrose.

### 1.4.1 Static in vitro Blood-Brain Barrier Model

Static in vitro models have been employed for decades (e.g. [88, 95, 97, 98]). They are based on the insertion of a microporous membrane filter into the well of a culture plate (Fig. 4). Brain endothelial cells are cultured on the membrane in the insert, hence forming a monolayer which models an intact BBB.

![Endothelial cells in culture](image)

**Figure 4** A hanging cell culture insert inserted into a well of a culture plate. A microporous membrane forms the bottom of the insert. In the insert BCECs can be cultured in a monolayer.

Astrocytes can be cultured in the well underneath the inserts, which corresponds to the abluminal side of the BBB, in a non-contact co-culture. The astrocytes can also be seeded on the membrane on the outside of the inserts, which ensures direct contact between the astrocytes and endothelial cells through the microporous membrane and is therefore called a contact co-culture. In both co-culture forms the TEER can be measured with the aid of two electrodes. One electrode is inserted into the well and the other into the insert. The two electrodes are separated by the endothelial cell layer and its electrical resistance is measured. TEER measurements of the static in vitro BBB models are generally lower than in vivo conditions although a few studies have reported on high TEER values reaching those of TEER values in vivo [87].

### 1.4.2 Dynamic in vitro Blood-Brain Barrier Model

Dynamic in vitro BBB models are based on the fact that they are able to create shear stress. Shear stress is able to induce most features of the BBB phenotype e.g.
BBB tightness. In vivo shear stress is the mechanical pressure generated by the blood flow exerted on the luminal surface of the endothelial cells [99, 100]. Shear stress is not created in the static in vitro BBB models.

In this thesis the emphasis will be on the dynamic in vitro blood-brain barrier model “Flocel” made by Flocel Inc., USA [100, 101, 102, 103, 104, 105, 106, 107, 108, 109]. The Flocel model (Fig. 5) consists of a cartridge with two compartments.

![Diagram of Flocel model](image)

**Figure 5** The dynamic in vitro BBB model. The model consists of a cartridge placed in a TEER measurement system. The DIV-BBB cartridge has an inner compartment consisting of 19 hollow fibers made of a microporous membrane. The cartridge has four sampling ports and four electrodes for measuring.

The inner compartment is made up of hollow fibers which mimic brain capillaries. On the outside of the hollow fibers, the outer compartment constitutes the surrounding space mimicking the brain extracellular space. The cell media is pumped via CO$_2$/O$_2$ permeable tubing through the hollow fibers creating shear stress along the inner surface. Sampling ports connect to both the inner and outer chambers from where media can be collected. The bottom side of the cartridge contains four electrodes that allow for measurement of TEER values of the cells placed inside the hollow fibers to form a barrier. BCECs can be seeded in the inner chamber of the hollow fibers. Furthermore astrocytes can be seeded in the outer chamber at where they can grow to cover the entire abluminal side of the hollow
fibers and form direct contacts with BCECs through micro pores in the fiber walls. TEER values in the Floigel model have been measured to values around 1200 \( \Omega \cdot \text{cm}^2 \), which is near the TEER of the BBB in vivo (e.g. [109]).
2 Objective of the thesis

Over all the objective of this study is to find applicable drug carriers for delivery to BCECs. Furthermore the objective is to establish the best possible *in vitro* BBB model for testing the application of drug carriers. For further description the objective can be divided into three separate aims for further description.

1) The first aim is to investigate a novel non-viral carrier pullulan-spermine for its abilities to function as a transfection agent at the BBB. Pullulan-spermine has been proved to be able to carry cDNA into various cell types and therefore this part of the thesis aims at exploring, if the carrier also has capabilities of gene delivery to BCECs. If pullulan-spermine could successfully deliver DNA to the BCECs then it would be interesting to detect whether the DNA also would be transcribed and expressed by the BCECs. It would also be interesting to investigate whether the BCECs would be able to produce and secrete the DNA encoded protein.

2) SPIOs can potentially be used for targeted delivery and the second aim of the thesis is to investigate if SPIOs would be able to enter into and cross the brain capillary endothelial cells. This would involve the application of an external magnetic force that can pull the SPIOs towards the source of the magnetic field. The particles could therefore potentially be very precisely delivered. Therefore the aim in this part of the thesis is to test the ability of magnetic particles to pass through BCECs cultured in an *in vitro* BBB model with and without an external magnetic source. The impact of SPIOs and the external magnetic source on BBB integrity and BCEC vitality is also investigated.

3) The third aim is to characterize a new dynamic *in vitro* model of the BBB, which can be used for testing the ability of various drug and gene carriers to penetrate the BBB. The model has been claimed to exceed the abilities of other models in the field to replicate the BBB. The dynamic model will therefore be compared with a well-established static model. A good model should be able to express as many BBB characteristics as possible and therefore give reasonable indications of the abilities of the carriers to penetrate the *in vivo* BBB.
3 Results

3.1 STUDY I

GENE DELIVERY BY PULLULAN DERIVATIVES IN BRAIN CAPILLARY ENDOTHELIAL CELLS FOR PROTEIN SECRETION

Louiza Bohn Thomsen, Jacek Lichota, Kwang Sik Kim and Torben Moos

The manuscript was published in Journal of Controlled Release, Vol. 515, Issue 1, 45-50, 2011.

Reprinted with permission.
Gene delivery by pullular derivatives in brain capillary endothelial cells for protein secretion

Louiza Bohn Thomsen 1,2,*, Jacob Lichota 3, Kwang Sik Kim 4, Torben Mose 4

1 Department of Health Science and Technology, Morslevvej, Aalborg University, 9220 Aalborg, Denmark
2 Division of Molecular Neuroradiology, Department of Health Science and Technology, Aalborg University, 9220 Aalborg, Denmark
3 Division of Molecular Neuroradiology, Department of Health Science and Technology, Aalborg University, 9220 Aalborg, Denmark
4 Division of Molecular Neurobiology, Department of Health Science and Technology, Aalborg University, 9220 Aalborg, Denmark

ARTICLE INFO
Article History:
Received 2 July 2011
Accepted 9 August 2011
Available online 19 August 2011
Keywords:
Blood-brain barrier
Pullular
Gene therapy
Gene expression
Spermicides

ABSTRACT
The blood-brain barrier (BBB) formed by brain-capillary endothelial cells protects the brain against potentially harmful substances present in the circulation, but also restricts exogenous substances such as chemotherapeutically acting drugs or proteins from entering the brain. A novel and rather unchallenged approach to allow proteins to enter the brain is gene therapy based on delivery of genetic material into brain capillary endothelial cells. In theory, in vitro transfection will allow protein expression and secretion from brain capillary endothelial cells which enter the brain via transfection of transport proteins across the BBB. The aim of this study was to investigate the potential to use brain capillary endothelial cells as carriers for recombinant protein production. Neural gene carriers were prepared from human brain capillary endothelial cells. Human brain capillary endothelial cells were transfected with a reporter gene (Luciferase) and a control plasmid DNA. We were able to transfect rat brain capillary endothelial cells (BBB) and human brain microvascular endothelial cells (BBBE) with pullular–protein conjugates containing plasmid DNA bearing hCG (human chorionic gonadotropin (hCG)) and LUC (luciferase) reporter proteins. In vivo, pullular–protein conjugates mediated gene expression in brain capillary endothelial cells and may have potential for application in multiple disease states.

1. Introduction
The blood-brain barrier (BBB) is made of non-fenestrated brain capillary endothelial cells connected by tight junctions that restrict paracellular diffusion of solutes or drugs into the brain [1]. Thus, the blood-brain barrier is a major barrier to the transport of new exogenous molecules including polypeptides [2,3]. Polypeptides such as brain-derived neurotrophic factor (BDNF), erythropoietin (EPO), growth hormone (GH) and fibrinolytic growth factor (KGF) have been proved to have neuroprotective and neurorestorative effects [4-11]. These microvascular agents would otherwise be useless in the treatment of CNS injuries and disorders. However, these macromolecules are progressively transported from the brain in an active manner to exert their therapeutic effect (4-13). A novel and rather unchallenged approach to allow polypeptides to enter the brain is gene therapy based on delivery of genetic material into brain capillary endothelial cells (BBB). The in vivo transfection allows proteins created from plasmids and further into the brain and exposes a new paradigm for therapy to transport polypeptides across the BBB [12]. There are two general classes of carriers for gene delivery: viral and non-viral [13]. Viral carriers are highly efficient, but they are associated with a risk of not being biologically safe. The non-viral carriers have the disadvantage of being biologically safe, but they exhibit low transfection efficiency and carry large DNA fragments [14]. Pullular–protein conjugates containing plasmid DNA has been shown to be a unique carrier system for transfection of parenchymal [4-13]. Pullular is a water soluble polysaccharide (24,000,000 D) and a naturally occurring polysaccharide present in all endothelial cells and is involved in basic cellular metabolism [14,20]. Pullular–protein is known to undergo cellular endocytosis via clathrin- or caveolin-mediated endocytosis and the mechanism leading to cellular uptake and further internalization are not known. Pullular might be recognized by the adenosine triphosphate (ATP) and/or ATPase (ATP) found within the bone cells which do not express ATRP and are able to influence pullular–protein conjugates [16]. In this study, we were able to construct both osteogenic and angiogenic pullular–protein conjugates and demonstrate the capability of the cationic liposome to transfer immortalized human brain microvascular endothelial cells (BBBE) and rat brain endothelial cells (BBB)
Non-viral delivery strategies into/across the brain capillary endothelial cells

2. Materials and methods

2.1. Materials

Polyplexes were transfected into Neuro-2a cells, human neuroblastoma cells (a type of neuron cell line), using Fugene 6 Transfection Reagent (Promega, Madison, WI, USA). Neuro-2a cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO2. After reaching subconfluence, cells were trypsinized and reseeded in 6-well plates at a density of 3 x 10^6 cells/well. Polyplexes were prepared by mixing equal volumes of pDNA and polyplex, followed by incubation at room temperature for 1 h. The polyplex solution was then added to the cells and incubated for 24 h. The efficiency of transfection was determined by measuring the expression of a reporter gene using a fluorescence assay.

2.2. Preparation of polyplexes

Polyplexes were prepared by mixing equal volumes of pDNA and polyplex, followed by incubation at room temperature for 1 h. The polyplex solution was then added to the cells and incubated for 24 h. The efficiency of transfection was determined by measuring the expression of a reporter gene using a fluorescence assay.

2.3. Preparation of plasmid DNA

The plasmid DNA was purified using the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA was dialyzed against PBS and stored at -80 °C until use.

2.4. Electrophoresis of plasmid DNA

The purified plasmid DNA was analyzed by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and visualized under UV light. The size of the DNA was estimated by comigration with molecular weight markers.

2.5. Characterization of polyplexes

The polyplexes were characterized by measuring their size, charge, and transfection efficiency. The size of the polyplexes was determined by dynamic light scattering (DLS). The charge of the polyplexes was measured using a ZetaPALS instrument (Brookhaven Instruments Corporation, Holtsville, NY, USA). The transfection efficiency was determined by measuring the expression of a reporter gene using a fluorescence assay.

2.6. Cell culture

Neuro-2a cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO2. After reaching subconfluence, cells were trypsinized and reseeded in 6-well plates at a density of 3 x 10^6 cells/well. Polyplexes were prepared by mixing equal volumes of pDNA and polyplex, followed by incubation at room temperature for 1 h. The polyplex solution was then added to the cells and incubated for 24 h. The efficiency of transfection was determined by measuring the expression of a reporter gene using a fluorescence assay.

2.7. In vitro transfection of brain endothelial cells

The pDNA and polyplexes were prepared according to the protocol described in Section 2.6. The cells were transfected with the polyplex solution using a transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The transfection efficiency was determined by measuring the expression of a reporter gene using a fluorescence assay.

In conclusion, we have demonstrated that non-viral delivery strategies can be used to deliver genes into the brain capillary endothelial cells. These strategies have the potential to be used for gene therapy and for the delivery of therapeutic molecules to the brain.

2.8. Conclusion

Non-viral delivery strategies have the potential to be used for gene therapy and for the delivery of therapeutic molecules to the brain.
stored for short term at –20°C for further use in an immunoprecipitation assay. None of the cells with transduced cells, neither transfected with pulskal-polyamine or TurboFect™ co-encapsulated with pCMV-βGal, were hypotonic stained with Trypan Blue (Sigma-Aldrich) and viable cells were counted.

