Development of gold nanocarriers to deliver proteins and peptides to the CNS

A THESIS SUBMISSION TO THE OPEN UNIVERSITY FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

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Declaration

I hereby declare that the work presented in this thesis is a result of my own academic and experimental enquiry; contributions made by other researchers are fully acknowledged in relevant parts of the text. Moreover, this work does not contain any material submitted for award of any other degree.

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Abstract

Treatment of many CNS disorders, specifically neurodegenerative disorders are severely limited at present, and with an increase in the aging population, research into an effective treatment is necessary. A major issue is that many therapeutic agents cannot access the brain, due to a structure termed the blood brain barrier (BBB), which excludes many potential therapeutic drugs from the CNS of the drugs that could possibly treat these disorders. Cellular delivery of bioactive molecules e.g. antibodies, peptides and cytokines are a growing area of research due to their possible therapeutic potential. There have been several cytokines which have been successfully used in experimental models of many neurodegenerative diseases but have had difficulty in being translated into clinical trials. These cytokines do not cross the BBB by themselves but if attached to an effective nanocarrier that is able to cross this barrier, could be translated into a much-needed treatment. Gold Nanoparticles (AuNP) have been selected due to their numerous useful characteristics, such as easy production, biological compatibility and chemical stability. Previous research has shown that glucose derived-coated gold nanoparticles can bind to, and cross human brain endothelial cells in vitro and rat brain endothelial cells in vivo.

15 formulations of ligand coated AuNPs were investigated using a simple model of the BBB, to determine which formulation could cross the brain endothelium most efficiently. PEGamine/GalactoseC2 and GlucoseC2 ligand coated AuNPs were able to cross most effectively and were taken forward for further experimentation; GalactoseC2 coated AuNPs were used as a control ligand coating.

BDNF, the chosen cytokine of interest for attachment to AuNP, was mutagenically altered (Histidine $\rightarrow$ Cysteine) in the His tag region of a plasmid used to produce recombinant human BDNF to produce a free thiol group on the Cysteine allowing a place-exchange reaction onto the surface of the AuNP. We optimized the production of our modified BDNF (BDNF-H9C) and developed effective techniques to measure the quantity and biological activity of the protein produced (ELISA and TrK-B Assay). However insufficient quantity of BDNF-H9C could be produced from transfected cells and purified to perform an exchange reaction.

A series of peptides was analysed for their ability to bind the transferrin receptor expressed on brain endothelium in vitro (hCMEC/D3 cells). One strongly-binding peptide was selected for attachment to AuNPs and this was found to increase the amount of AuNP that crossed a BBB model.
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Abbreviations
Aβ- Amyloid Beta
ABC- ATP-binding cassette
AD- Alzheimer’s disease
ALS- Amyotrophic lateral sclerosis
ANOVA- Analysis of variance
Au- Gold
AuNPs- Gold nanoparticles
BBB- Blood brain-barrier
BDNF- Brain derived neurotrophic factor
BMECs- Brain micro-vessel endothelial cells
BSA- Bovine serum albumin
CBP- CREB-binding protein
CEF- Chicken embryo fibroblast cells
CHO-K1- Chinese hamster ovary cell line-K1
COS-7 CV-1 cell line
CNS- Central nervous system
CNTF- Ciliary neurotrophic factor
CSF- Cerebrospinal fluid
DALYs- Disability-adjusted life years
DLS- Dynamic light scattering
DMSO- Dimethylsulfoxide
ECL- Enhanced Chemiluminescence
ECM- Extra-cellular matrix
ECL- Enhanced chemiluminescence
EDTA- Ethylenediaminetetraacetic acid
ELISA- Enzyme linked immunosorbent assay
FACS- Fluorescence-activated cell sorting
FBS- Fetal bovine serum
FPLC- Fast protein liquid chromatography
FRET- Fluorescence resonance energy transfer
GFP- Green fluorescence protein
GLUT1- Glucose transporter 1
1. Introduction

1.1. Neurodegenerative disorders

Neurodegenerative disorders (ND) are of growing global concern and are considered a major threat to human health. There are numerous examples of neurodegenerative disorders such as Alzheimer’s, Parkinson’s, Huntington’s disease, Amyotrophic lateral sclerosis (ALS), Frontal lobe dementia etc. for which due to their diverse pathophysiology, there are no effective treatments, to date. These diseases not only have a huge impact on the patient themselves but their families, and society at large. Globally, in 2016 neurological disorders were the leading cause of disability-adjusted life years (DALYs; the sum of years of life lost and years lived with disability) and were the second leading cause of death with approx. 9 million globally (Bourassa et al., 2019).

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder with cognitive, functional, and behavioural alterations, and the development of an effective therapy is a necessity. The Global cost of AD on its own in 2010 was over £470 billion, and this is only likely to increase due to the rapidly ageing population (Nichols et al., 2019).

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, after AD and is characterized by loss of dopamine secreting neurons of the substantia nigra in the basal ganglia of the brain (Del Rey et al., 2018). PD is a multifactorial disease which has genetic factors that are either causative or susceptibility variants as well as unknown environmental cues that can lead to initiation of the disease (Del Rey et al., 2018). Treatment of PD has had a fragmented past, side effects of treatment can often be worse than the disease symptoms. Dopamine itself does not cross the blood brain-barrier (BBB) by itself so a precursor L-Dopa, that can cross the BBB and increase the dopamine levels inside the brain is the most common treatment for PD. Sadly due to the varied number of side effects, clinicians’ attempts to limit use of L-Dopa and other drugs such as Requip/Ropinirole have had serious issues and lead to large lawsuits (Nichols et al., 2019).

Amyotrophic lateral sclerosis, also known as motor neuron disease (MND), is characterised by a progressive degeneration of both upper and lower motor neurons, resulting in muscle atrophy, gradual paralysis, and death, usually resulting from respiratory failure (Tortarolo et al., 2017). ALS has no cure and treatments are only designed to lessen the symptoms.

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) for which the cause is unclear and again there is no cure. Progressive MS leads to the loss of neurons and is suggested to be a result of an overactive immune response, a failure of myelin producing cells or a combination of the two (Goldenberg, 2012).

As of 2018, there was 112 agents in the current pipeline for AD treatment alone, with 26 agents involved in 35 phase III trials (63 agents in 75 trials in phase II, and 23 agents in 25 trials in phase I)
(Cummings et al., 2018). Although this is promising, from 1998 to 2017 there have been 146 failed attempts to treat AD with only 4 treatments being approved (donepezil, rivastigmine, galantamine, and memantine) which can temporarily, improve symptoms but do not stop the disease itself (Cummings et al., 2018).

All of these disorders are linked with some form of disfunction to the healthy processes in the brain, most have some form of protein aggregation and all lead to loss of cells and tissue within the brain. With this in mind some form of therapy where the aggregating proteins are removed and the surviving tissue is stimulated to repair and regrow using anti-aggregation peptides like R1-OR2 and cytokines like neurotrophic factors could be part of a future therapy (Gregori et al., 2017).

However there are a number of reasons why treatments have failed, one major issue is that therapeutic agents find it difficult to access the brain, due to a structure termed the BBB, which excludes or severely limits more than most of the drugs that could possibly treat these disorders access to the brain and completely excludes proteins and most peptides (B. Brazil, 2017).
1.2. Blood brain Barrier

The blood brain barrier (BBB) is a complex, dynamic, adaptable interface that is the principal regulator of transport of molecules and cells into and out of the CNS. The BBB is essential for regulating the neural microenvironment and constitutes the largest surface area for exchange between the blood and the brain. The BBB is a complex system made up of a structurally distinct, continuous endothelial cell layer separating the blood from the extracellular fluid of the brain (Blanchette and Daneman, 2015; Geevarghese and Herman, 2015).

1.2.1. Neurovascular Unit

The subunit of the BBB is the neurovascular unit (NVU) as defined by Harder in 2002, “structure formed by neurones, interneurones, astrocytes, basal lamina covered with smooth muscular cells and pericytes, endothelial cells and extracellular matrix”. Its primary component is the endothelial cells, which form the actual barrier with the blood, but the endothelial cells need interaction with the other cells in order to develop the phenotype responsible for the barrier’s function.