2.4. Immunoprecipitation, gel-electrophoresis and Western blotting: isolation and detection of FLAG-tagged δN-PD

To isolate the FLAG-tagged human growth hormone that was secreted from the transfected cells and into the media, a FLAG® Tagged Protein Immunoprecipitation Kit (Sigma-Aldrich) was used according to the manufacturer’s protocol. In short, anti-FLAG affinity resin was washed: first to remove the storage buffer and secondly to remove unbound anti-FLAG antibody from the resin suspension. The resin was incubated with the media from the transfected cells, negative and positive control cells and controls for the assay. All reactions were left overnight at 4°C with agitation. The resins were then washed and the FLAG-tagged protein was eluted from the resin with ELUTION Sample Buffer and run on a 12% NaPh/Pi/m-Na/Tris-Tris-gel in NuPAGE MOPS SDS Running Buffers (Thermo) and transferred to a PVDF-membrane with the iBlot® Dry Blotting System (Invitrogen). Proteins were run on 1% or 4% gels and visualized with the anti-FLAG® 52 monoclonal antibody-polyverose conjugate in PBS containing 0.1% Tween-20. Thereafter the membrane was washed three times and incubated with 3,3',5,5'-tetramethyl-benzidine (TMB) solution for approximately 20–30 min until the intense bands were clearly visible. The membrane was washed with destilled water, dried on blotting paper, scanned and stored in the dark.

2.5. RT-PCR

RNA was purified using Nucleospin RNA I kit (Macherey-Nagel) followed by cDNA synthesis using Scripto Kit (Molinsa, USA). 1 μg of total RNA was used for every first strand cDNA synthesis with random hexamer primers. PCR was performed using DreamTaq Green master mix (Thermo) at 1 μl of each cDNA sample and 2 μl of each of the primers (Table I). PCR was run as follows: 1 cycle 95°C (5 min), 30 cycle 95°C (30 s), 62°C (30 s), and 72°C (30 s), 1 cycle 95°C (5 min). The PCR products were run for 10 min at 120 V on a 2% agarose gel with 10 μg/ml ethidium bromide and visualized on the Kodak Image Station 4400MM Pro (Carestream Health).

3. Results and discussion

An efficient non-viral gene delivery depends on carriers that can electrostatically bind to a genetic material. The present study demonstrates in vitro gene segregation after transfection of plasmid DNA-pulsed-polyamine derivative into BEB4 and HMBCC

| Table I: Primers used for RT-PCR |
|-----------------|-----------------|
| Name            | Forward primer  | Reverse primer  |
| Human GAPDH     | GAGAAAGATCATGC | TACTCCAGGAGCG |
| Human βGal      | CTCCAGAGCTGAC | TGTGCTGCTGGAT |
| Mouse pCMV-βGal | TTCTCCAGAGCT | TGGTGCTGCTGG |
| GFP              | CCTGTGCTAGCT | TGTGCTGCTGG |

3.1. Preparation and characterization of PCLs

Complication of plasmid DNA with spermine-polyamine derivatives was chosen as a method to manufacture a carrier for gene delivery into brain endothelial cells in vivo. The spermine-polyamine derivatives were prepared as described in the literature [14]. The PCLs of pulskal-polyamine or plasmid DNA were run in an agarose gel in order to prove efficient complexation of DNA with pulskal derivatives at various DNA to polyamine w/w ratios (Fig. 1).

After electrophoresis and visualization it has been concluded that PCLs have not migrated into the agarose gel due to their size. Instead, the complexes were coeluted at the edge of each well. It is interesting to note that the PCLs migrate towards the cathode or anode depending on the amount of DNA complexed, hence the total charge of PCLs (Fig. 1), which demonstrates that both ratio- and anisomes have been formed. In order to confirm this observation the q-potential of PCLs was measured. The measurements have indicated that calciplates had a positive charge of approximately 10 μC, whereas the anisomes were approximately -20 μV (Fig. 2). These results are in good agreement with previous reports describing both types of PCLs, where calciplates were measured to be approximately 10 μC, whereas anisomes were negatively charged reaching -48 μV [20]. To further characterize the PCLs, DLS measurements (a non-invasive back-scatter analysis) were performed. The size of the pulskal-polyamine derivative was very difficult to measure and resulted in several peaks suggesting an irregular shape of the conjugates. Interestingly however, both a regular form and compact structure were observed upon addition of DNA (Fig. 2a). The calciplates were usually small in size (approximately 30–80 nm) whereas anisomes reached a size of 800–3000 nm (Fig. 2a), as they were complexed with approximately 1:10 more DNA. Similarly, PCL sizes were reported previously for the calciplates [14] whereas smaller size (340 nm) was reported for anisomes [20]. The total N content was measured for the anisomes and calciplates and the N/P ratios were calculated to be 4 and 2 respectively. Based on the DLS measurements, a fraction of calciplates were isolated and exclusively used for the remaining experiments. These calciplates had an N/P ratio of 2.4.
Non-viral delivery strategies into/across the brain capillary endothelial cells

![Image](image-url)

**Fig. 3.** Measurement of size and charge of plasmid DNA: (a) size of plasmid DNA alone (control); (b) size of plasmid DNA and complexes (control). 

3.2. In vitro transfection of brain capillary endothelial cells

pHRed-C1 plasmid containing iDNA for the red fluorescent protein Red as a reporter gene was complexed with poly-L-lysine to form cationic complexes. These complexes were then used to transfect the HRECs. Forty-eight hours after the cells had been transfected, the transfection efficiency was measured using fluorescence microscopy. As shown in Figure 3, HRECs and HRECs with transfected cells were observed using fluorescence microscopy. The transfected cells were identified by the presence of red fluorescence, which is characteristic of HRECs. The transfection efficiency of the pHRed-C1 plasmid was found to be high, with a transfection efficiency of approximately 80%. This high transfection efficiency is attributed to the use of poly-L-lysine as a transfection reagent. The poly-L-lysine-mediated transfection results in the formation of positively charged complexes that interact with negatively charged cell membranes, facilitating the entry of the plasmid DNA into the cell. The transfection efficiency is further increased by the use of poly-L-lysine in the form of nanoparticles, which can enhance the uptake of plasmid DNA by the cells.

In vivo experiments on the transfection efficiency of the pHRed-C1 plasmid were conducted in mice. The transfection efficiency of the pHRed-C1 plasmid was found to be high, with a transfection efficiency of approximately 80%. This high transfection efficiency is attributed to the use of poly-L-lysine as a transfection reagent. The poly-L-lysine-mediated transfection results in the formation of positively charged complexes that interact with negatively charged cell membranes, facilitating the entry of the plasmid DNA into the cell. The transfection efficiency is further increased by the use of poly-L-lysine in the form of nanoparticles, which can enhance the uptake of plasmid DNA by the cells.
In order to further characterize NGH1 expression, the total RNA was purified from both transfected and non-transfected cells. cDNA was synthesized and RT-PCR performed (Fig. 4a, b). The PCR reaction confirmed the presence of a high amount of NGH1 transcript in the transfected cells. Another interesting observation has been made concerning expression of NGH1 in non-transfected cells; even though endothelial cells initially are not a main source of NGH1 in vivo, a small amount of NGH1 transcript was detected in the control cells, which is in accordance with previous studies [24]. Expression of a transgene has previously been proved by Thaker et al. [25] after transfections of DBCs, rat dental root ganglia, with pulsolin-spermine complexed with plasmids expressing human bone matrix growth factor (NGF). A significant increase in the outgrowth of neurites was seen in dental root ganglion cells in the SC after the transfection [26].

In this study we wanted to investigate the possibility to use BCCs as small factories for recombinant protein production. An immunoprotective assay was developed for detection of secretion of a gram protein directly into culture media after pulsolin-spermine mediated transfection. pCMV5-GH1I (GH1I cloned in vector bearing a DNA for a FLAG-tagged human growth hormone; constructed in my laboratory) in NGH1 is readily synthesized as a pro-hormone in the anterior pituitary in vivo. During processing through the endoplasmic reticulum and the Golgi, small signaling peptides are removed, and the mature NGH1 is stored in secretory granules. GH is also synthesized in small volumes in the remaining brain [24]. GH promotes growth, tissue repair and cell regeneration and has been proven to have a neuroprotective effect. Therefore it can be used for treatment of CNS injuries [6, 7]. The secretion products of NGH1 and shape of FLAG-tag immunoreactivity and detection were employed in this study. The pulsolin-spermine complex delivered plasmid with CMV-expressing NGH1 into the mononucleate of brain endothelial cells thereby making them transient transgenomic factories for production and secretion of NGH1. Gene expression by BSCF, CSP and EPO could be assessed for their ability to transfuse the brain capillary endothelial cells using the pulsolin-spermine conjugate technique and evaluate their ability to act as a transgene in disease models.

For in vivo use, the delivery of PKI containing gene material including growth factors, targeted delivery to the brain capillary endothelial cells is likely needed. We aim in the process of blocking a physiological pathway of angiogenesis for in vitro engendering by encapsulating it with a lipid coat that can be attached to an antibody that targets the transferrin receptor. The capillary endothelial cells of the CNS are the only endothelial cells in the body to contain transferrin receptors [27] and this receptor is a probable target for the delivery of vectors intended for non-viral gene therapy to the brain using the vascular route [28]. The anti-vitron transferin receptor antibody gets internalized by BCCs in vivo and therefore represents a reasonable tool for in vivo targeting of pulsolin-spermine complexes [28, 29].

4. Conclusions

In this study antisense and cardiac pulsolin-spermine/DNA complexes were prepared and characterized. Complexes have a positive charge of approximately +10 mV and had a size of approximately 300 nm whereas the antisense was approximately -20 mV in charge and 400-500 nm (i.e. HBBOS and BSCF were successfully transfused with the fluorescent reporter gene phd/sel-ch/l-E2 with good transfection efficiency and low toxicity. Secretory NGH1 protein was detected after in vivo transfection of HBBOS with pulsolin-spermine complexed with pCMV5-GH1I. We conclude that the pulsolin-spermine delivery system is a very promising method for delivering DNA to the brain endothelial cells and for using these cells as factories for protein secretion.

Acknowledgements

We would like to thank Mette Fretheim for technical assistance in the laboratory and Professor Thomas Jensen for his input in the project. The work has been kindly supported by the Danish Medical Research Council (Grant no. 271-06-0212), the Sapce Fund, and the Uhleke Foundation.

References

Non-viral delivery strategies into/across the brain capillary endothelial cells
3.2 STUDY II

IN VITRO DELIVERY OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES THROUGH BRAIN ENDO THELIAL CELLS

Louiza Bohn Thomsen, Thomas Linemann, Jacek Lichota, Kwang Sik Kim, Gerben Visser and Torben Moos

The manuscript has been submitted to Journal of Controlled Release
IN VITRO DELIVERY OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES THROUGH BRAIN ENDOTHELIAL CELLS

L.B. Thomsen\(^a\); T. Linemann\(^a\); J. Lichota\(^a\); K. S. Kim\(^b\); G. M. Visser\(^c\) and T. Moos\(^*\)

\(^a\)Department of Health Science and Technology, Aalborg University, Denmark
\(^b\)Division of Pediatric Infectious Diseases, Johns Hopkins University, School of Medicine, Baltimore, USA
\(^c\)Department of Infectious Diseases & Immunology, Utrecht University, The Netherlands

* Correspondence:
Louiza Bohn Thomsen,
Section of Neurobiology, Biomedicine
Department of Health Science and Technology
Fr. Bajers Vej 3B
Aalborg University
DK-9220 Aalborg East, Denmark
Phone: +45-99443731
E-mail: lbt@hst.aau.dk

**Keywords:** Blood-brain barrier, SPIOs, drug delivery, TEER, SPIO

**Abbreviations**

BBB – blood-brain barrier
CNS – central nervous system
BCEC - brain capillary endothelial cells
TEER – trans endothelial electric resistance
SPIO – superparamagnetic iron oxide nanoparticle
MDM – magnetic dextran microspheres

**ABSTRACT**

The blood-brain barrier (BBB) constitutes a physical, chemical and immunological barrier making the brain accessible to only a few percent of potential drugs intended for treatment inside the central nervous system (CNS). A new approach with the purpose of overcoming the restraints of the BBB by enabling transport of drugs, siRNA or DNA into the brain is to use superparamagnetic iron oxide nanoparticles (SPIOs) as drug-carriers. The aim of this study was to investigate the
ability of fluorescent SPIOs to cross the BBB facilitated by an external magnetic force. The capability of SPIOs to penetrate the barrier was shown to be significantly higher in the presence of an external magnetic force in a static in vitro BBB model of the BBB. Particles added to the luminal side of the in vitro BBB model were found in astrocytes co-cultured in remote distance on the abluminal side, indicating that particles were transported or drawn through the barrier and either taken up by or forced into the astrocytes by the external magnetic field. The SPIOs did not negatively affect the viability of the endothelial cells as revealed by a live/dead assay and by trypan blue uptake. The magnetic force-mediated dragging of SPIOs through the BBB may denote a novel mechanism for drug delivery to the brain.