![Figure 1.1 The structure of the Neurovascular unit](image)

The NVU is a functional structure at the level of brain microvessels, the schematic shows the cells involved in producing the correct phenotypical response. Endothelial cells are connected by tight junction and together with pericytes are ensheathed in the basal lamina. Astrocytes end feet are in regular contact with the endothelial cells helping to produce the barrier function with microglia and neuronal interactions all helping to produce a tight barrier.

Figure modified from (Abbott et al., 2010)

1.2.2. Astrocytes

Astrocytes are the most common cell type in the brain and are essential for the formation and maintenance of the BBB by providing secreted factors that lead to the formation of strong tight junctions between the endothelial cells (Sweeney, 2015). Astrocytes, through their end-feet
establish the link between the endothelial blood flux and neurons and are important regulators in the formation and maintenance of the BBB (Cabezas et al., 2014). Astrocytic secreted molecules are important for the regulation of interactions between the other NVU cells. Astrocytes produce antioxidative molecules like GSH, ascorbate and superoxide dismutase (SOD) and a great number of growth factors and neurotrophins, important for brain cell survival particularly during neurodegenerative processes (Cabezas et al., 2014).

1.2.3. Pericytes
Pericytes are cells which wrap around the endothelial cells via cytoplasm processes that extend along the abluminal surfaces of the endothelium covering 30-90% of the abluminal surface of endothelial cells in the CNS (Bertrand, Cho and Toborek, 2019)(Geevarghese and Herman, 2015). Pericytes display contractile, cytoskeletal, and surface proteins; however, no single or specific pericyte marker is currently available, rather detection is determined by a series of markers, similar to the markers expressed by vascular smooth muscle cells (Vanlandewijck et al., 2018). Pericytes play a critical role in regulation of capillary blood flow and interactions between endothelial cells and pericytes are necessary for the proper formation, development, and maintenance of the BBB. Dysfunction or loss of pericytes leads to BBB breakdown contributing to the pathogenesis of several diseases that are associated with microvascular instability and blood vessel ruptures (Bertrand, Cho and Toborek, 2019).

1.2.4. Basal Lamina
The basal lamina is part of the extra-cellular matrix (ECM), an important structural element of the BBB as it serves as an anchor for the endothelium through the interaction of the endothelial integrin receptors and matrix proteins such as laminin (Abbott et al., 2010; Cabezas et al., 2014). Disruption of the ECM is strongly associated with an increase in BBB permeability during pathogenic states and expression of matrix metalloproteinases (MMPs), which break down the ECM and activate the microglial cells, is increased in numerous neurological disorders (Yamazaki and Kanekiyo, 2017).

1.2.5. Brain Micro-vessel Endothelial cells (BMECs)
The brain micro-vessel endothelial cells (BMECs) in the CNS have a unique set of characteristics which differentiate them from those found in the periphery. BMECs have reduced pinocytosis compared to other endothelial cells in the body and tight junctions which tightly ‘knit’ the cells together (Helms et al., 2015). The endothelial cells of the brain microvasculature also have a series of specialized transporters, efflux transporters (P-gp-1, BCRP, Mrp) and enzymatic components (cytochrome P-450) which help regulate the passage of molecules in and out of the CNS (B. Brazil, 2017; Nam et al., 2019).
The BMECs are in contact with astrocytic end-feet and pericytes through the basal lamina, which produce and maintain numerous barrier functions of the BBB. BMECs are important in the bidirectional transport across the BBB through ion transporters, protein and peptide carriers and active efflux transporters (Pulicherla and Verma, 2015).

BMECs transcytosis activity is known to be extremely low compared to peripheral ECs, however BMECs facilitate the transport of several specific molecules across the BBB, using a number identified pathways (B. Brazil, 2017). Passive diffusion allows for the influx of small, nonpolar, and lipophilic molecules into the CNS across the BMECs while glucose, hormones, amino acids and nucleotides can pass through the BBB by carrier-mediated transport (Pulgar, 2019).

The CNS is described as an immunologically privileged area of the body and the BMECs express low levels of leukocyte adhesion molecules (LAM), which support migration of phagocytes and lymphocytes from the blood into the target tissue (Vanlandewijck et al., 2018).

Tight junctions are critical for the formation of a physical barrier by sealing the paracellular route between the EC’s. They are composed of several types of transmembrane proteins such as claudins, and occludin and intracellular components including zona occludeins proteins. The Tight junctions interfere with paracellular transport of molecules and ions between cells creating a high transendothelial cell electrical resistance (TEER) (Blanchette and Daneman, 2015).
1.2.5.1. Transport systems

BMEC employs many different transport systems in order to meet the needs of the brain for nutrients. The transport systems can be classified as passive diffusion, carrier-mediated transport, and receptor-mediated and adsorptive endocytosis/transcytosis (figure 1.2).

Paracellular diffusion of hydrophilic compounds is highly restricted due to the tight junctions. Lipophilic compounds no larger than 400-500 Da in size are able to cross the BBB by transcellular diffusion, along with gases such as oxygen and carbon dioxide (Abbott et al., 2010). Molecules that can form six or more hydrogen bonds at the BMEC cell membrane and have a polar surface area greater than 80 Å² are far less likely to be able to cross (Clark, 2003).

Carrier-mediated transport is used for the transport of specific molecules such as glucose, amino acids, nucleotides and small peptides. This form of transport also works for the efflux ATP-binding cassette (ABC) transporters such as P-gp1 and BCRP. Which are located on the luminal side of the BMEC. These transporters remove lipophilic molecules, potential harmful substances and drugs from the CNS and the BMECs and pump them back into the blood (Helms et al., 2015).

Figure 1.2 Physical and molecular properties of endothelial cells contributing to BBB integrity and function

Tight junction and adherens junction complexes between endothelial cells restrict paracellular flux across the BBB. In addition, some nutrients and essential molecules are selectively transported from luminal to abluminal membranes by specific influx transport systems, such as insulin, which is transported by receptor-mediated endocytosis/transcytosis. Most of the small lipophilic molecules that have passively diffused across the lipid bilayer are returned to the blood by ATP-dependent efflux transporters.

Figure take from (Yamazaki and Kanekiyo, 2017)
There are a number of carrier mediated and receptor mediated transporters that are expressed at the BBB that help regulate trafficking of different molecules.

1.2.5.1.1. Glucose Transporter 1 (GLUT1)
Glucose transporters at the BBB maintain the high energy demands of the brain by the continuous supply of glucose across the capillary endothelium (Pulgar, 2019). There are numerous sodium dependent and independent glucose transporters, but the major one that has been investigated as a target for drug delivery is GLUT1 (Patching, 2017). GLUT1 is a highly specific transporter for D-glucose, and targeting GLUT1 with novels drugs is difficult, chemotherapy agents conjugated to glucose have been largely unsuccessful, with few exceptions (Stenehjem et al., 2009).

1.2.5.1.2. Low density lipoprotein receptor-related protein 1 and 2 (LDLR)
Lipoproteins are proteins that transfer lipids (fats) around the body in the extracellular fluid, and bind to endothelial LDL-receptors to initiate their transcytosis across the BBB (Georgieva, Hoekstra and Zuhorn, 2014). LDLR is of a family of single transmembrane glycoproteins that are generally recognised as cell surface endocytic receptors whose intracellular trafficking was described for the LDL particles and are expressed with some degree of tissue specificity.

Nanoparticles which were able to absorb apolipoproteins on their surface were shown to have increased transcytosis across the BBB, but the need for surfactant in the nano-formulation, did have some toxicity issues (Huang, Cambre and Lee, 2017).

1.2.5.1.3. Insulin Receptor
The insulin receptor is highly specific and the conjugation of molecules to its substrate, insulin, has had mixed results (Mainprize et al., 2019). However, using antibodies against the insulin receptor has been more fruitful, making it a possible candidate for the delivery of drugs and recombinant protein across the BBB (Tortarolo et al., 2017).