INTRODUCTION
Drug delivery to the brain has proven to be a difficult task mainly due to the presence of the blood-brain barrier (BBB) formed by tightly interconnected brain capillary endothelial cells (BCECs). The impermeability properties of the BCECs are supported by astrocytes, pericytes and neurons which together form the so-called neurovascular unit [1]. The BBB excludes most molecules from entering the central nervous system (CNS) [2] and molecules must be preferably small in size and lipophilic to enter the brain [3]. In spite of being in possession of these qualities many of the carriers however fail to deliver their cargo to the brain in an amount adequate for treatment without allowing unacceptable high off-target affection. Many drug-carriers have been created, e.g. liposomes or polyplexes, which fulfill the demands of being lipophilic and/or at the nano-size scale. A relatively new approach in the field of drug delivery is the use of magnetic nanoparticles. Hence, magnetic nanoparticles are currently being used for various purposes such as a contrast agent for magnetic resonance imaging (MRI) [4], induction of hyperthermia for tumor therapy [5], cell labeling/cell separation [6, 7], targeted therapeutics [8, 9] and magnetofection [10].

Superparamagnetic iron oxide nanoparticles (SPIOs) is a subtype of magnetic nanoparticles which are highly magnetizable and have a core of iron oxide particles composed of magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) [11]. The SPIOs typically have a mean diameter of 50-100nm [11], and their iron oxide core exerts low toxicity, as it is gradually degraded to Fe²⁺ and Fe³⁺ in the body and enters the pool of body iron [11]. SPIOs have been shown to induce oxidative stress in murine macrophage (J774) cells, but only in doses higher than 100µg/ml [12]. Their magnetic core can be coated with lipophilic fluorescent dyes for visual detection. Furthermore, the particles can be protected by a biocompatible polymeric shell, like dextran, polysorbate or starch, or coated by phospholipids or polyethylene glycol (PEG) to prolong their presence within their circulation due to a lower capture of the particles by the mononuclear phagocyte system [9, 13, 14, 15]. A proper coat also prevents aggregation of the particles, which they otherwise tend to due to a strong magnetic dipole to dipole attraction [13, 17]. Furthermore a
protective coat enables conjugation of e.g. various proteins, DNA and drugs to the surface of the SPIOs [13, 15, 16, 17].

A major advantage of the properties of SPIOs is their ability to precisely deliver their cargo to a given target organ when drawn there to by the force of a magnetic field provided by an external or implanted magnet [13, 17]. Under the influence of the magnetic field, the SPIOs are drawn towards the magnet to concentrate near its location. Delivery of SPIOs will therefore benefit from being very local and its dosing can be minimized to reduce off-target effects [13, 17].

In this study the ability of SPIOs to function as drug carriers is investigated in an in vitro BBB model. The SPIOs are taken up by endothelial cell and increasingly pass the intact brain endothelial cell monolayer with the aid of an external magnet to end up in a layer of astrocytes cultured in remote distance on the “brain side” of the endothelial cells.

**MATERIALS AND METHODS**

**Materials**

Transwell membrane culture inserts and plates (Corning, Thermo Fisher Scientific), fluorescent SPIOs “nano-screenMAG-D” composed of magnetite (Chemicell, Germany), mouse-anti-ZO-1 and Alexa Fluor 488 goat-anti-mouse, live/dead cell viability assay (Invitrogen, UK), Trypan Blue stain, 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Germany), mounting media and mouse anti-cow glial fibrillary acidic protein (Dako, Denmark).

**Cell cultures**

Immortalized human brain microvascular endothelial cells (HBMEC) were cultured in Medium 199 with L-Glutamine and HEPES (Invitrogen) with 10 % Fetal Calf Serum (Invitrogen), 10 % Nu Serum IV (BD Biosciences, USA) and 100 U/mL Penicillin G Sodium and 100µg/mL Streptomycin sulphate (Invitrogen) [18]. Immortalized rat brain astrocytes (DI-TNC1) (ATCC, Sweden) were cultured in DMEM/F12 (Lonza, Switzerland) with 10 % fetal calf serum and 100 U/mL Penicillin G Sodium and 100µg/mL Streptomycin sulphate.

**Establishment of an in vitro BBB model in Transwell membrane plates**

HBMECs were seeded as monocultures in inserts of twelve wells Transwell membrane culture plates in a density of 150.000cells/insert. The HBMECs were cultured in an astrocyte conditioned media (ACM) consisting of a mixture of 50% DI-TNC1 media aspirated from astrocytes after 24 hours incubation and 50% HBMEC media. When mentioned DI-TNC1s were seeded in the wells of the 12 well culture plates with 100.000cells/well. The astrocytes cultured in DI-TNC1 media were grown overnight in a humidified incubator with 5% CO2 to ensure proper cell attachment. Then the inserts containing HBMECs were re-inserted into the Transwell culture plate’s containing the DI-TNC1 astrocytes to form a non-contact co-culture. The medium was replaced every day to avoid high media changes in the pH.
Non-viral delivery strategies into/across the brain capillary endothelial cells

**Trans Endothelial Electrical Resistance (TEER) measurements**

TEER measurements were conducted with a Millicell™ ERS-2 apparatus (Millipore, USA) and an STX-1 electrode (Millipore). To calculate the TEER $R_{\text{blank}}$ was subtracted from $R_{\text{sample}}$ and then the product was multiplied by the well area. The TW in this study had a well area of 1.1 cm$^2$; therefore the equation was as follows: $(R_{\text{sample}} - R_{\text{blank}}) \times 1.1 \text{ cm}^2 = \Omega \cdot \text{cm}^2$

The TEER was measured every second day up until seven days, and thereafter every day. Just before the TEER measurements were made the culture media was changed and cells and media were allowed to reach room temperature. Three measurements were made on each well from which an average TEER value was calculated.

**Fluorescent SPIOs**

The SPIOs used in this study is commercially available magnetic iron oxide nanoparticles with a hydrodynamic diameter of 100nm. They consist of a magnetic magnetite core surrounded by a lipophilic fluorescence dye covered by a polysaccharide matrix of starch consisting of α-D-glucose units (Fig. 1). Both red and blue fluorescent SPIOs were used in this study. The blue fluorescent nanoparticles have maximal excitation at 378 nm and emission at 413 nm and red nanoparticles have excitation wavelength at 578 nm and emission wavelength at 613 nm.

![Figure 3-1](Image) The SPIOs consist of a magnetic core, surrounded by a lipophilic fluorescence dye layer covered by a polysaccharide matrix of starch consisting of α-D-glucose units.

**Size and charge of the SPIOs**

Size (DLS/Non-Invasive Back-Scatter (NIBS)) equivalent to particle diameter and charge/ $\zeta$-potential were measured on a Zetasizer Nano (Malvern, UK). 20 µg of SPIOs was diluted in 1 ml double distilled water and tested in triplicate. The size of the SPIOs was analyzed based on the Culmulants method by the computer software which calculated the $R_s$ values and provided the apparent size of the SPIOs. The $\zeta$-potential was likewise calculated by the software tested tree times.

**Application of SPIOs on the BBB model**

When the TEER of the HBMEC’s reached a plateau, indicating that the highest TEER had been reached and the endothelial cells had formed a barrier, the fluorescent SPIOs (Fig.1) were added to the inserts in doses of 35, 70 and 140
µg/insert in three replicas of each concentration. The process of addition of SPIOs to the cell culture is demonstrated in Fig. 2. The external magnetic force was supplied by a ferrite block magnet with field strength of 0.39 Tesla.

![Image](image_url)

**Figure 3-2** Drawing of the method employed for addition of SPIOs to the culture inserts with the aid of avoiding paracellular transport. SPIOs are depicted in blue. 1) The inserts were moved (2) to other twelve well plates (3). After addition of the nanoparticles (4), the endothelial cells (pink) were incubated for 24 hours. Afterwards, the media from the inserts was changed, and the inserts washed three times with PBS to remove nanoparticles that had not been taken up by the HBMEC’s (5+6). The inserts containing endothelial cells in PBS (light blue) (6) were reinserted containing cells in the twelve well plates (7) and placed on a ferrite block magnet for 5 hours at 37°C to draw the magnetic particles towards the bottom of the well (8) in where astrocytes (green) were cultured in remote distance from the endothelial cells. A control plate was also kept at 37°C in secure distance from the magnet. After 5 hours the media in upper and lower chambers of the wells was collected and stored at 4°C. A control plate was also kept at 37°C in secure distance from the magnet. After 5 hours the media of the upper and lower chambers of the wells were collected and stored at 4°C.

**Immunostaining**

After terminating the experiment, the HBMEC’s of the control and the experimental plates were washed three times in PBS, fixed in 4% paraformaldehyde for 4 minutes and washed three times in PBS. The cells were incubated overnight with mouse-anti-zonula occludens-1(ZO-1), and binding of the primary antibody was visualized using Alexa Flour 488 goat-anti-mouse. DI-TNC1s were incubated overnight with mouse-anti-glial-fibrillary acidic protein (GFAP), and binding of the primary antibody was visualized using Alexa Flour.
488 goat-anti-mouse. The cell nuclei of both DI-TNC1s and HBMECs were stained with DAPI for 5 minutes. The membrane of the inserts containing HBMECs was cut out of the insert, mounted on a slide with fluorescent mounting media and observed under a fluorescence microscope.

**Cytotoxicity**

To examine if the cells get impaired by the SPIOs or by the application of the external magnetic field, the cell viability was visualized using a live/dead cell viability assay. The assay was performed according to the recommendations from the vendor. In brief, two working solutions were prepared: Solution one containing 50 µM C12-resazurin in Dimethylsulfoxide (DMSO), solution two consisting of 1 M SYTOX Green stain in DMSO. The culture medium was aspirated from the TW inserts, and 0.25 ml of PBS added to each well. The working solutions were added to the wells to reach a final concentration of 5 µM C12-resazurin and 50 nM SYTOX Green dye in the two solutions respectively. The cells were then incubated at 37°C in an atmosphere of 5% CO2 for 15 minutes and afterwards they were kept on ice, rinsed three times with PBS and observed under a fluorescence microscope.

Dead and viable cells were counted on the basis of a counting of Trypan blue-labeling, as trypan blue only enters dead cells. Cells were cultured in monoculture in six wells culture plates until 100 % confluence was reached. Then SPIOs was added to half of the wells in a concentration of 1170 µg which corresponded to the highest dose (140µg/insert) added in amount per square centimeter in the experiment described above. The cells were incubated with or without SPIOs for 24 hours and placed on the plate magnet for 5 hours. The cells were then trypsinized and mixed with Trypan blue. An appropriate amount of cell suspension containing Trypan blue was then filled in a hemocytometer and dead and living cells counted. The total amount of dead and vital cells were calculated and a student’s T-test was performed to test, if there were any differences in the amount of vital and dead cells between the control wells and experimental wells subjected to the magnetic force. A p-value at p<0.05 was considered statistically significant.

**Quantification of SPIOs crossing the BBB in vitro**

The well plates with the presence of DI-TNC1 astrocytes were investigated under a fluorescence microscope with the medium remaining in the wells. The fluorescent SPIOs were counted using a counting mesh with an area of 0.054mm² that was inserted inside of the microscopes ocular. Counts were made at randomly picked areas 10 times per well to obtain a statistical correct counted average of the amount of nanoparticles in the wells. By the use of a student’s t-test, it was examined if there were any differences between the amounts of particles in the wells of control versus experimental plates. A p-value was considered statistically significant at p<0.05.
RESULTS AND DISCUSSION
In this study we wanted to investigate whether SPIOs are able to enter and cross BCECs and if an external magnetic force could be applied to aid the penetration rate and efficiency. We also wanted to test if the particles have a toxic effect on the cells and will obstruct the barrier when passing the endothelial cell layer.

Size and charge of the SPIOs
The hydrodynamic diameter of the SPIOs was determined by DLS, which is a back scatter analysis. The SPIOs had a mean diameter of 117.5 nm which is a little larger than proclaimed by the manufacturer. Furthermore the $\zeta$-potential of the SPIOs was measured to be -16.8 mV. Starch coated SPIOs have previously been found to be of similar anionic charge [19].

SPIOs enter into and cross though endothelial cells
Using immunofluorescence, the HBMECs were investigated for their expression of ZO-1, a marker of tight junctions, before and after exposure to SPIOs and subsequent magnetic force (Fig. 3). Clear signal and equal intensity of the ZO-1 marker protein provide morphological evidence that tight junctions were present between HBMECs in both the experimental and control plates.
Figure 3. The presence of fluorescent SPIOs (a,b), ZO-1 (c,d), and DAPI (e,f) in inserts with cultured HBMECs in absence (b, d, f, h) or presence (a, c, e, g) of magnetic force. Overlay of a, c, e in g and overlay of b, d, f in h. A magnification of h can be seen in i. The ZO-1 expression is prominent in both the experimental and control plates (a and b). Notice that there are fewer SPIOs present in the insert exposed to a magnetic force (a) compared to that of the control insert (b). (The red fluorescent SPIOs are shown in white for better visualization) (The insert membranes tend to bulge on the slides which results in different levels of focal points within a frame and “out of focus areas” can be observed) ((a-h) scale bar = 50µm, (i) scale bar = 5µm)

Before applying the external magnetic force, SPIOs were located inside the HBMEC monolayer (Fig. 3). After exposition to the magnetic force fewer particles
were detected in the HBMEC monolayer (compare Figs. 3a and 3b), indicating that the nanoparticles were drawn through the cells by the magnetic force and entered the abluminal (“brain side”) chamber of the microporous membrane. These findings suggest that the SPIOs are taken up by the HBMECs even without the addition of an external magnetic force. They also indicate that SPIOs do not need any further chemical/physical changes on their surface to interact with the BCECs and can subsequently be internalized by BCECs.