Ulbrich et al used human serum albumin with covalently attached insulin or anti-insulin receptor monoclonal Abs and bound loperamide. Loperamide exerts an anti-nociceptive effect when directly injected into the brain, but it is a strong P-gp1 substrate and so is usually stopped from crossing the BBB in pharmacologically significant amounts. Ulbrich et al were able to show that the loperamide was able to show an effect when conjugated to either the insulin or the anti-insulin receptor antibody (Ulbrich, Knobloch and Kreuter, 2011). Interestingly insulin is able to cross the BBB largely independent of glucose, which may be useful to exploit in targeted delivery to the CNS (Banks, Owen and Erickson, 2012)
1.2.5.1.4. Transferrin Receptors

In vertebrates iron is transported within the organism between sites of absorption, storage and utilization by transferrin. Cellular iron uptake from transferrin is mediated by the transferrin receptor (TfR), a cell membrane associated glycoprotein. TfR is ubiquitously expressed at low levels on mammalian cells but is highly expressed on cells which require large amounts of iron (e.g. maturing erythroid cells), rapidly dividing cells (intestinal epithelium and cancerous cells) and on the endothelium of the BBB (Daniels et al., 2006, 2012).

TfR targeting allows for the possibility for RMT drug delivery and recently TfR mediated transcytosis demonstrates great potential for increasing the targeting to the BBB for clinically significant molecules that could be therapeutic within the CNS and this has been demonstrated in various pre-clinical studies, in vitro and in vivo (Macdonald et al., 2017a; Webster et al., 2017a).
1.3. Targeting the TfR

The most studied receptor system shown to facilitate CNS delivery of clinically relevant doses in vivo is the TfR which is widely agreed to be more highly expressed in brain endothelial cells in vivo, than the Insulin receptor and the LDLR, which makes the TfR a good target for receptor mediated-transport (RMT) across the BBB (Helms et al., 2015). It also appears that TfR levels do not decrease in the micro-vessels of AD patients compared to healthy patients, even with BBB disruption (Bourassa et al., 2019).

1.3.1. Antibodies

A recent promising avenue for targeting the TfR was the use of antibodies against TfR such as OX26 antibody, which was shown to bind to the capillaries in rat brains but not the vasculature of other organs. It was however shown to localise to maximum of 0.44% of the injected dose of OX26 detectable in brains with capillaries removed (i.e antibody had crossed the BBB and entered the brain parenchyma) (Paterson and Webster, 2016). Interestingly Yu et al have shown that the affinity of the antibody was affecting the antibody’s ability to transverse the BBB i.e they investigated a panel of low affinity antibodies at a therapeutic level and showed that the level of antibody detectable in the brain after 24hrs was inversely correlated with the antibody’s affinity for the transferrin receptor. The higher affinity antibodies appeared to adhere to the capillary but were not getting released on the abluminal side and ended up in lysosomes (Yu et al., 2011).

1.3.2. Aptamers

Aptamers are oligonucleotide based targeting molecules that can bind molecular targets at high affinity and specificity via the three-dimensional hairpin structures they form through folding. Oligonucleotide aptamers consist of RNA or single stranded DNA (ssDNA) and can bind to a large range of potential targets. There is a number of aptamer based drugs in clinical trials and Macugen an aptamer based anti-angiogenic medicine for the treatment of macular degeneration has been very promising (Trujillo et al., 2007).

Aptamers are relatively stable molecules that have a much smaller size compared to antibodies, which should mean increased tissue penetration compared to the larger antibodies, as well as relatively low immunogenic potential as the immune system generally does not raise an immune response against nucleic acids (Macdonald et al. 2017). However nucleotides are rapidly degraded by serum and intracellular nucleases via catalysis of hydrolysis reactions.

MacDonald et al created bifunctional aptamers which bound to both the transferrin receptor and to Epithelial cell adhesion molecule (a possible target for cancer treatment), and were able to cross the BBB in vivo (Macdonald et al., 2017b).
1.3.3. Transferrin mimics

Lactoferrin is a glycoprotein which is part of the transferrin family and has been known to cross the BBB via the TfR. Due to the lower concentration of endogenous lactoferrin than Transferrin, lactoferrin was observed to exhibit better BBB uptake than Transferrin, because of less competitive inhibition. The results from Huang et al suggest that by conjugating their NPs (liposome based) with lactoferrin they increased the NP in vitro uptake by 3 fold and in vivo uptake 2 fold (Huang et al., 2013).

1.3.4. Peptides

Despite the relative success of using Abs for targeting the BBB and the transferrin receptor, peptides have numerous advantages. Peptides are relatively low cost compared to antibodies and other small drugs, they can be highly specific with lower immunogenicity compared to Abs. Another advantage of peptides is that they are better suited to chemical synthesis and modification, making them ideal for attachment to NPs.

Melanotransferrin, another of the Transferrin family has been utilized to produce a 12- amino acid peptide sequence which seems to mimic the binding of melanotransferrin and has been shown to have positive results when conjugated to an interleukin-1 receptor antagonist in a neuropathic pain model (Thom et al., 2018).
1.3.5. Transferrin targeting Peptides

1.3.5.1. Pasqualini and Ruoslahti experiments with brain targeting cyclic peptides

Pasqualini and Ruoslahti used in vivo panning with a T7 phage library in Balb/c mice, using tail vein injection and isolated the bound phage from the brain. Peptide sequences CENWWGDVC, CLSSRLDAC, WRCVLREGPAGGCAWFNRHRL and as well as CNSRLHLRC (which we have designated as Pep-R1, 2, 3, 4 respectively for our work). Pep-R2 was conjugated to red blood cells resulting in an accumulation of red blood cells in the capillaries of the brain, with all 4 peptides shown to bind to the brain vasculature. When tested as isolated phages the Pep-R1 and Pep-R2 each targeted the brain several fold more effectively than the kidney. (Pasqualini and Ruoslahti, 1996)

![Figure 1.3 Uptake of the Pasqualini and Ruoslahti peptides](image)

**Figure 1.3 Uptake of the Pasqualini and Ruoslahti peptides**
Uptake of the Pasqualini and Ruoslahti isolated peptides phages (Pep-R1, -R2, -R3) through brain endothelial cells in the presence (grey bar) or absence (black bar) of pure cyclical Pep-R2 peptide. Figure amended from a figure in Pasqualini and Ruoslahti 1996.

1.3.5.2. Lee experiments with TfR targeting linear peptide

Lee et al used a phage display library to find peptides specific for binding to and internalization by the human TfR. Their phage display selection system was based on alternating rounds of selection on chicken embryo fibroblast cells (CEFs) either lacking or expressing hTfR. This process isolated two peptides, HAIYPRH and THRPPMWSPVWP the latter of which we used as Pep-L (Lee et al., 2001). To show that Pep-L as well as HAIYPRH are internalized Lee et al conducted a peptide mediated cell entry experiment which used green fluorescence protein (GFP) fusion proteins tagged with either of the peptides. They were able to show with immunofluorescence that the GFP-Pep-L was being internalized by the CEF-hTfR cells when the cells were washed with acid (to remove any protein bound at the cell membrane) at 37°C but not at 4°C (when clathrin-coated endocytosis is stopped). They were also able to demonstrate a similar pattern of internalization by transferrin
fused protein, but this did not seem to compete against the selected peptides fused to GFP proteins (Lee et al., 2001).

1.3.5.3. Daas experiments with TfR targeting cyclic peptides

Dr Daas, worked with MedImmune and the Open University, with the goal of producing aptamers and cyclic peptide domains which could be used in a drug delivery system to deliver macromolecular drugs into the CNS.

Three receptors were characterised for use as targets on the cell surface of hCMEC/D3 cells, the TfR, LDLR and LRP1. Aptamers and cyclic peptide domains were then selected via in vitro selection techniques. He selected 13 peptides that demonstrate species cross-reactivity to human, mouse and rat TfR as determined by phage ELISA, and then confirmed by binding to hCMEC/D3 cells and mouse endothelial cell bEnd.3s. Three of these Tfr binding peptides were chosen in this project for study of their potential to target brain endothelium, two, (Pep-1 and -10) as they bound to mouse, rat and human TfR greater than the others and Pep-2 as a negative comparison.
1.4. Overcoming the BBB

BBB disruption is a common occurrence in numerous degenerative neurodegenerative disorders, with a ‘leaky’ BBB frequently being seen in AD and PD. This initially may seem beneficial for the treatment of these disorders, but BBB disruption is often seen late in the disease progression, meaning an effective treatment would need to cross a healthy BBB, at the earlier stages of the disease, to have the best chance of being effective.

1.4.1. Mechanically overcoming the BBB

There have been numerous approaches to overcome the healthy BBB, one of the earliest area of research is a direct mechanical approach to locally circumvent the BBB using catheters, implantable devices including microspheres, microchips, direct injection and nasal drugs.