SPIOs very similar to those used in the present study coated with starch and of a size of ~110nm have been shown to enter the brain of Fischer 344 rats when injected intravenously without the presence of a magnetic force [13]. This supports the findings in the present study and it seems as there are to some extent an extravasation of the SPIOs. These results imply that a targeting strategy towards BCECs is needed if these are to be the only target. If systemically injected the SPIOs used in this study would probably also interact with other cells than BCECs. Therefore a new strategy is necessary for targeting the particles to the BCECs only. It has been shown that SPIOs can be coated with substrates that can bind e.g. ligands or antibodies [15]. With such modified SPIOs, BCECs can be directly targeted and exclusive uptake by in BCECs can be achieved. The results seen in Fig. 3 also suggest that SPIOs can be drawn out of the HBMEC monolayer by an external magnetic force, which contributes the targetability aiding their passage towards their intended destination. This phenomenon was therefore explored further in this study.

**Exposure to SPIOs and magnetic force does not lead to cytotoxicity of the endothelial cells.**

SPIOs exhibit a generally low, but concentration depended cytotoxic action [11, 12]. Our study revealed no signs of lost vitality of the HBMECs after the cells had been incubated for 24 hours with various concentrations of SPIOs (35µg, 70µg and 140 µg per ml) (Fig. 4). Hence, a trypan blue stain conducted to count the amount of dead cells in wells incubated with or without 140µg/ml SPIOs revealed no statistical difference (p<0.05) between cell viability in the two conditions. Naqvi et al. (2010) observed the toxicity of SPIOs with a Tween 80 coat and 30nm in diameter increases in a concentration-dependent manner [12]. In their measurements, the toxicity seen as a marked change in cell viability was observed when between 100 and 200µg/ml SPIOs were added to cultures of murine macrophage cells (J774), indicating that SPIOs are non-toxic to cells in concentrations of 100µg/ml or less [12]. These data are in good accordance with the results of the present study even though the concentration of 140µg/ml lies within their range of a toxic concentration, but does not exhibit any toxic effect on cells in our study. Furthermore, it has been shown that SPIOs with an anhydroglucose polymer coat and 50-150nm in diameter did not affect the mortality of Sprague-Dawley rats when injected in the tail vein in a dose of 5% of the estimated blood volume [20]. The rats were monitored for up to 65 days and it was detected that the amount of magnetic particles found in the animal decreased over time [20]. These data indicates that magnetic nanoparticles can be
administered systemically without exerting toxicity on cell cultures or animals. It also seems that when given in vivo the particles are cleared probably by deposition into iron stores in the cells. Therefore the particles should be safe to administer in small doses in vivo.

![Figure 4](image.png)

**Figure 4.** Live/dead cell viability stain of HBMEC’s in Transwell membrane inserts. Lower row, HBMEC’s in inserts from experimental plates subjected to magnetic force. Upper row, HBMEC’s in inserts from controle plates. 70 µg of SPIOs were added to the insert on both plates. Dead cells are visualized with Sytox green stain/uptake (a+d)(indicated by white arrows). Live cells are visualized with resazurin staining (b+e). Overlays of the two stains are seen on c) and f). There are no differences in the viability between the cells of the two plates. (scale bar = 50µm)

**The integrity of the in vitro BBB model**

A commonly used in vitro model of the BBB consisting of a microporous membrane insert to form a static model of the BBB was employed in this study. In Figure 5 the TEER values measured on HBMECs of a control and experimental plate can be seen. The TEER values was rather low but did reach a threshold TEER value of 43.6±0.7 Ω•cm²±SE which is not unusual for human brain microvascular cell lines [21]. The TEER depends on how tightly the BCECs are interconnected via tight junctions and a low TEER could indicate that there are open areas in between BCECs [21]. Although the immunostaining of ZO-1 showed the presence of tight junctions between BCECs we wanted to eliminate the possibility of SPIOs passing the BCECs paracellularly.

To secure exclusive transcellular transport of SPIOs by the HBMECs and exclude paracellular transport, the cells were cultured in culture inserts and removed from the culture plates (empty or containing astrocytes) while adding the SPIOs (see description in the results section and Fig. 2). After incubation with the SPIOs for 24 hours the HBMEC monolayer was washed to remove excess nanoparticles and the inserts were returned to their original culture plate (Fig. 2). By performing this step it was ensured that any particles detected in the abluminal side of the well-
chamber under the inserts could only derive from the magnetic force drawing the particles through the HBMECs or from their secretion.

The TEER of the HBMECs reached a plateau after approximately 6 days of culture (Fig. 5). The TEER values were also measured after magnetic force had been applied to the HBMECs containing SPIOs (Fig. 5). The stable TEER values after the exposure to the magnetic field indicate that the integrity of this *in vitro* BBB was not harmed by the magnetic-field-aided penetration of the SPIOs. This observation is in good agreement with the findings in Saiyed et al. (2010) who showed that magnetic particles encapsulated in liposomes were taken up by monocytes and drawn through an *in vitro* BBB model with an external magnet without affecting TEER values [17].

![Graph showing TEER values over time](image)

**Figure 5** The graphs shows the measured TEER values over time from both the control (circle) and experimental plate (triangle) in HBMEC monocultures grown in the presence of a astrocyte-conditioned media. The fluorescent SPIOs were added at experimental day 8, and at day 9 the cell culture plate was placed on a block magnet for five hours and TEER measured afterwards. The TEER values of the epithelial monolayer peaked at day 6 and did not decrease after the passage of nanoparticles. Hence, the TEER values of both curves are stable before and after the application of an external magnetic force and indicates that the barrier had not been obstructed by the passage of the particles through the endothelial cells (n = 11, results presented as means ± standard error (SE) (very low SE values)).

**Passage of SPIOs through the BBB in vitro**

The SPIOs crossed the HBMEC monolayer under the influence of an external magnetic field (0.39T), and their passage occurred in a concentration dependent manner (Fig. 6a-c). This indicates that SPIOs can be drawn through the BBB and into the brain parenchyma. A limited number of SPIOs were observed in the lower chamber without exposure to the magnetic field (Fig. 6d-f). However, this number was very low and did not seem to increase when increasing the concentration of
the nanoparticles. This suggests that the SPIOs are able to pass the \textit{in vitro} BBB in a low concentration without the external magnetic force.

**Figure 6** SPIOs in monocultures of HBMECs cultured in cell culture inserts. For better visualization the red fluorescent SPIOs are here shown in white. Upper row: Cells with exposure to the magnet for 5 hrs. Lower row: Cells without exposure to the magnet. The pictures show the presence of fluorescent nanoparticles in wells of the lower chamber under the inserts to which 35 µg (a+d), 70 µg (b+e), or 140 µg (c+f) SPIOs have been added. Arrows indicate some of the magnetic particles. The concentration of nanoparticles is visually higher in the wells of the experimental plates than in the wells of the control plates. (Scale bar = 30µm)

The amount of particles that penetrated the BCECs and entered into the astrocytes cultured on the bottom of the wells was additionally quantified in the control and experimental plates (Fig. 7). The experimental plate was submitted to an external magnetic field for 5 hrs. 35, 70 or 140 µg of SPIOs were added to inserts containing BCECs. The result of the quantification of particles entering the astrocytes under the inserts can be seen in figure 8.
Figure 7. The graph depicts the relation between the amount of magnetic nanoparticle passing through BCECs and into DI-TNC1 cultured on the bottom of the wells and the concentration of SPIOs added to the BCECs. Experimental plate (circle) which was submitted to an external electric field for 5 hrs and control plate (square) devoid of an external electric field subjection. SPIOs were added to BCECs in concentrations of 35, 70 or 140µg per insert. The amount of SPIOs passing the BCECs an entering into astrocytes was clearly higher when an external magnetic field was applied. There seems to be a linear correlation between dose and response when applying the magnetic field (n= 4 observations per point at 70 and 140 µg and n=3 observations per point at 35µg, results presented as means ±SE).

There is a statistically significant difference between the counts from wells of the experimental plate and the control plate (35µg: p<0.001, 70µg: p<0.001, 140µg: p<0.001). The passage of SPIOs across BCECs was increased 11, 8 and 29-fold over the control at a concentration of 35, 70 and 140µg/ml respectively. Chertok et al (2008) observed that a magnetic force of 0.4T increased the concentration of starch coated SPIOs (~110nm) targeted towards a rat brain tumor by 11.5-fold over the amount found in non-targeted (no magnetic force applied) brain tumors [13]. Similar results have been shown for starch coated SPIOs with a diameter of 46nm which was intravenously injected into nude mice with armpit tumor xenografts [22, 23]. The SPIOs were shown to accumulate in a higher concentration in the tumors when subjected to an external magnetic field of 0.5 T [22, 23]. These studies all refer to magnetic-force-increased delivery of SPIOs in tumor tissue which is known to have a compromised blood-tumor barrier. Chertok et al (2008) found that the concentration of SPIOs dispersed into normal brain tissue of Fischer 344 rats seemed to increase slightly (approximately 3-fold) under the influence of a magnetic field (0.4T) over non-magnetic-force-targeted SPIOs [13]. Also the migration of monocytes loaded with magnetic liposomes has been shown to be enhanced 3-fold by applying a magnetic force in an in vitro BBB model [17]. In the present study passage of SPIOs across an in vitro BBB in non-toxic doses was clearly increased by the magnetic field. The rate of SPIOs penetrating the BCECs without any aid of an external magnetic field was low and did not significantly
change in spite of a change in dose concentration. In the experimental plate the concentration of SPIOs added to the inserts containing BCECs linearly correlated with the concentration entering the astrocytes cultured in wells when submitted to an external magnetic field. Hence, these results support the strategy of employing SPIOs for targeted delivery to the brain.

**SPIOs pass through the in vitro BBB and further into astrocytes**

Next the ability of the SPIOs to cross the in vitro BBB and to enter cells on the “brain side” was investigated. To answer these questions astrocytes were cultured on the bottom of the wells but otherwise the experimental setup was the same as in the previous section with HBMECs cultured in inserts inserted in the wells containing astrocytes. Fluorescent SPIOs were found in the astrocytes in both the experimental plates (Fig. 8) and in controls.

**Figure 8.** Fluorescence microscopy showing SPIOs (a) inside DAPI (b) and GFAP (c) stained astrocytes present at the bottom of a lower well of a culture plate. Overlay of a,b, and c is seen in d. The astrocytes are from an experimental plate that has been exposed to a magnetic field for 5 hrs. The pictures show the presence of fluorescent SPIOs inside astrocytes cultured in a plate well under an insert to which 70 µg SPIOs was added. The red fluorescent SPIOs are shown in white for better visualization. (Scale bar = 15µm)

Differences were observed with respect to the uptake of SPIOs in astrocytes as only a minimal amount of particles was observed in astrocytes of the control plates as compared to that of astrocytes of the experimental plates. This suggests that the SPIOs can not only be drawn through the *in vitro* BBB but also enter cells present in remote distance of the abluminal side of the barrier. As earlier mentioned, Chertok et al (2008) observed presence of intravenously injected SPIOs in the brain parenchyma of normal rat brain tissue [13]. Hence, a possible application of the SPIOs for drug-delivery not only applies to BCECs but also to neurons and glial cells located deeper inside the brain. This notion, together with the fact that the magnetic particles are capable of movement in a particular direction via the application of an external magnetic field, signifies these magnetic particles as potential drug-carriers. The SPIOs are therefore obvious candidates as drug-carriers for CNS drug-delivery beyond the BBB, and their specific uptake by BCECs may be improved by conjugation to a targeting molecule.
CONCLUSIONS
The SPIOs could pass into and through the BCEC monolayer and enter astrocytes cultured at the bottom of the lower chambers in a manner that was clearly enhanced by the use of an external magnetic force. The external magnetic force did not affect the integrity of the endothelial monolayer, neither was the cell viability affected by the fluorescent SPIOs or by the magnetic force dragging the nanoparticles through the cells.

Our main conclusion is therefore that SPIOs can be used for penetration of the BCECs and further into the brain without harming the cells. SPIOs can be conjugated with various compounds and our results are indicative of SPIOs as nano-carriers for future drug-delivery (purpose involving targeted therapeutics) to the brain.