Catheters can be surgically implanted into the brain to directly infuse drugs, and have been successfully used to help treat cancer symptoms (Thurel et al., 2011; Nature Editorials, 2013; Bors and Erdö, 2019).

Invasive approaches have a number of issues, primarily they require a surgical procedure to an already compromised patient, that can easily lead to the introduction of infection. The procedure itself can lead to damage to the brain tissue surrounding the device and can lead to long term issues.

1.4.1.1. Focused Ultrasound and Microbubbles

Focused ultrasound can be used to disrupt the BBB, and when combined with intravenously injected microbubbles can be used as a delivery mechanism to cross the BBB. These microbubbles consist of heavy gasses in a protein or lipid shell, between 1 to 10µm in diameter. When used with ultrasound, the acoustic wave produced by the ultrasound generator causes the cavitation of the microbubbles, transforming the energy from the ultrasound wave to kinetic energy in the microbubble’s local environment, leading to transient permeabilization of membranes (Song, Harvey and Borden, 2018).

This process can affect the tight junctions between the BMECs increasing the inter-endothelial cleft temporarily allowing the passage of drugs/therapeutics circulating in the blood temporary access to the CNS (Bors and Erdö, 2019).

A phase-1 studying using focused ultrasound and microbubbles to open the BBB allowing a chemotherapy agent into the CNS, for treatment of glioma, found the procedure to be tolerable, repeatable and the BBB was closed within 24hrs after opening (Arvanitis et al., 2018; Mainprize et al., 2019).

The major disadvantage with these approaches is that prolonged opening of the BBB, even if it is under 24hrs, can lead to the influx of numerous molecules which can lead to immune response,
infection, nucleation of protein aggregation etc. While this can be acceptable in the treatment of brain tumours, the BBB disruption with Focused ultrasound has been accompanied by red blood cell extravasations, indicating a mild vascular injury, and this would be unacceptable in a long term administration (Vykhodtseva, McDannold and Hynynen, 2008; Mainprize et al., 2019)

### 1.4.1. Small molecules

The BBB is a highly selective and dynamic interface between the CNS and blood capillaries, many molecules are able to freely diffuse through the BBB, small lipophilic molecules (<500 Da) as well as O₂, CO₂, nicotine, steroid hormones and alcohol via the transcellular lipophilic pathway.

However most small molecule drugs that have been designed show a logarithmic decrease in concentration in the brain with each mm of distance from the CSF surface (Pardridge, 2012). There was an ~10 -fold decrease in drug concentration of small molecules with each mm of distance removed from the brain surface (Pardridge, 2012; Tang et al., 2019).

#### 1.4.1.1. Modification of drugs

The majority of drugs that act on the CNS are small molecules drugs that have been engineered to increase their ability to cross the BBB and still be effective. The engineering of these molecules usually revolves around a few key aspects of the design, the size, charge, amount of hydrogen bonds and the lipid solubility of the molecule.

Size is important and has been historically the main area of engineering molecules to cross the BBB. If a molecule such as a cytokine is too big then small peptides that can mimic the binding of these cytokines to the target receptors inside the brain (Gregori et al., 2017).

Charge is also highly significant, especially with small molecules. Positively charged moieties will interact with the negative surface charge on the endothelial cells of the BBB, theoretically getting ‘stuck’ and not crossing the plasma membrane (B. Brazil, 2017).

Minimizing hydrogen bonding groups, reducing the number of possible rotational bonds is important to decrease the chance of creating hydrogen bonds with other molecules creating aggregates. These aggregates would no longer be able to cross the BBB and/or be therapeutic (Petrov et al., 2017).

Adding lipid-like molecules to a drug and thereby making it more hydrophobic so that the drug can cross the BBB passively has often been used to cross the BBB (B. Brazil, 2017; Del Rey et al., 2018).

The CNS multiparameter optimization algorithm was devised by Pfizer® researchers who developed a simple design algorithm that has helped to increase the percentage of compounds nominated for clinical development, and is an example of how all these parameters need to be considered when designing a system to circumnavigate the BBB (B. Brazil, 2017; Del Rey et al., 2018).
And finally, prodrugs have been developed, molecules which can be metabolized enzymatically or chemically into a drug in the cell or in the extracellular space of the CNS. Dopamine is a neurotransmitter and is decreased in diseases, such as PD. Dopamine itself does not cross the BBB by itself but L-DOPA a precursor to dopamine, can cross the BBB and lead to an increase in dopamine in the CNS (Wager et al., 2016; Tang et al., 2019).

1.4.2. Trojan Horse

For larger molecules, it is more difficult to cross the BBB. One possible system is the Trojan horse approach. The Trojan horse system uses a therapeutic cargo that cannot cross the BBB and attaching or encapsulating the cargo in something that is easily transported across the BBB. This often involves the attachment of peptides such as RVG29 and attaching them to therapeutic cargo, smuggling them across the BBB into the CNS (Oller-Salvia, Teixidó and Giralt, 2013; B. Brazil, 2017). Other Trojan horse peptides have been developed including venoms found in nature which can cross the BBB, like Apamin. Apamin is an 18 amino acid peptide which is found in bee venom and has shown promising results crossing cellular models of the BBB, and is being developed into a Trojan horse style treatment (Oller-Salvia, Teixidó and Giralt, 2013).

1.4.3. Antibodies

Monoclonal antibodies (mAB’s) have been at the forefront of therapeutic research, showing positive development in cancer and inflammatory disease (Tsumoto et al., 2019). However, in the treatment of neurodegenerative disorders it has been less than fruitful. mABs specificity has been overshadowed by their large size (150kDa) which has led to poor tissue penetration. Added to this, issues with clearance, cost due to storage and poor human trial results, has meant that traditional mABs engineering is not enough for a successful BBB penetrating treatment (van Dyck, 2018).

1.4.4. Nanoparticles

One rapidly growing area of research in the delivery of therapeutics across the BBB is the use of nanocarrier systems, using Nanoparticles (NPs). NPs are being developed not only for the treatment of the currently uncurable ND but also for the treatments of cancers, diagnostics of disease and medicine in general. NPs are objects in the size range between 1 and 100nm, and due to their diminished size, have unique properties that are distinct from their bulk counterparts. Table 1.1 Shows a summary of common NPs with their advantages and disadvantages as possible nanocarriers for drug delivery of therapeutics across the BBB. Note non-biodegradability is an issue for some NP designs due to the possibility that they can lead to toxicity in and in vivo setting.
<table>
<thead>
<tr>
<th>Nanocarrier Type</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Surface functionalization Strategies</th>
</tr>
</thead>
</table>
| Polymeric NPs (PLGA) | - Biocompatibility  
- Biodegradability  
- Drug protection  
- Ease of preparation  
- Good tolerance  
- Controlled pharmacokinetics  
- Tunable physicochemical properties | - Neurotoxicity | - Polysorbate 80  
- RVG29 peptide  
- Anti-A B1-42 antibody  
- Monoclonal antibody (OX26)  
- Anti-A β (DE284)  
- G7 ligand  
- TGN peptides  
- QSI peptides  
- L-valine  
- Chlorotoxin  
- Apolipoprotein E |
| Solid-lipid NPs | - Biocompatibility  
- High physical stability  
- Bioavailability  
- Drug protection  
- Strict control of release  
- Ease of preparation  
- Good tolerance  
- Biodegradability without generating toxic by products  
- No neurotoxic effects reported  
- Hydrophobic drug entrapment efficiency  
- Lipophilicity  
- Possibility of passively cross the BBB | - Reduced hydrophilic  
- Drug entrapment  
- Efficiency  
- Sterilization difficulties | - Apolipoprotein E |
| Liposomes | - Possibility of entrapping both hydrophilic and hydrophobic compounds  
- Improved drug protection and targeting  
- Efficiency  
- Lipophilicity  
- Possibility of passively cross the BBB | - Neurotoxicity  
- Physicochemical instability  
- Tendency of fusion  
- Rapid clearance  
- Sterilization difficulties | - Phosphatidylserine-targeting antibody  
- Polyethylene glycol transferin  
- PFVYL peptide  
- Penetratin peptide  
- Glucose-vitamin C complex  
- Phosphatidic acid  
- Apolipoprotein E |
| Dendrimers | - Possibility of entrapping both hydrophilic and hydrophobic compounds  
- Biodegradability  
- Stimuli-responsive  
- Enhanced targeting efficiency | - Neurotoxicity  
- Synthesis variability  
- Rapid clearance  
- Organ accumulation | - Polyethylene glycol  
- Glioma homing peptides  
- Sialic acid  
- Glucosamine  
- Concanavalin A |
| Micelles | - No neurotoxic effects reported  
- Improved drug bioavailability  
- Physicochemical stability  
- Sustained and controlled release | - Use only for lipophilic drugs  
- Low drug loading capacity | - Tween 80 |
| Inorganic NPs | - Unique optical, electrical, and magnetic properties  
- Tunable size, shape, composition, structure, and porosity  
- Prolonged enhanced permeability and retention effect  
- Enhanced on-demand drug release by applying external stimuli (near-infrared radiation and magnetic field) | - Neurotoxicity  
- High tendency of aggregation  
- Non-degradable-organ accumulation  
- Functionalization for BBB crossing | - Cyclo RGD peptides  
- Phosphonate polyethylene glycol  
- Bovine serum albumin  
- Folic acid  
- CBP4 peptide  
- KLVFF and LPPFD peptides  
- CLPFFD peptides  
- L-DOPA  
- Hif-prolyl hydroxylase 2 silencing |
| Carbon nanotubes | - Unique structure  
- Exceptional electrical, mechanical, optical, and thermal properties  
- High surface area | - Neurotoxicity  
- Need further functionalization for BBB crossing | - Pittsburgh compound B  
- Polysorbate and phospholipid coating |
| Quantum dots | - Exceptional optical and electrical properties | - Neurotoxicity  
- Need further functionalization for BBB crossing | - Polyethylene glycol  
- Asparagine-glycine-arginine peptides |

Table 1.1 Nanocarriers
The main advantages, disadvantages, and surface functionalization strategies for the organic and inorganic nanocarriers
Figure adapted from (Teleanu et al., 2019)
1.4.5. NP uptake mechanisms

Endothelium cells at the BBB have a series of transport systems which could be exploited for the transport of NPs across the BBB and into the CNS.

**Passive diffusion** allows small molecules that are lipid soluble with size <400Da enter the brain, this would be difficult to take advantage of with NP carrier systems in general as the therapeutic cargo in question and the NP would have to be under the 400Da cut off size and lipid soluble (Partridge et al., 2010).

**Carrier-mediated (solute) transportation** can work via facilitated diffusion or may employ the use of sodium or proton gradient for the exchange and is used for transport of glucose, amino acids, small peptides etc (Abbott et al., 2010). While theoretically this system could be hijacked by attaching the target of the specific carrier transporter, it is a complicated process and it also must be noted that there are efflux transporters (ABC transporters) which can flush out foreign molecules from the brain endothelium back into the blood (Gromnicova et al., 2017).

Larger molecules (macromolecules) that are needed by the CNS are transported across the BBB via active endocytotic processes which are traditionally categorized as **receptor mediated** or **adsorptive transport**. Receptor mediated endocytosis involves a specific receptor (e.g. insulin, transferrin etc) while adsorptive endocytosis involves binding of charged molecules that initiate transport (Abbott et al., 2010). Historically these systems are the most exploited process for NP delivery of therapeutic cargo across the BBB (Georgieva et al., 2014).

1.5. AuNPs

Gold nanoparticles (AuNPs) have a set of unique characteristics including their optical, electrical and biological properties, which make them of keen interest in the development of a nanocarrier system. AuNPs can be synthesised in several shapes (rods, cages, stars, shells etc) however the most commonly used is spheres (Liu et al., 2014; Nam et al., 2019).

NP size is an important aspect of developing nanocarriers, with most AuNPs being in the diameter size range of 1.5-50nm (Gromnicova, 2016). This small size relates to the amount of surface area the NP has for the attachment of potential ligands. While not all atoms on the surface can form bonds and the characteristics of the ligand in question can alter the amount that can bind to the AuNP. Generally smaller AuNPs, have greater surface to volume area, allowing for the theoretical delivery of more therapeutic cargo (Thambiraj, Hema and Ravi Shankaran, 2018; Khongkow et al., 2019).
1.5.1. Synthesis of AuNPs

AuNPs are formed when gold salts are reduced in the presence of a stabilizing agent. The AuNP used in this project were supplied from Midatech Pharma®, which use a modified form of the ‘Brust-Schiffrin’ method to synthesis AuNPs with a diameter size of 1-3nm (Gromnicova, 2016).

The Brust-Schiffrin method produces small stable NPs, using sodium borohydride as a reducing agent and alkanethiols as stabilizing agents. The reaction results in AuNPs of 1-3nms with thiolated molecules attached to the gold core (Male, Gromnicova and McQuaid, 2016).

AuNPs stability and the stability of the ligands can be modulated by the way in which they are attached, either covalently or by non-covalent electrostatic interaction. The use of a covalent bond results in a more stable ligand attachment and nanocarrier.

1.5.2. Ligand exchange reaction

Murphy’s group (Hostetler et al., 1996) first described a very simple exchange reaction that occurs with thiol-bound ligands to the gold core that is coated with thiolated ligands. When an excess of free thiolated molecules is added to the mixture of thiol-coated gold nanoparticles, this excess of free thiols reacts with the gold core and some exchange with the already attached ligand to bind covalently to the core (Figure 1.4). This reaction is particularly useful for coating of gold nanoparticles with ligands that may be sensitive to the reducing environment required for the Brust-Schiffrin reaction. In order for the exchange reaction to be favoured, the free thiolated ligand must be in molar excess to the gold.
This reaction has been used both for coating nanoparticles as well as release of ligands from nanoparticles in cells. An example was the fluorescent dye alkanethiol-BODIPY which was attached using the ligand-exchange reaction to 2nm gold nanoparticles (Atukorale, 2015). Furthermore, this thiol-induced release of thiolated ligands from the nanoparticle has also been demonstrated to occur within cells. Cells contain a relatively high cytosolic concentration of glutathione, which has been shown to release ligands from the gold core (Hong et al., 2006; Pulicherla and Verma, 2015).

![Figure 1.4 Ligand binding to gold core via ligand exchange (or place-exchange) reaction.](#)

If thiol-coated gold nanoparticles are incubated in the presence of a molar excess of free thiolated ligands, an exchange reaction occurs which results in some free thiols binding onto the gold core, displacing and thus releasing the original thiolated ligand. This exchange reaction can be used for the attachment of therapeutic modified proteins as well as peptides and other ligands.
1.6. Therapeutic Cargo

If an effective delivery system was created to overcome the BBB issue, the next step would be the attachment of the correct therapeutic molecule for the specific disorder.

There are numerous approaches currently being developed and employed for the treatment of neurodegenerative disorders, one is to target the aggregating molecule. In nearly all major neurodegenerative diseases, including AD and PD, they are defined and diagnosed by the presence of one of four proteins that have formed aggregates, tau, amyloid-beta (Aβ), alpha-synuclein (α-syn) and TDP-43 (Neumann, 2007; Robinson et al., 2018). These proteins misfold and accumulate destroying neurons, and there has been considerable efforts into targeting the aggregation process, such as using antibodies which bind to the Aβ plaques marking them for degradation or the tau tangles, but not only has the mechanism for action been questioned but in some of the mouse models that demonstrated clearing of the aggregates, the neurons do not regrow, and the brain remains damaged (De Vincenti et al., 2019).

This has led to development of other possible therapeutic strategies, such as targeting the regrowth and repair of the damaged tissue, primarily the neurons, using bio-molecules such as proteins, cytokines and peptides.

1.6.1. Cytokines

Cytokines are secreted small glycoproteins that exert a variety of biological responses, by acting on a specific receptor and help coordinate many physiological functions from tissue repair to immune function. Cytokine dysregulation is found in most disease states including cancer, autoimmune disorders and ND (Azodi and Jacobson, 2016).

Cytokines including many interleukins and neurotrophins, offer a promising avenue for research and the development of pharmacological therapies for these diseases, one of considerable interest is BDNF.