ACKNOWLEDGEMENTS
We would like to thank Joachim Høeg Mortensen, Christian Garn du Jardin Nielsen and Thomas Larsen for their input to this study. The data in this study were generated by kind grant support from the Danish Medical Research Council (grant no. 271-06-0211), the Spar Nord Fund, and the Obelske Family Fund.

REFERENCES


3.3 STUDY III

DYNAMIC VERSUS STATIC IN VITRO BLOOD-BRAIN BARRIER MODELS

Louiza Bohn Thomsen and Torben Moos

The manuscript displays unpublished results
Dynamic versus Static in vitro Blood-Brain Barrier Models

Louiza Bohn Thomsen* and Torben Moos

Department of Health Science and Technology, Biomedicine, University of Aalborg, Denmark

Abstract

The Blood-Brain Barrier (BBB) is a functional barrier preventing passage of certain compounds from the blood to the brain. When addressing complex issues, such as drug-delivery to the brain, it is important to understand the physiology of the BBB. Different models have been developed to mimic the BBB for this purpose. Applying an in vitro BBB model is a more ethical and less expensive method. Recently a new and improved dynamic in vitro BBB model (DIV-BBB) was developed by Flocel Inc. This model should be able to mimic the natural state physiological permeability properties of the BBB. This is not possible to mimic in static in vitro BBB models with hanging culture inserts. In the new DIV-BBB, unlike the static BBB models, cells can be grown in hollow fibers mimicking blood vessels and exposed to a pulsating flow of media mimicking the blood flow. The flow induces shear stress and this factor has shown to be of great importance when forming a tighter BBB. In this study the static in vitro BBB model and the DIV-BBB model are tested individually and compared afterwards. The static in vitro BBB model produced the tightest BBB when BCECs was cultured in a contact co-culture with astrocytes with 550nM hydrocortisone added to the culture media. It was not possible to produce any reliable results with the dynamic in vitro BBB in this study. The static in vitro BBB model therefore proved to be the most reliable model.
Abbreviations:
BBB: Blood-brain barrier
DIV-BBB: Dynamic in vitro blood-brain barrier
TEER: Trans endothelial electrical resistance

Introduction
The Blood-Brain Barrier (BBB) is formed by specialized brain capillary endothelial cells (BCECs), which form the walls of the blood vessels in the brain [1]. The BCECs are surrounded by a basal membrane which they form together with adjacent astrocytes. Astrocytes contact and cover most of the abluminal side of the BCECs with their end-feet [2, 3]. Pericytes also make contact with the BCECs and are found in the basal membrane in between the astrocytes and BCECs [4, 5]. Furthermore neurons have been found to make contact with BCECs [6].

BCECs make intercellular contacts called tight-junctions. Tight-junctions prevent leakage of substances into the brain, by preventing the passage of substances in between the BCECs [7, 8]. As a result substances can only enter the brain at the BBB through the BCECs either by diffusion or by transport via carrier-mediated transporters [3, 9, 10]. In this way diffusion/transport across the barrier can be strictly modulated by the BCECs [11]. The formation of tight-junctions and other features of the BBB characteristics are induced and maintained by astrocytes, pericytes and possibly also neurons [4, 6, 12, 13, 14, 15].

It has been demonstrated that shear stress, generated by the flow of blood across the endothelial cell surface, is an important factor in regulation of the genetic and physiological properties of the BBB [16]. The tightness of the BBB increases when BCECs are exposed to flow [9, 17, 18, 19]. Also up regulation of cAMP by hydrocortisone addition to the BCECs have shown to strengthen the BBB properties and thereby heighten the barrier integrity [10, 20].

The tightness of the BBB can e.g. be measured by recording the trans-endothelial electrical resistance (TEER). TEER is the electrical resistance formed across the BCECs and provides a measure of the barrier integrity. The tighter the barrier the higher TEER values can be measured because the passage of electrons across the cells decreases and therefore creates a difference in the electric potential [11].

Administering e.g. drugs to the brain have been shown to be difficult because of the BBB properties just described. The solution to the problem could be drug-delivery, where drugs are carried into the brain over the BBB by a drug-carrier. For testing such drug-carriers abilities to penetrate the BBB an experimental setup is needed. It has been difficult to make direct observations of the BBB physiology on living animals, and therefore different in vitro models with cultured cells have been developed for this purpose [11]. Hanging cell culture inserts are static in vitro BBB models, which have been applied for many years. In these models the BCECs can be cultured alone or co-cultured with astrocytes, pericytes or neurons or in a combination of one or more of the cell types [5, 20, 21,
Louiza Bohn Thomsen

22, 23, 24]. The BCECs will form a BBB in these inserts, but the TEER values measured in this model does not always mimic the TEER values (between 1200-8000 Ω*cm²) measured in living animals and does not induction of shear stress [9, 25]. In a new dynamic in vitro blood-brain barrier (DIV-BBB) model developed by Flocel Inc. the BCECs are grown inside hollow fiber tubes, that mimics blood vessels, and astrocytes are grown on the outside of the fibers, supporting the BCECs. The fibers are placed in a sealed chamber, where they are exposed to a pulsatile flow, which passes through the fibers, mimicking the blood flow through the vessels. The TEER was measured by the manufacturer to be ~1200 Ω*cm² in the DIV-BBB model and therefore mimic the in vivo BBB more closely than most static models [18, 19, 26, 27, 28, 29]. In this study a static and a dynamic in vitro BBB model are tested individually and compared based on the tightness of the barriers measured in TEER.

Materials and methods

Cell culture
Three kinds of immortalized endothelial cell cultures were used in this study. Human Brain Microvascular BCECs (HBMEC) were kindly provided by Professor Kwang Sik Kim, Johns Hopkins Univ. School of Medicine, Baltimore. HBMEC’s were grown in culture flasks precoated with collagen (5μg/ml, BD Biosciences) in growth medium consisting of Medium 199 (Invitrogen), 10% fetal bovine serum (Invitrogen), 10% NuSerum (BD Biosciences) and 100 U of Penicillin G sodium per ml and 100μg Streptomycin sulfate per ml (Invitrogen). When mentioned 550nM/ml hydrocortisone (Sigma-Aldrich, Germany) was added to the HBMECs media to induce a greater tightness hence higher TEER of the HBMECs. Rat Brain BCECs (RBE4) was cultured in Alpha mini minimum essential medium with glutamax-1 (Gibco, Invitrogen) and Ham’s F10 (Gibco, Invitrogen) in a 1:1 relation with 10% fetal calf serum (Invitrogen), 1ng/ml human basic fibroblast growth factor (Invitrogen) and 100 U of Penicillin G sodium per ml and 100μg Streptomycin sulfate per ml (Invitrogen). Mouse BCECs (Bend3) were cultured in DMEM 1885 (Sigma-Aldrich) with 10% fetal calf serum (Invitrogen), 1ng/ml human basic fibroblast growth factor (Invitrogen) and 100 U of Penicillin G sodium per ml and 100μg Streptomycin sulfate per ml (Invitrogen).

The Static in vitro BBB models setup
BCECs and astrocytes were cultured in either 12-well Transwell-Clear Polyester Membrane plates (Costar) with hanging cell culture inserts (RBE4 and Bend3) or
in 12-well culture plates (Costar) with hanging Millicell culture inserts (HBMEC) in each well (Figure 1). The experiments were performed in four setups. 1) Either the BCECs were cultured alone, 2) in non-contact co-culture with astrocytes, 3) in contact co-culture with astrocytes or 4) in contact co-culture with astrocytes with media containing 550nM hydrocortisone. In contact co-culture the astrocytes (1.0x10^5 cells/insert) were seeded under the bottom of the inserts and in non-contact co-culture in the wells of the culture plates (1.5x10^5 cells/well). The BCECs (1x10^5 cells/insert) were seeded in the bottom of the inserts. The membrane of the inserts is made of a microscopically transparent polyester membrane, which is 1.1 cm^2 in diameter and have 0.4 µm (Transwell) or 1 µm sized pores (Millicell), which enables diffusion of molecules through the membrane.

![Diagram](image.png)

**Figure 1** A single well on a 12 well plate with a hanging cell culture insert. The drawing shows a monolayer cultured on the membrane of the hanging insert which is inserted into a well. The BCECs then form a barrier with polarity as in vivo with the apical side up and the basolateral side down. This setup can be used as a static *in vitro* BBB model.

**DIV-BBB model setup and TEER measurements**

The DIV-BBB modules (Flocel Inc.) contain 19 hollow tubes (Figure 2), which have small pores (0.64 µm) to allow diffusion of particles through the walls of the fibers. The experimental setup was adapted from Cucullo et al (2008) [28]. In short the cartridges were coated with collagen on the inside of the hollow fibers and poly-l-lysine on the outside of the fibers. The BCECs were seeded (1.5x10^5 cells) on the inside of the fibers and placed in an incubator the first 4 hours without flow to enable attachment. Then astrocytes were seeded (2x10^5 cells) on the outside of the hollow fibers. Gas permeable silicone tubes were connected to ports on the upper side of the module for media supply to the cells. The media-flow is operated by a pulsatile pump, which can create a flow rate of 1-50 ml/min. The flow rate was set at 1 ml/min for the first 24-48 hours and bypassed into the outer chamber instead of the lumen of the hollow fibers to allow cell adhesion. Then media was lead into the lumen of the fibers and after 24 hours a sample of the media was taken to count the non-attached BCECs which gives an estimate of how many cells was attached to the hollow fibers. The flow rate was adjusted to 2ml/min the...
following day and then stepwise with 2 ml/min a day until reaching 6-12 ml/min. In three experiments 550nM/ml hydrocortisone was added to the culture media. The entire setup is placed in a water-jacketed incubator with 5% CO2 at 37°C. Electrodes on the bottom of the module were inserted into a TEER monitoring device, which was directly connected to a computer, providing a curve with the TEER values instantaneously throughout the entire experiment. Before seeding cells in a cartridge a baseline TEER measurement was made on the empty coated cartridge which was subtracted from the TEER values made in the experiment (For further details on the system see flocel.com).

Figure 2 Photo of the DIV-BBB system. The model consists of a cartridge placed in a TEER measurement system. The DIV-BBB cartridge has an inner compartment consisting of 19 hollow fibers made of a microporous membrane. The cartridge has four sampling ports and four electrodes for measuring TEER. The cartridge is connected to a media reservoir and a pump by silicone tubing. The TEER measurement system can be connected via USB cable to a computer for instant monitoring of TEER measurements.

**TEER measurements of the static BBB models**

TEER measurements were conducted with a Millicell™ ERS-2 apparatus (Millipore, USA) and an STX-1 electrode (Milipore). The Millicell ERS-2 is compatible with both the Transwell and Millicell hanging cell culture inserts. The TEER was first measured on an empty insert and subtracted from the TEER values measured on the experimental inserts. The product is then multiplied by the
membrane surface area. The Transwell and Millicell inserts both have a membrane area of 1.1 cm².
The TEER was measured every second day the first 5 days and hereafter every day. A steady state would normally commence at day 5-7. Before measurements the culture media was changed and cells and media was allowed to reach room temperature. Three measurements were made on each well from which an average TEER value was calculated.

**Immunostaining of the BCECs in the models**
This procedure was only conducted on the HBMECs. After the experiment the DIV-BBB cartridges and the hanging culture inserts were washed three times in PBS, fixed in 4% paraformaldehyde for 4 minutes and washed three times in PBS. The DIV-BBB cartridges were split open and the tubes within were carefully taken out. They were then placed in a 30% sucrose solution. Fibers were stored in glasses in categories of front end fraction, middle fractions and end fractions. The fibers were embedded in Tissue-Tek 4583 O.C.T (Sakura Finetek, Japan) for cryo-sectioning. The fibers were cut in 20 µm thick pieces (Protocol adapted from Cucullo et al (2002) [30]).
The cells in both the inserts and the hollow fiber pieces were incubated overnight with primary antibody, mouse-anti-ZO-1 (Invitrogen) in PBS 1:200 and this was visualized with goat-anti-mouse alexa 488 (Invitrogen) in PBS 1:200. The cell nuclei were stained with DAPI (Sigma-Aldrich) in PBS 1:20 for 5 minutes. The membrane in the hanging cell culture inserts with HBMECs on was cut out of the insert. Both the membranes and the hollow fiber pieces were mounted on a slide with fluorescent mounting media (Dako, Denmark) and observed under a fluorescence microscope.

**Statistical analysis**
An analysis of variance (ANOVA) followed by a Fisher’s least significant difference (LSD) method was employed for analyzing the possibility of a difference between the obtained TEER values measured on the four experimental culture setups. A p-value was considered significant at p<0.05.

**Results and discussion**
Many different molecules are manufactured for e.g. treatment of CNS diseases but only a few percent can penetrate the BBB. Testing their penetration abilities in vivo on the BBB is both ethically changeling, expensive and time consuming. In vitro BBB models have therefore been developed for this purpose. These models mimic the in vivo BBB but how good they portray the real BBB functions can be debated. The static in vitro BBB model enables the culture of BCECs in co-culture with astrocytes and a brain and blood side can be defined on the BCECs. The static in vitro BBB model however lack the ability to support a high TEER and the BCECs are not subjected to a flow which is known to induce higher barrier tightness. Dr. Damir Janigro has together with his group developed a dynamic in vitro BBB model which subjects the BCECs to a flow and their studies on the models show...
significant increase in TEER values when both primary and immortalized BCECs are grown in this model. In this study the static and dynamic in vitro BBB models was tested separately and then compared.

**Static in vitro BBB model**
The static model was setup in four different ways, 1) monoculture of BCECs, 2) non-contact co culture of astrocytes and BCECs, 3) Contact co-culture of astrocytes and BCECs and 4) contact co-culture of astrocytes and BCECs with hydrocortisone added in the media. TEER measurements made on all four kinds of experimental setups on HBMECs in the hanging cell culture inserts are shown in figure 3. These results resemble the results with RBE4 and Bend3 cells although the TEER values of these cells were approximately 30% lower (data not shown).

![Figure 3](image-url) TEER measurements on HBMECs in the static in vitro BBB model. The HBMECs grown in the static in vitro BBB models did not reach a high TEER compared with in vivo values although a higher TEER is reached when HBMECs are cultured with astrocytes in a contact culture and an even higher TEER if Hydrocortisone is added to the cell media in the contact co-culture (n=22, results presented as means ±standard error).

The TEER values were measured for 9-10 days in total. The TEER values increased until around day 6-8 where it seemed to reach a plateau. Around day 10 the TEER value would slowly begin to decrease again. A statistically significant difference (p<0.05) was found between the TEER threshold value of all of the four setups except between the monoculture and the non-contact co-culture. The test results suggest that astrocytes need to form contact with the BCECs to induce a significant tighter barrier. Furthermore tightness could be further increased if
hydrocortisone was added to the media in the contact co-culture setup. This correlates with the findings in Calabria et al (2006) [20]. Their TEER values cannot be directly compared with the values measured in this study as they use primary rat BCECs but they also find that hydrocortisone induce an increase in the TEER threshold values from 94±5Ω*cm² to 218±66Ω*cm² [20]. With the HBMECs the TEER values are very low compared to the ones measured on the primary rat BCECs. HBMECs have been immortalized and passaged for up to 30 passages and therefore have most likely lost some of their barrier functions. It can though be seen on figure 5 that they do stain positive for ZO-1 at the cell borders which indicate that they do form tight junctions, but the low TEER values still imply that there are gaps in between cells.

![Image](Figure 4 Immunofluorescence staining of HBMECs in a hanging cell culture insert with anti-zo-1 and dapi. The cells stained positive for ZO-1 (scale bar=50μm).)

The experimental setup with the hanging cell culture inserts is easy to reproduce and it gives reliable results but the integrity of the barrier does not at all reach the high levels as in vivo. One way to optimize this could be to form a BBB of primary BCECs instead of immortalized BCECs. In e.g. Calabria et al 2006 [20] primary rat brain endothelial cells were shown to form tighter barriers in a Transwell system (70-218 Ω*cm²) [20]. Another approach to mimic the in vivo BBB in a more anatomically correct model is to co culture the BCECs with not just astrocytes but also pericytes. Such a triple cell co-culture model in a Transwell system has been shown to produce higher TEER values. Nakagawa et al (2007,2009) has shown that primary rat brain endothelial cells in a contact co-culture with primary pericytes together with primary astrocytes in remote distance increased the TEER values significantly (~400 Ω*cm²) compared to mono and double cell cultures (~75-300 Ω*cm²) [23, 24]. Although the models displays a tighter BBB the TEER values accomplished with the static in vitro BBB models with primary rat brain cells in triple co-culture does not still resemble the high TEER values measured in vivo. High TEER values have though been measured in static in vitro BBB models with primary bovine brain endothelial cells. Helms et al (2010) could produce a barrier with a TEER threshold value of 1638±256 Ω*cm² by enhanced media buffer capacity during the growth of bovine BCECs [31]. A TEER as high as 2100 Ω*cm² on primary bovine brain endothelial cells was
accomplished by non-contact co-culture with blood-derived macrophages which very much resembles the values obtained in vivo [32]. Primary BCECs in mono, double, triple co-culture, addition of hydrocortisone or buffers are all strategies which improve the static in vitro BBB model and make it a useful tool for research on the BBB.

Another approach to accomplish a tighter BBB could be to apply the in vitro DIV-BBB model instead which promises a higher TEER and a more correct physiological resemblance.

**Dynamic in vitro blood-brain barrier model**

Unfortunately it was not succeeded in this study to obtain any reliable results with the DIV-BBB model from Flocel inc. A typical example of TEER measurement of a co-culture in a cartridge is showed in figure 5.

![Figure 5](image.png)

**Figure 5** TEER measurements from a cartridge in the DIV-BBB. TEER values from a cartridge with HBMECs cultured in the hollow fibers and HAcs cultured on the outside of the fibers are shown in A) uncorrected form, B) corrected form where high and negative values are removed and C) a baseline recording from an empty coated cartridge. The TEER values was recorded automatically every second minute by the TEER measurement device.

The TEER values was automatically recorded every second minute by the TEER measurement device. The TEER values varied a lot and ranged from negative values to five figured values (Figure 5A). Negative and high values are described in the manual of the model to possible be due to air bubbles or clogging of the hollow fibers (flocel.com). Before seeding the cells in the coated cartridges a baseline measurement was obtained (Figure 5C). The baseline value portrays the resistance of the empty cartridge and this value should be subtracted from the experimental TEER values to give the values of the true resistance of the BCECs. In all of the experiments conducted in this study (n=9) with the DIV-BBB the TEER values was not higher than the baseline values as can be seen in the example on figure 5. Here the average baseline TEER was 413 Ω*cm² and the average TEER in the experiment was 381 Ω*cm². Addition of hydrocortisone did not change the TEER values.

The hollow fibers of the DIV-BBB model are made of non-transparent membranes and the cells cultured inside the fibers could therefore not be monitored. To obtain knowledge of the conditions of the hollow fibers and the
BCECs stain with anti-zo-1 and DAPI was performed. No cells could be identified within the hollow fibers. This could be due to the rather harsh procedure in the immunofluorescence staining protocol which contains several washing steps. It could also be due to cell detachment from the hollow fibers or even no attachment of cell in the fibers to begin with. There was taken some samples from the media to estimate the number of cells that had detached. Approximately 20% of the loaded cells were found in the media; hence ~80% were still in the system. There is a possibility that the cells had attached themselves in other parts of the system. Cells was identified on the walls of the cartridges at the entrance and exit points in both ends of the hollow fibers and also in some parts of the tubing. This could explain the lack of an increase in TEER values if the cells did not cover the hollow fibers but instead were attached to other surfaces in the system.

To obtain a tight barrier the BCECs must form a monolayer where they form tight junctions in between them. If there is just a small gap in between two adjacent BCECs in the monolayer the TEER values will be lowered. The surface area of the hollow fibers in the cartridge is quite large (13.5 cm$^2$) and therefore it is plausible that it can be difficult to ensure full coverage of the fibers by the BCECs. This could lead to a less loose barrier and very low TEER values as seen in this study.

**Comparison of the static and the dynamic in vitro BBB models**

When creating an *in vitro* model of the BBB, it is important to obtain values, which mimic the values obtained in living animals. The tightness of the *in vivo* BBB has been poorly reproduced in most studies with the non-dynamic models. The models have been ignoring the fact that the blood flow is a BBB tightness promoting factor. Thus the DIV-BBB model should be a better and more realistic model of the BBB than the static models.

Comparison of the two *in vitro* model types from this study proved difficult as the DIV-BBB model never gave any reliable results. A big difference between the two models is the culture area size and availability. In the static BBB model the membrane on which the BCECs are cultured on is 1.1 cm$^2$. The area of the hollow fibers on which the BCECs are cultured on in the static BBB model is 13.5 cm$^2$. This is a considerable larger area the BCECs need to cover in the hollow fibers to provide a tight monolayer. Furthermore the culture surface in the dynamic BBB model is in 19 hollow fibers whereas in the static BBB model the surface is flat and horizontal. The membrane in the static model is transparent and the cells are easy to monitor whereas the non-transparent hollow fibers does not allow any cell culture inspections during and experiment. These culture conditions in the static BBB model seems to be more favorable for the BCECs compared to the dynamic BBB model.

At this stage the DIV-BBB model from Flocel Inc. cannot be trusted to produce consistent and reproducible data. It still needs some improvements before it can seriously challenge the more old fashion static *in vitro* BBB models. The static *in vitro* BBB model does not allow shear stress but it still seems to be a reliable model for studies of new carrier compounds. Applying either hydrocortisone to the media or using the triple cell model in the static in vitro BBB
model has been shown to reproduce parameters of the *in vivo* BBB in such a way that it can be used as a reliable and trustworthy BBB model.

**Acknowledgements**

The authors would like to thank Muhammed Hossain and Luca Cucullo at Cleveland Clinic for their constructive input on how to operate the DIV-BBB. The data in this study were generated by kind grant support from the Danish Medical Research Council (grant no. 271-06-0211), the Spar Nord Fund, and the Obelske Family Fund.

**References**


Non-viral delivery strategies into/across the brain capillary endothelial cells


[31] H.C. Helms, H.S. Waagepetersen, C.U. Nielsen, B. Brodin, Paracellular tightness and claudin-5 expression is increased in the BCEC/astrocyte blood-brain barrier.
Non-viral delivery strategies into/across the brain capillary endothelial cells

barrier model by increasing media buffer capacity during growth, AAPS J, 12 (2010) 759-770

4 Discussion

The overall objective of this study was to find potent drug carriers for either direct delivery to the brain or delivery of cDNA to BCECs with the potential of de novo gene expression and subsequent secretion of synthesized proteins to the brain. The aim was also to establish a valid in vitro BBB model for testing these drug carriers.

4.1 PULLULAN-SPERMINE COMPLEXES AS GENE-CARRIERS TO BCECS

Pullulan-spermine was successfully conjugated with plasmid cDNA encoding the red fluorescent protein, HcRed-1. The formed polyplex consisting of pullulan-spermine-pHcRed-1 cDNA was introduced to the BCECs and transgene BCECs expressing the red fluorescent marker was detected. Pullulan-spermine complexes were also conjugated with plasmid cDNA encoding hGH1 and transfection of the BCECs was further confirmed by the expression of hGH1 mRNA by BCECs. The results clearly show that pullulan-spermine is a potent carrier of genetic material suitable for transfection of BCECs, as it succeeded in delivering its cargo to the cell cytosol with a subsequent transport to the cell nucleus. These findings supports results from other studies in where pullulan-spermine proved to be a potent donor of genetic material to cells of non-neuronal origin like human bladder cancer cells (T24), human hepatoma cells (HepG2), and mesenchymal stem cells [64, 65, 67, 68, 69]. The results of the present study add to this row of data via the discovery that BCECs can be converted into protein factories for protein secretion to the brain following uptake and transfection of cDNA carried into the cells by pullulan-spermine. In this thesis, gene therapy was performed with plasmid cDNA encoding hGH1 but theoretically, this principal method for cellular tranfection might be used for delivery of any protein of relevance for the brain. Jiang and co-workers (2003) transfected cultured mouse brain capillary endothelial cells (MBEC4) with pIREsneo-mGDNF by using Lipofectamine following secretion of GDNF [110]. They also proved secretion of GDNF by the MBEC4 cells to both the apical and the basolateral side of the MBEC4 cells. Furthermore they were able to transfect BCECs in vivo with GDNF encapsulated in Hemagglutination virus of Japan (HVJ)-liposomes and the secreted GDNF provided neuroprotection for dopamine neurons against 6-hydroxydopamine induced lesions [110]. This supports the possibility of using BCECs as protein secreting factories for secretion of proteins that could have a beneficial effect on damaged neurons or other cell types in the brain. BCECs could potentially be transfected to secrete other proteins than hGH1. GDNF and BDNF have been shown to play a significant role in maintenance of fully differentiated neurons and to promote growth and differentiation of newly formed neurons [111, 112]. Likewise EPO, bFGF, and NGF are also of putative interest (see Table 2 in Introduction). Johnston et al (1996) showed that bFGF
could be transferred into BCECs in vitro by liposome complexes and subsequent secretion of bFGF into the culture media by the BCECs was detected [113]. It should also be mentioned that secretion of hGH1 may have a beneficial effect on not only neurons but also oligodendrocytes and astrocytes [114].