1.6.1.1. BDNF

Neurotrophins are a family of growth and survival cytokines that induce the survival, development and functions of neurons. Neurotrophins form homodimers of about 27kDa and have a common structural motif, including 3 disulphide bridges and this family includes nerve growth factor (NGF), neurotrophin-3 (NT3), neurotrophin-4/5 (NT4/5) and Brain-derived neurotrophic factor (BDNF) (Géral, Angelova and Lesieur, 2013; De Vincenti et al., 2019). BDNF is widely produced in the cortex throughout life, where it influences neuronal function and is the most abundant neurotrophin in the CNS, yet has also been located in heart, spleen and skin tissue, blood platelets and circulating plasma, indicating that the protein affects non-neuronal populations (Nagahara and Tuszynski, 2011; Manley, 2013).
BDNF is essential for life and its knockout was found to be embryonically lethal. It has a multitude of functions including synaptic plasticity, long term potentiation, learning, memory, hippocampal neurogenesis and regeneration after injury (Chen et al., 2017). Taking into account the essential role that BDNF exerts in the CNS in physiological conditions, it is evident that alteration of the levels, distribution, structure, and activity of this protein can result in neuronal dysfunction (Travaglia and La Mendola, 2017).

The ability of BDNF to cross from the blood into the brain is controversial with Pan et al using ~100µg radiolabelled BDNF being injected in the jugular vein of mice which appeared to cross the BBB, with Poduslo et al having similar results in rats (Poduslo and Curran, 1996; Pan et al., 1998). However others have found that BDNF did not cross the BBB (Pardridge, Wu and Sakane, 1998; Pardridge, 2007; Price et al., 2007; Numakawa, Odaka and Adachi, 2018). If BDNF does cross the BBB and has some unknown mechanism of transcytosis across the BBB, it seems clear that it does not cross in significant quantities to have a therapeutic effect and therefore needs assisted transport (Petrov et al., 2017).

1.6.1.2. Structure, function and mechanism of action of BDNF

BDNF is synthesized as a 32kDa N-glycosylated and glycosulfated pro-form (Pro-BDNF) which is proteolytically cleaved to the 14kDa mature form (mBDNF) (Manley, 2013; Wang et al., 2018). ProBDNF can be cleaved intracellularly by action of furin or other proconvertases in the trans-golgi network or in secretory granules to yield the mature dimers, yet the majority is secreted uncleaved into the extracellular environment (De Vincenti et al., 2019). Subsequently, a portion of the pro-protein is cleaved to the mature form by the serine protease plasmin and matrix metalloproteinases. Findings suggest that the mature form of BDNF is predominant and of greatest physiological relevance in the adult CNS. However ProBDNF is important for proper folding, dimerization, and targeting of the mBDNF, and ProBDNF may also elicit distinct effects of its own which may oppose those of mBDNF (Manley, 2013; De Vincenti et al., 2019).

All neurotrophic proteins operate by binding to two types of receptors: a common receptor, p75-NTR, which is a member of the family of the tumour necrosis factor receptors, and a second receptor of a high affinity, the tropomyosin-related kinase (Trk) receptor, belonging to the large family of tyrosine kinase receptors; in the case of BDNF it is Trk-B. However Trk-B can also be activated by NT4/5 leading to different biological effects (Allen et al., 2013; Wang et al., 2018).

1.6.1.2.1. Trk-B

It has been established that the interaction of the Trk-B receptor with BDNF is mediated by multiple contacts (Géral, Angelova and Lesieur, 2013). Trk-B exists in two isoforms, a full length form (154kDa) and a truncated form (95kDa); the full length form of Trk-B is involved in the major
biological signalling which is associated with BDNF activated Trk-B. Trk-B has an extracellular domain with many sites of glycosylation, which upon activation by BDNF results in activation of different intracellular signalling pathways (Bathina and Das, 2015).

One of these signalling pathways activated by Trk-B is the RAS/MAPK/ERK pathway. It is essential for neurogenesis and promotes survival by induction of pro-survival genes and inhibition of proapoptotic proteins (Géral, Angelova and Lesieur, 2013; Meng et al., 2019).

Another is IRS-1/P13K/AKT pathway which when activated, supresses apoptosis and plays a crucial role in activation of pro-survival genes responsible for cell survival (Géral, Angelova and Lesieur, 2013; Bathina and Das, 2015).

BDNF signalling pathways can activate one or both of the transcription factors CREB and CREB-binding protein (CBP) that regulate expression of genes encoding proteins involved in neural plasticity, stress resistance and cell survival (Bathina and Das, 2015).
1.6.2. BDNF in health and disease

In healthy volunteers the mean plasma BDNF level was found to be ~92.5pg/ml. Levels are higher in women but decrease with advancing age in both genders (Bathina and Das, 2015). BDNF is abundant in the cortex as well as the hippocampus which are critical areas of the brain involved in memory related functions and has been shown to modify long-term potentiation, a form of neuronal plasticity which acts as the cellular basis of learning and memory (Habtemariam, 2018). BDNF stimulates and controls growth of new neurons from neural stem cells and alteration of BDNF levels in the CNS can cause or has been linked to multiple pathologies; levels of BDNF are decreased in PD (Paldino et al., 2019), HD (Paldino et al., 2019), AD (Giuffrida, Copani and Rizzarelli, 2018).

BDNF has been tried to treat neurological disorders but have had issues, carrier free administration of BDNF have had little clinical success, e.g. subcutaneous or intrathecal administration of rBDNF in ALS patients was well tolerated but showed a lack of efficacy due to short half-life in the blood and lack of large scale penetration through the BBB (Geral et al., 2013). A carrier system which can increase the stability of BDNF in the blood, while also facilitating transport across the BBB, and maintaining biological activity is needed to test the long term therapeutic potential of BDNF in humans.
1.7. Aims of thesis

As the introduction indicates there is a need for the development of an effective nanocarrier system that can deliver therapeutics across the BBB. AuNPs can act as the base of nanocarrier system but can be altered to increase targeting and delivery of therapeutic cargo it correctly designed.

- **Chapter 3**: Identifying the ligand coating of AuNPs that cross the BBB model most effectively
- **Chapter 4**: Design and production of the therapeutic cargo BDNF-H9C
- **Chapter 5**: Increasing the amount of AuNP crossing the BBB, by addition of targeting peptides
2. Material and methods

2.1. Gold glyconanoparticle

Gold nanoparticles of about 2nm gold core coated with ligands including glucose, maltose, PEG-amine/galactose, glutathione etc (modified to have a linker, usually C2) were obtained from Midatech Pharma. These nanoparticles were prepared as stated previously (Lund et al.2011); they were suspended in water and were characterized by Midatech and myself before their arrival, using HPLC (high performance liquid chromatography) and DLS (dynamic light scattering) on a Nano ZSP zetasizer (Malvern instruments). FPLC on a G200 gel filtration column was used to separate and identify the nanoparticles’ ligands based on their size and DLS was used to determine the size of nanoparticles via light scattering.

2.1.1. Ligand Exchange reaction

Ligand exchange reaction occurred when AuNPs (with base coating of C2-sugar residues) were mixed with ligand solutions containing a free thiol. In short, 10.2nmol of NP (defined as 100 gold atoms/core) was combined with the appropriate ratio of ligand, i.e in the 1:1.5 exchange reaction mixture, 15.3nmol of peptide, Pep-L (1722MW) = 2.6µl of 10mg/ml stock and Pep-L+FITC (2080MW) =3.2µl of 10mg/ml stock, in a 15ml falcon tube. This mixture was vortexed 15-30secs (note: this was not performed over nitrogen as Pep-L had not oxidized) and then incubated at 37°C at 600rpm for 3hrs in a PHMT Grant-bio thermos fisher heat/shaker block (Figure 2.1). All samples were then spun at 17,000G for ~30secs after which samples were spun-filtered 3 times in 15ml 10kDa MWCO vivaspins at 4000G and resuspended in ddH₂O.

![Figure 2.1. Ligand Exchange reaction](image)

Coated AuNP when mixed with a solution of ligands (including targeting peptide, therapeutic protein or other ligands) with free thiol can exchange onto the surface of the AuNP.
2.1.2. Analysis of gold concentration by spectrophotometric assay

Samples of AuNP were measured against a standard (gold standards for AAs 10µg/ml, Sigma) that was applied at 3 concentrations in triplicates (1mg/ml, 5mg/ml and 10mg/ml). The assay was performed in a 96 well plate, each sample was tested in triplicate. A total volume of 10µl of sample was applied to the well. To this, 30µl of 50% fresh, cold Aqua Regia (mixture of conc. nitric acid and conc. hydrochloric acid in a 1:3 Molar ratio) was applied. The liquid was mixed by gentle tapping and left to stand for 1 minute. Next, 150µl of 2M NaBr was added. The absorbance was read on a plate reader OPTIMA FluoSTAR at 382nm.

2.1.3. TEM size determination of nanoparticles

Transmission electron microscopy was performed by air drying 2µl of AuNP solution (1:10) on a copper mesh grid (with pioloform film, carbon coated), left for a few seconds to adhere to the film and then blotted off with a filter paper. The grid was then air-dried overnight. The nanoparticles were observed on a JEM 1400 or JEM 2100, with acceleration voltage of 120kV and 200kV, respectively.
2.2. Cell culture related Methods

2.2.1. Cell cultures

Table 2.1 Culture conditions for the maintenance of cell lines used in this study

<table>
<thead>
<tr>
<th>Cell type/name</th>
<th>abbreviation</th>
<th>Source/supplier</th>
<th>Culture medium/supplier</th>
<th>Serum content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain endothelial cell line</td>
<td>hCMEC/D3</td>
<td>Weksler et al. 2005</td>
<td>EBM-2 MV* (Lonza) Grown on Collagen coated culture plates</td>
<td>2.5%</td>
</tr>
<tr>
<td>Chinese hamster ovary (K1)</td>
<td>CHO-K1</td>
<td>(OU stock)</td>
<td>RPMI-1640</td>
<td>10%</td>
</tr>
<tr>
<td>CV-1 cell line</td>
<td>COS7</td>
<td>(OU stock)</td>
<td>RPMI-1640</td>
<td>10%</td>
</tr>
<tr>
<td>human embryonic kidney cells</td>
<td>Hek293</td>
<td>(gift from Dr. Allman)</td>
<td>DMEM</td>
<td>10%</td>
</tr>
<tr>
<td>Chinese hamster ovary (TrKB-NFAT-bla)</td>
<td>TrkB-NFAT-bla</td>
<td>Thermofisher (Catalog no. K1491)</td>
<td>DMEM +Glutamax ** (Thermofisher, 2019a)</td>
<td>10%</td>
</tr>
</tbody>
</table>

*EBM-2 MV medium for hCMEC/D3 contained 0.025% VEGF, IGF and EGF, 0.1% bFGF, 0.1% (v/v) rhFGF, 0.1% (v/v) gentamycin, 0.1% (v/v) ascorbic acid, 0.04% (v/v) hydrocortisone.
**TrkB-NFAT-bla CHO-k1 cells assay media is the same formulation as growth media without Blasticidin or Zeocin.

The medium was prepared according to the manufacturer’s instruction unless otherwise stated. All media contain antibiotics (penicillin/streptomycin) at manufactures recommended concentration. All cell lines were split at 90% confluency, with medium being changed every 2-3 days

2.2.2. Determination of cellular respiration of gold nanoparticles with MTT assay

To assess the affect the AuNPs can have on cellular respiration, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed on cells cultured in 96 well plates, with 4 replicates per treatment. Nanoparticles were applied to the confluent cells at varying concentrations, from 0 to 50µg/ml as Au weight, not AuNP + ligand weight. incubation time was 20-24hrs at 37°C. Digitonin was used as a positive control of toxicity at 30µg/ml for 30mins. The nanoparticles or digitonin solution were removed, the cells carefully washed once in HBSS (Hank’s balanced salt solution, Sigma), fresh medium (50µl/well) was added followed by MTT (10µl/well of 5mg/ml MTT) and the cultures were incubated for 3.5-4hrs at 37°C. the MTT solution was carefully aspirated, 100µl of DMSO (dimethylsulfoxide) added per well and the plate was shaken for 5-10mins. The absorbance was read at 540nm on a plate reader OPTIMA FLuoStar.
2.2.3. Determination of cellular respiration of gold nanoparticles with Alamar Blue assay

To assess the affect the AuNPs can have on cellular respiration, an Alamar Blue assay (Thermofisher Cat.DAL1025) was performed on cells cultured in 96 well plates, with 4 replicates per treatment. Nanoparticles were applied to the confluent cells at varying concentrations, from 0 to 50µg/ml. The incubation time was 20-24hrs at 37°C. Digitonin was used as a positive control of toxicity at 30µg/ml for 30mins. The nanoparticles or digitonin solution were removed, the cells carefully washed once in HBSS (Sigma), fresh medium (50µl/well) was added followed by Alamar Blue (5µl/well stock solution) and the cultures were incubated for 3.5-4hrs at 37°C. The absorbance was read at 570nm and compared to fluorescence at ex.560/em.590nm on a plate reader OPTIMA FLuoStar.

2.2.4. Nanoparticle transport assays

To assess the rate of transport of nanoparticles into and across the cells, the assay was performed using a collagen type 1 gel (0.5ml volume) on which a monolayer of endothelial cells was formed. The endothelial cells cultured in the system were hCMEC/D3’s. The cells were seeded at a density of 80,000-100,000 cells per inset and incubated for 2-3 days in their respective culture medium at suitable conditions. After a monolayer was formed, the endothelial cells were switched to a medium without VEGF for 3 days to form tight junctions. The nanoparticles were applied to the top of the chamber (into the insert part) at a defined concentration. The culture temperature was kept at 37°C and incubated for 3hrs. After the incubation, the media was removed, cells washed with HBSS (to remove any unbound NPs) and the cells removed with 0.5ml of 0.25% collengase digestion, (9mins to remove cell monolayer at 37°C). The collagen gel is then further digested (22mins at 37°C) by the remaining collagenase and stored at -20°C before thawing and running in ICP-Q-MS for gold assay.

2.2.5. Trk-B Cho Assay

TrK-B Cho cells were harvested from growth medium and resuspended in assay media in a black-walled clear bottom, 96 well plate at 30,000 cells/well unless otherwise stated. The assay always contained at least 4 wells with no cells, just media as control as well as 4 wells completely blank. A range of rBDNF concentration was used (0-250ng/ml) to act as comparison. Cells were exposed to rBDNF or sample media (media harvested from CHO-K1 producing BDNF-H9C) and incubated in a humidified 37°C/5%CO₂. Wells were loaded with LiveBLAzer™-FRET B/G Substrate (CCF4-AM) substrate mixture composed from (Thermofisher, 2019b). The plate was covered from light and evaporation and incubated at room temperature for 2hrs. Detection was measured using an OPTIMA FLuoStar using excitation filter 405/20nm, emission filters 460/40nm and 530/30nm. Background was subtracted and the Blue green ratio was calculated in comparison to the known rBDNF values (Thermofisher, 2019a).
2.2.6. Cell surface TfR ELISA

10,000 hCMEC/D3 cells were seeded and grown to confluence in a 96 well cell culture plate and were fixed using 0.1% glutaraldehyde in 1x PBS. Non-specific binding was then blocked using Tris-HCl at 0.1M and pH 6.8/7.5 and washed with ELISA wash. The primary anti-hTfr antibody solution was then added at 70µl/well and kept for a minimum of 2 hours at room temperature. The cells were then washed again, before a 1µl/ml solution of the secondary biotinylated goat anti-mouse IgG was added at 100µl/well, and kept at room temperature for no longer than 1 hour. The cells were washed in ELISA wash again 3 times before 100µl of 1.42µl/ml streptavidin peroxidase was added and kept at room temperature again for 45mins. The cells were washed for a final time before 100µl of chromogen (Thermofisher Cat. 002023) was added. When the cells appeared to turn sufficiently blue in colour, the chromogen reaction was stopped with the addition of 25µl/well 10% sulfuric acid. Optical density was measured on the plate reader at A450.

2.3. ICP-Q-MS

2.3.1. TMAH solubilization

Tetramethylammonium hydroxide (TMAH) is an ammonium salt, which will solubilize biological material, and will strip the ligands from the surface of the AuNP but does not digest the Au core. Performed in a ventilated hood, 2.5ml of a 3% TMAH (diluted in ddH2O) (SIGMA Cat. T7505) is added to 50µl of sample, Incubate at room temperature for at least 15mins (usually 30mins-1hr). The sample was then vortexed and neutralised with 2.45ml of 3% HCL +Ir 2ng/ml internal standard solution. Sample was then run on ICP-Q-MS.