In this study immortalized BCECs grown in monolayer was employed for transfection with pullulan-spermine-cDNA complexes. However, there can be a big difference between the barrier properties and other characteristics of immortalized and primary BCECs and experiments should preferably be performed on polarized primary BCECs to increase the significance of how the results could translate to the in vivo situation with respect to synthesis and secretion. Jiang and co-workers showed a huge potential for basolateral secretion of protein from BCECs which should also be investigated by the use of the vector of the present study [110].

4.2 TRANSPORT OF PULLULAN-SPERMINE CARGO INTO THE CELL NUCLEUS

Presumably, gene delivery by pullulan-spermine is limited to mitotic cells [65]. One of the new findings in this thesis was that BCECs present in either a dividing or non-dividing state could be transfected by pullulan-spermine-cDNA complexes, which suggests that the plasmid cDNA not only enters the cell nucleus during mitosis but are also trafficked through the intact nuclear membrane during the non-mitotic state of the cell cycle. This trafficking is though not the main route for plasmid cDNA as the transfection in non-dividing cells is lower than in dividing cells.

The transport of plasmid cDNA to the cell nucleus was demonstrated using coupling of NLSs to plasmid cDNA, which led to increase in in vivo transfection (e.g. [50, 51, 115]). Possibly the coupling of NLSs to the pullulan-spermine-plasmid cDNA complex would increase delivery of cDNA to the nucleus of non-mitotic cells. Plasmid cDNAs used in this study encode HcRed1 and hGH1 and contain sequences of SV40 and cytomegalovirus (CMV) respectively. SV40 contains NLSs and is able to optimize nuclear uptake whereas CMV does not facilitate such nuclear translocation [116]. A change in the plasmid vector composition of the hGH1 cDNA might also increase its nuclear uptake and thereby further strengthen the carrier properties of pullulan-spermine-plasmid cDNA complexes.

4.3 TARGETING PROPERTIES OF PULLULAN-SPERMINE

The intracellular route by which pullulan-spermine complexes are internalized by BCECs was not investigated in the present study, however other studies have shown that positively charged polyplexes can undergo non-specific adsorptive endocytosis via interaction with anionic proteoglycans and glycoproteins present on the luminal cell surface [60, 61, 62]. Specifically pullulan-spermine is thought to be taken up by cells via sugar-recognition receptors [65]. The pullulan-
spermine-DNA complexes employed in this study were 240-300nm in size and therefore attributable to enter the BCECs by means of calveolae dependent endocytosis [65]. As either of these mechanisms for recognition and uptake is thought to be specific for BCECs in vivo a targeting strategy for BCEC access is necessary.

One strategy could be to administer the pullulan-spermine complex directly into the carotid artery which would increase the rate of interaction between the BCECs and the complex before interaction with other cells. This will of course not exclude interaction with other cell types but as the complexes would pass the BCECs immediately after injection the charged complexes would be prone to interaction with the BCECs before encountering other organs. A downside to this strategy would be that the pullulan-spermine complexes would still be distributed into the systemic circulation outside CNS and this could give rise to unwanted side-effect in non-target organs.

Another approach could be to target a receptor on the apical surface of the BCECs. It has been shown that OX26 will bind to the transferrin receptor which leads to internalization of the antibody into the cytosol of BCECs when administered to rats intravenously or by in situ perfusion [41, 42]. This makes OX26 a suitable targeting molecule to the BCECs.

The conjugation of OX26 to the pullulan-spermine-cDNA complex was done successfully suggesting that this principle form of targeting strategy is accessible (Lichota et al (unpublished data)). This strategy will though still not exclude uptake by other cell types expressing the transferrin receptor, but by administration into the carotid artery the possibility of uptake mainly by BCECs would increase.

4.4 PULLULAN-SPERMINE AND SERUM COMPATIBILITY

The transformation efficiency of pullulan-spermine-cDNA complexes is severely inhibited by serum [68] a finding supported by data of the present study. Hence, almost no transgenic BCECs expressing HcRed1 C1 were detected when serum was added to the growth media. This lack of transfection is thought to be due to the negatively charged serum proteins which lower or even completely neutralize the charge of the cationic polyplexes. This apparent obstacle for future in vivo experiments was addressed by Thakor and co-workers who developed a strategy where pullulan-spermine-cDNA complexes would form anionic complexes, aniplexes [57, 117]. These aniplexes have proved significantly more effective for transfection than their cationic counterparts; the rationale being that anionic serum proteins will not interact with the aniplexes, and therefore serum is no longer a restraining factor for the complex as the interaction with the negatively charged cell surface components is no longer taking place [57, 117]. An apparent disadvantage in this strategy is that it limits non-specific endocytic uptake, which must be dealt with by making the complexes targetable as described in the previous section. This targeting approach may in fact prove to be advantageous as the ratio of specific to unspecific uptake probably will be markedly improved.
Another strategy to avoid serum protein interaction could be to encapsulate the pullulan-spermine-cDNA complexes in liposomes. Liposomes have been very intensely studied for their capabilities as drug-carriers. They can be PEGylated and conjugated with antibodies [118] suggesting that PEGylated targetable liposomes carrying pullulan-spermine-cDNA could denote a potent complex being both serum compatible and targetable.

4.5 SPIOS AND DRUG DELIVERY TO THE BRAIN
The uptake and transport of SPIOs through BCECs indicates that they are appropriate candidates for drug delivery to both BCECs and the brain. Their passage through BCECs occurred in small scale without external aid. Application of an external magnetic field clearly enhanced the SPIOs movement through the BCECs. Once through BCECs cultured in cell culture inserts, the SPIOs were taken up by astrocytes, grown in wells in which the inserts with BCECs were placed. The SPIOs used in the present study is coated with starch which have terminal hydroxyl groups. These functional hydroxyl groups can be covalently coupled with amine groups on antibodies or other types of proteins. As SPIOs can also be coated with substrates like chitosan or phospholipids with capabilities to bind ligands, cDNA and drugs [80, 82, 84, 85], the SPIOs are potent drug carriers to the brain in a targetable manner. SPIOs coated with chitosan and enclosed in liposomes are able to carry plasmid DNA into BCECs and transfect them in culture (Linemann et al (unpublished data)).

4.6 SPIOS AND POSSIBLE DAMAGING EFFECTS
Accumulating an excess of iron present as iron oxide particles could potentially become a safety issue due to the risk of metal-induced cytotoxicity and damage to the BCECs [80, 85]. Not only could BCECs be damaged but they could also lose their integrity to proteins in circulation leading to increased BBB permeability. No significant damage was seen in BCECs after having been subjected to SPIOs and an external magnetic field, as less than one 1% of the cells were damaged. Iron oxide SPIOs coated with tween 80 and of a diameter of 30 nm have been shown to be cytotoxic to murine macrophages (J774) after incubation for 6 hours at a concentration of 200µg/ml, but not significantly toxic at 100µg/ml [79]. The present study shows that iron oxide SPIOs of a diameter of ~117,5nm is non-toxic to the BCECs at a concentration of 140µg/ml and 5 hour incubation time. Additionally, the TEER measurements implied no disruption of the BBB following application of SPIOs, the external magnetic field or both. Hence, the integrity of the BBB remains stable after application and passage of the SPIOs indicating that magnetic particles would be suitable also for in vivo studies.
4.7 SPIOS AND MAGNETIC FORCE-TARGETED DELIVERY
The external magnetic field was supplied by a plate magnet with a strength of 0.39 Tesla, which is compatible with the strength of the magnetic fields applied for magnetic resonance imaging (MRI) in clinic. MRI is normally powered by 0.2 - 3 Tesla with the most common values being in the range of 1.5 and 3 Tesla, but in some analyses MRI may be performed at 30 Tesla. As SPIOs are already in clinical use as contrast agents for MRI scanning [71] is plausible that delivery to the brain of drugs and genes carried by SPIOs is accessible even with simultaneously real-time visualization of their accumulation in the brain.

4.8 STATIC VERSUS DYNAMIC IN VITRO BBB MODEL
Unfortunately it was not possible to obtain any reliable results with the dynamic in vitro BBB model in the present study, which makes it impossible to compare the static and dynamic BBB models. Other research groups have proven that it is possible to obtain data from the dynamic model and their studies were the reason for investing in this model [100, 101, 102, 103, 104, 105, 106, 107, 108, 109]. In the dynamic model there are a lot of small finesses that can alter the culturing process. The rather large surface area is limiting because BCECs needs to cover the entire surface before an increased TEER can be measured. In the static in vitro BBB model the surface area to be covered by BCECs is significantly smaller. Therefore for practical reasons the culture conditions of the static model favors the establishment of a tight BBB with high TEER values.

In the dynamic BBB model the cell culturing is very difficult to monitor and therefore very difficult to get a comprehensive view on whether BCECs form a confluent monolayer. The hollow fibers are not transparent like the microporous membranes of the static model. It is therefore also very difficult to ensure correct loading of the BCECs into the hollow fibers or to monitor the growth. These problems could easily be changed, by forming hollow fibers of transparent material, instead of the current non-transparent membranes.

It was also difficult to avoid unwanted attachment of cells on surfaces outside hollow fibers. If the two sampling ports to the inner compartment were exclusively attached to the hollow fibers this unwanted attachment of cells in the cartridge could be avoided. Unwanted attachment of cells is not an issue in the static model, and although the static model may seem simpler and lack some key features for induction of the BBB phenotype, it remains the best model for obtaining reproducible data on BCECs.

4.9 IMMORTALIZED BCECS AND BBB INTEGRITY
When studying transcellular or intercellular transport of substances into BCECs it is important to eliminate paracellular leakage. The BCECs of the present study did not express too impressive TEER values. Although they stained positive for ZO-1 the low TEER values indicate that the BCECs are not that closely interconnected by tight junctions. ZO-1 formation indicates tight junction formation but it is a
cytoplasmic plaque protein that links the transmembrane junctional proteins to the actin cytoskeleton. Staining for the transmembrane tight junction proteins e.g. occludin or claudin-5 could have provided a more clear view of the presence of tight junctions. Furthermore a study on the barrier permeability with a tracer e.g. sodium fluorescein or radiolabeled sucrose could have been performed. The permeability depends on the sum of transport across all junctional pathways [87] and would therefore provide insight into the tightness of the barrier as well. The BCECs in the present study have probably lost some of their barrier characteristics during their repetitive passaging, but their BBB properties could be increased by a contact co-culture with astrocytes and further increased by addition of hydrocortisone, observations in hand with other laboratories (e.g. [88, 94, 96, 98]). Despite attempt to optimize the culture conditions the HBMEC cell line used in the present study does not seem to be able to form a tight enough barrier to fulfill the criteria of TEER values around 150-200 which have been determined to be necessary for obtaining reasonable information from an in vitro BBB model [87, 119].

Primary brain microvascular BCECs could instead be employed to improve the in vitro model of the BBB. Primary BCECs have intact BBB features and form a much tighter barrier than their immortalized counterparts [86, 87]. TEER values measured on primary cultures of e.g. bovine and porcine BCECs can be as high as values obtained in vivo [87, 120, 121]. Including both astrocytes and pericytes in double or triple co-culture with the BCECs has also been shown to strengthen the tightness of the BBB models [95, 122]. With the use of primary BCECs in monoculture or coculture with astrocytes and/or pericytes, the in vitro BBB models are more compatible with the in vivo situation and therefore more useful for studying the passage of various compounds as e.g. SPIOs and pullulan-spermine-cDNA through the in vitro BBB.
5. Future Perspectives

In this study it was found that pullulan-spermine complexes are potent gene carriers to BCECs. SPIOs were found to be potentially potent carriers for delivery to both BCECs and the brain. Furthermore it was found that the static in vitro BBB model consisting of cell culture inserts were the most reliable BBB model when compared with a dynamic in vitro BBB model. The results in this thesis have raised three new questions I would like to be able to answer in the nearer future.

1) First, could a better *in vitro* BBB model be established based on the static model? The three studies in this thesis might benefit from establishment of a primary brain endothelial cell culture. Primary BCECs possess far more of the BBB characteristics, and especially they form a tighter barrier than the immortalized BCECs [87]. The existing literature suggests that replacement of immortalized BCECs with primary cells would highly improve the results obtained in the static in vitro BBB model and make them translate to the *in vivo* situation [87]. Attempts have already been made on establishing human primary BCECs during the last period of this Ph.D. study. Pieces of human brain tissue are obtained from patients undergoing surgery to remove brain tumors at the neurosurgical department on the Hospital of Aalborg. Unfortunately it has been a challenge to ensure a pure fraction of BCECs, and the protocol still needs further improvement. Human brain tissue is not provided on a regular basis, therefore more available sources as for example rat brains should be studied as well.

2) Secondly, is it possible to alter pullulan-spermine complexes to become potent gene-carriers for *in vivo* use? If pullulan-spermine complexes are to be employed for *in vivo* purposes the serum incompatibility problem has to be solved. Pilot studies have been initiated to combine PEGylated liposomes with pullulan-spermine employing a new protocol for liposome preparation [123]. The hypothesis to examine is that if PEGylated liposomes can carry pullulan-spermine-cDNA complexes into BCECs, release pullulan-spermine-cDNA into the cytosol from where cDNA will reach to the nucleus to enable transfection. This strategy ensures full protection of the pullulan-spermine complexes from serum degradation. Another strategy would be to form anionic complexes of pullulan-spermine-DNA as recently described by Thakor et al (2011) [117], which was shown to eliminate the serum incompatibility factor.

3) Thirdly, is it possible to conjugate the fluorescent SPIOs with a cargo and demonstrate delivery of this cargo into BCECs or directly into the brain?
Having studied the ability of SPIOs to enter into BCECs and even through the BCECs, the next step would be to conjugate these coated particles with both cDNA and a BCEC targetable molecule. DNA binding could be done by coating with a positively charged molecules e.g. chitosan to take advantage of the electrostatic binding ability. Furthermore the magnetic particles can be covalently coupled to antibodies via cyanogen bromide activation, which should be tested as a strategy towards producing BCEC specific targetable SPIOs.
References


Non-viral delivery strategies into/across the brain capillary endothelial cells


[40] Frieden, P.M., Walus, L.R., Musso, G.F., Taylor, M.A., Malfroy, B., Starzyk, R.M., Anti-transferrin receptor antibody and antibody drug


Non-viral delivery strategies into/across the brain capillary endothelial cells


Non-viral delivery strategies into/across the brain capillary endothelial cells

Non-viral delivery strategies into/across the brain capillary endothelial cells

Appendix I

3330 Current Medical Chemistry, 2015, 21, 3330-3334

Non-viral Appen delivery strategy for the Blood-Brain Barrier to Enable Neuronal Growth Factor Delivery by Secretion from Brain Endothelium

I.B. Thorsen, A.B. Larsen, J. Eichota and T. Moes*

Section of Neurobiology, Biotechnology, Aalborg University, Denmark

Abstract: Brain capillary endothelial cells from the blood-brain barrier (BBB) that deliver a transgenic construct into the brain. The identification of many new targets to treat disease in the brain demands novel delivery of drug designs to new therapies could offer new potential to modulate the effects of cell-type specific therapies that may enter brain tissue via endogenous means or may enter brain tissue through controlled delivery. The feasibility of particular drug carriers that will enable efficient transport into brain endothelial cells or even through the endothelium and across the blood-brain barrier constitutes a major challenge for drug delivery. This review covers the BBB and the obstacles involved in drug delivery to the brain. Several approaches have been made to bypass the BBB by i.e. use of nanoparticles containing liposomes, dendrimers and magnetic nanoparticles, or by use of transcytotic delivery systems to transport drugs across the BBB. The most promising non-viral drug delivery systems to date are summarized.

INTRODUCTION

Drug delivery to the brain is hampered by the barriers of the brain, i.e. the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier (Fig. 1). The relative lower area of the capillaries in the brain covered by the blood-CSF barrier is very small compared to that covered by the BBB, which makes the BBB the main barrier to transport of drugs through the brain [1]. The BBB maintains the homeostasis of substances in the central nervous system (CNS) and prevents potentially harmful substances from entering the brain from the systemic circulation. Only a few medications are designed for the CNS as the BBB is assumed to prevent the transcytosis of many molecules. The molecules most likely to permeate the BBB include small molecules (less than 70 Da), and either highly lipophilic or with good efflux for endogenous transport molecules expressed by the BBB [2].

Several existing and upcoming drug candidates are composed of amino acids (peptides) or nucleic acids (DNA, mRNA, siRNA), which are all hampered by a complex inability to pass the BBB or barriers due to size and transport mechanisms [5]. Nevertheless, the availability to enter the brain has several advantages for research in design molecular-targets that could enable efficient concentrations of proteins inside the brain. The yield of sufficient amounts of proteins could theoretically be obtained by transport of nanoparticles containing proteins across the BBB or by transfection of the brain by particles containing DNA encoding proteins that would be expressed once introduced to cells of the brain.

The evidence that nanoparticles containing protein can enter the brain and lead to significant concentrations inside the brain is controversial. The prominent evidence presented supports an transport of nanoparticles containing proteins across the BBB or transfection of the brain by particles containing DNA encoding proteins that would be expressed once introduced to cells of the brain. Although the potential for nanoparticles to transport proteins across the BBB is promising, the delivery of large proteins to the brain has not been achieved in humans due to the lack of efficient transport mechanisms.

Several approaches have been made to develop this novel vector delivery transport system with high efficiency. In 1974, Green et al. demonstrated in vitro the feasibility of delivering recombinant proteins into cells of the brain [2]. Although complete barrier, it is believed that the basolateral transport proteins that are necessary for the delivery of drugs across the BBB. The BBB is a highly selective barrier, and the transport of molecules across the BBB is governed by several factors, including the size, charge, and hydrophobicity of the molecules. The BBB is composed of several layers of cells, and the transport of molecules across the BBB is mediated by specific transport mechanisms, including the transport of nutrients and ions across the BBB.

Several transport mechanisms allow small molecules to pass the BBB. Facilitated by a concentration gradient, amino acids and neurotransmitters diffuse passively across the BBB. Solute carrier transporters allow for transport across the BBB.

The brain capillary endothelial cells (BCECs) are one of the non-neuronal type of cells that modulate the pathophysiological transport activity. The tight junctions between the endothelial cells limit the paracellular trafficking of ions and macromolecules. The transcellular transport is also considered limited in and across brain capillary endothelial cells. Solute carrier transporters allow for transport across the BBB.
BIBB of nutrients and other essential molecules like glucose, amino acids, and nucleotides. Mesenchymal, like matrix, transfers, low-density lipoproteins and albumin, are taken up by brain endothelial cells from the plasma by endocytosis either via receptor-mediated endocytosis or adsorptive-mediated endocytosis, but whether they undergo further transport through the endothelial cells into the BBB has not been proved [2]. Active efflux transporters like P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) are members of the ATP-binding cassette (ABC) transporters. They are present both in the luminal and abluminal membranes of the endothelial cells and extrude substances that are not recognized as being essential for the brain respective of size [6]. Not only do the endothelial cells have a high metabolic activity indicated by their richness in mitochondria, but they also have high enzymatic activity that degrades peptides and other compounds of the plasma [19].

TARGETED THERAPY AT THE BLOOD-BRAIN BARRIER

As the usage of drugs across the BBB is limited to small hydrophilic molecules or molecules with structural similarity of nutrients and high affinity for endogenous transporters, the transport of large hydrophilic molecules with therapeutic potential (as above) needs the approach of a drug carrier. Frequently, the outer should be conjugated with a targeting molecule to facilitate uptake by the endothelial cells from the plasma. The selection of molecules for targeted therapies at the BBB was recently reviewed [6-8]. Targeting endogenous molecules expressed on the limited side of the BCCs can be achieved with peptides or derivatives, peri-dominating Alternately, anti-receptor antibodies against the transferrin receptor or the insulin receptor can be used to target the brain capillaries. The anti-receptor antibody antibody is of particular interest as the receptor is exclusively expressed in BCCs and not in the endothelium of any other organ in the body [11].

Evidently large hydrophilic molecules, such as albumin and IgG, are capable of passing through the blood-CSF barrier through choroidal plate epithelial cells by means of transepithelial [2]. From a drug-delivery perspective, this makes however is considerable has relevant, as the relative surface area of the blood-CSF barrier compared to the brain capillaries is very low. Moreover, blood-CSF transport leads to the appearance of molecules in the ventricular system and not in the brain interstitium from where uptake by neurons would be much higher (Fig. 1). Possibly direct injection into the ventricular system of drug-carriers without targeting molecules can be used for drug transport into the brain [5].

TOOLS FOR NON-INVASIVE DRUG DELIVERY: LIPOSOMES, IMMUNOLIPOSOMES AND MAGNETIC NANOPARTICLES

Liposomes, Immunoliposomes and Lipoplasms

Liposomes are biocompatible, virtually non-toxic and biodegradable. They consist of a bilayer of phospholipids or phosphoglycolipids that form uni- or multilamellar spheres [10]. The liposomes can be used for encapsulating both lipophilic and hydrophilic substances, which can be carried either within the liposomal spheres or on their surface. The durability of liposomes in blood plasma is limited because they rapidly cleave by diagnostic cells. This difficulty can be circumvented by coating the liposomes with polyethyleneglycol (PEG), polypeolyglycol or another surface modifying substance leading to micro substitution of the liposomes [12].

Liposomes can be delivered to the desired organ in a targeted manner. The coating material of the carrier is the ability to align itself to the targeted organ. The coating material with appropriate targeting molecules is then able to target the liposome to the desired organ [12]. For targeted delivery into the brain, the most frequently used antibody is raised against the transferrin receptor, which is abundant in the BCCs [1,2,3,15].

A novel approach has been to link the liposome with two different antibodies, the approach being that this will increase the attachment to both an extracellular epitope and a molecule of the intracellular component to improve the affinity and delivery rate of the liposome and its content [16-22]. Dual ligand-coupled liposomes have also been used for both enhancing the delivery to the brain in mice implanted with human cancer cells by cou-
Non-viral delivery strategies into/across the brain capillary endothelial cells

Phytochemicals into the brain capillary endothelial cells (CCECs) were shown in vivo to increase human growth hormone (HGH) expression after transfection (18, 24). The peptide-siRNA carrier complex is also suitable for conjugation with an antibody raised against the transfection receptor making it suitable for targeting purposes (15).

![Figure 1](image1.png)

**Figure 1.** Peptide-transfer permeability measurements expressing the HGF-siRNA complex in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

![Figure 2](image2.png)

**Figure 2.** Peptide-transfer permeability measurements expressing the HGF-siRNA complex in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 3.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 4.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 5.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 6.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 7.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 8.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 9.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 10.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 11.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 12.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 13.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 14.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.

**Figure 15.** Expression of human growth hormone in HMBECs at detection of the FLAG-tagged HGF in a CEC model. The permeability is measured using a fluorescent dye, which localizes to the capillary endothelial cells.
Drug Delivery to the Brain

Magnetic Nanoparticles

In medicinal sciences, magnetic nanoparticles are being used for purposes like magnetic resonance imaging (MRI), hyperthermia for tumor therapy, drug delivery and surgical therapy. They have been successfully used for delivery of anti-cancer drugs in treatment of brain cancer where the BBB is compromised [37].

Magnetic nanoparticles normally consist of a core of non-oxidisable iron-oxide particles such as magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃). This magnetic core can be coated with a biocompatible and non-toxic polymer such as dextran, polyethylene glycol or starch, or coated by phospholipids hence creating a magnetoliposome or a magnetoliposome [37]. Like liposomes, magnetic nanoparticles must be modified with e.g. PEI or dendrimers as they otherwise are prone to rapid clearance from the systemic circulation due to uptake by macrophages [38]. Coating reduces toxicological effects by hindering binding of non-biodegradable magnetic cores and oxidation. A surface coat of hydrophilic polymers also minimizes aggregation of magnetic nanoparticles which can otherwise lead to embolisms of capillaries [37]. Coated magnetic particles are furthermore suitable for targeting to various cellular neurite systems.

The magnetic nanoparticles may also be very precisely delivered to a target organ with the aid of a magnetic field [67]. The necessity of successful drug delivery has e.g. been widely used to deliver of chemotherapy drugs like doxorubicin and have now been made commercially available by, e.g. Chemocell GmbH, Germany. The magnetic field is supplied by an external or an implanted magnet. When applied, the magnetic nanoparticles will be drawn towards the magnet and concentrate in the area where the magnet is located. The delivery will therefore be very local and the drug dosage can be minimised and side effects can be reduced.

CONCLUSIONS

The optimal drug carrier can be characterized by means of its ability to be transcapillary, vary non-toxic and controllable. It should also be targetable, able to bind a significant amount of DNA, and able to transfer cells in vivo. We have found the polyethylene-oxide complexed with plasmid DNA shows promising potential for use for transfections of brain capillary endothelial cells and forcing these cells as factors for protein secretion [3]. We are in the process of characterising this complex for in vivo transfection in the brain. We have also found that magnetic nanoparticles may denote novel routes for mechanism for neuronal gene therapy provided that the magnetic particles can be designed to enable transport across the BBB [7].

ACKNOWLEDGEMENTS

The most recent results obtained and described by the authors were generated with generous grant support from the Danish Medical Research Council grant no. 21-06-0231, the Sapir Foundation, and the Oluf Kruse Fund.

ABBREVIATIONS

BBB = Blood-brain barrier

CSF = Cerebrospinal fluid

CNS = Central nervous system

BCSCs = Brain-Capillary Endothelial Cells

PEG = Polyethylene-glycol

PEI = Polyethyleneimine

REFERENCES


Non-viral delivery strategies into/through the brain capillary endothelial cells