2.3.2. Aqua regia solubilization

Aqua regia is a common reagent for the preparation of biological samples for ICP-Q-MS. Aqua regia, is a mixture of 1 part nitric and 3 parts hydrochloric acid, it liquefies the sample and also breaks down gold nanoparticles. It is possible to speed up the break down process using heat/boiling Aqua regia. 2.5ml of Aqua regia was added to 50µl of sample in a Teflon coated reaction vessel and either allowed to react at room temperature or boiling for 30mins. The sample was then diluted in ddH2O and run in ICP-Q-MS.

2.4. Silver enhancement of cell culture plasticware

Silver enhancement of transwells, eppendorf and cell culture plates, was produced with silver enhancement kit (SIGMA-SE100-1KT). In short solution A and solution B were mixed in a 1:1 ratio, immediately before use and applied to the wells/Eppendorf etc. After 20mins, the wells, Eppendorf and plates were rinsed with ddH2O.
2.5. Mutagenesis of BDNF vector

2.5.1. BDNF Vector

Human BDNF transcript variant 4 ORF mammalian expression plasmid, with a His tag (10x histidine residues) attached to the C terminus was acquired from SinoBiogical INC. The initial vector used was pCMV3-BDNF-His, as Figure 2.2a/b shows.

QuikChange® Site-Directed Mutagenesis kit was acquired from Agilent Technologies. Plasmid DNA was isolated from DH5α (dam+ E.coli stain) and the modified primers were purified before starting (Table 2.2.) The sample reaction contained reaction buffer, BDNF plasmid, 125ng of each of the modified primers, 1µl of dNTP mix and ddH₂O to a final volume of 50µl, after which 1µl
of PfuTurbo DNA polymerase was added. 1st segment of the cycle was performed at 95°C for 30secs, the 2nd segment consists of 16 cycles of 30secs at 95°C, 60secs at 55°C and 6mins (1min/kb of plasmid length) at 68°C. Following temperature cycling the reaction mixture was placed on ice for 2mins.

1µl Dpn I restriction enzyme was added to each amplification reaction; the reaction mix was thoroughly mixed by pipetting and spun for 1min at 1000rpm and then was immediately incubated at 37°C for 1hr to digest. XL1-Blue supercompetent cells were used for transformation, 50µl of cell suspension was added to 1µl of the Dpn I-treated samples, and the mix was gently stirred and incubated on ice for 30mins. The transformation reaction mix was heat pulsed for 45secs at 42°C and then placed on ice for 2mins. NZY+ broth was preheated to 42°C and 0.5ml was incubated with the transformation reaction at 37°C for 1hr with shaking at 225-250rpm.

The reaction mixture was plated on LB-ampicillin agar plates containing 80µg/ml X-gal and 20mM IPTG and incubated at 37°C for >16hrs.
2.5.2.1. Design of Primers

The primers used for the Site-Directed Mutagenesis were designed Via Sigma nucleotide designer tool.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conor Mutagenesis Primer 1 (CMP1)</td>
<td>5’-gctctagactcagtttagctagctagctatggtaggtgatggtgg-3’</td>
</tr>
<tr>
<td>Conor Mutagenesis Primer 2(CMP2)</td>
<td>5’-ccaccatcatcaccacattggtaaactcagtcagttagctagctagc-3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Length (nt.)</th>
<th>Tm</th>
<th>Duplex Energy at 68 °C</th>
<th>Energy Cost of Mismatches</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP 1</td>
<td>40</td>
<td>79.10°C</td>
<td>-48.95 kcal/mole</td>
<td>5.80%</td>
</tr>
<tr>
<td>CMP 2</td>
<td>40</td>
<td>79.10°C</td>
<td>-52.24 kcal/mole</td>
<td>7.29%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer-Template Duplex</th>
</tr>
</thead>
</table>
| CMP1        | tcaccacccatcatcaccaccatcactcactaactcagttagctagctagcggc

| | 3’-ggtggttagtgtagtggtgtaacgatttgagctcagatctcg-5’ |
| CMP2        | 5’-ccaccatcatcaccaccatgtgaactaactcagtcagttagctagcgc

| | 3’-agtggtgttagtgtagtggtggtagtgatttgagctcagatctccg |

Site directed mutagenesis:
5’-gctctagactcagtttagcaatggtaggtgatggtggg-3’
5’-ccaccatcatcaccaccatgtgaactaactcagtcagttagctagccg-3’

Sequencing
5’-taatacgactcactataggg-3’
5’-tagaaggcagtcgcagg-3’

Highlighted section of CMP1 and CMP2 show the section which will alter the sequence of the plasmid to convert the histidine to a cysteine for the BDNF mutagenesis.

2.4.2.2 Sequencing

Sequencing was performed by SourceBioscience Sequencing using BGH and T7 primers for the plasmid supplied by SourceBioscience.

2.4.3 Protein purification

1000ml of supernatant media, spun in 250ml aliquots at 10,000rpm (18,900 RCF) for 30mins. Supernatant media was then sterile filtered through 0.2µm Nalgene filtration units.
The sample was then run through HisTrapHP (GE lifesciences Cat. 17524802) via an FPLC pump at 5ml/min with waste being passed back into the sample bottle, until the sample was cycled 5 times through the column. Sample bound to HisTrap column was washed with 6x column volumes (5mls) with washing Imizadole soln (50mM). The sample was then eluted with 6x column volumes with eluting Imizadole soln (300mM).

The sample was spun concentrated using 15ml falcon filters (10kDa MWCO at 4000xg for 1-2hrs) for 6mls, maximum volume concentrated to 0.5ml). All samples were resuspended twice in 5ml HBSS+ Mg/Ca and spun concentrated in 3kDA MWCO 14,000G 30mins 15ml spin columns washed with 500µl HBSS+ Mg/Ca and this was concentrated and resuspended to make a total volume of 70µl.

2.4.4 Peptide synthesize

All peptides investigated were obtained from Peptide Synthetics, Fareham, UK. Each peptide was dissolve to 10mg/ml in DMSO, aliquoted and frozen at -80°C, only being thawed for use. DMSO-dissolved aliquots were diluted further to the required concentrations in HBSS (+Ca²⁺) unless stated otherwise.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep-1</td>
<td>Fitc-GAWSIIDCSMNYYCLYIEG</td>
</tr>
<tr>
<td>Pep-2</td>
<td>Fitc-GAIHCHPQGDSVSFCWR</td>
</tr>
<tr>
<td>Pep-10</td>
<td>Fitc-GALHECTYYWWGLDCSFR</td>
</tr>
<tr>
<td>Pep-R1</td>
<td>Fitc-GACENWWGDVCGAGAG</td>
</tr>
<tr>
<td>Pep-R2</td>
<td>Fitc-GACLSSRLDACGAGAG</td>
</tr>
<tr>
<td>Pep-L</td>
<td>THRPPMWSPVWPCK-Fitc</td>
</tr>
</tbody>
</table>

Table 2.3 Peptide sequences used for targeting Tfr:

2.4.5 Cytometry analysis of Peptide binding

hCMEC/D3 cells were grown to confluency in a 12-well or 24-well plate, washed and treated with stated concentrations of each peptide according to the fluorimetry calculations. Treated cells were incubated at 37°C for approximately 3hrs. They were then washed twice with warmed HBSS and detached from their respective wells by adding 400-500µl of Trypsin + EDTA solution (Sigma-Aldrich, Dorset, UK, Cat No. T3924) and incubating at 37°C for approximately 5mins. The contents of each well was harvested into individual 15ml tubes. The wells were washed for remaining cells by adding another 1ml of HBSS, which was then also added to the respective tubes. The tubes were centrifuged for 5mins at 4°C and 1500 RPM. All but the cell pellet was then removed and another 1ml cold HBSS was added.
The cells were centrifuged under the same conditions and all but the cell pellet removed. The cells were resuspended in 300µl HBSS and the total solution transferred to chilled FACS tubes. The Cytometry machine was then set up to capture 10,000 cells in the FSC/SSC gate, and the FL1 voltage adjusted so that 90% of negative cells gave a FL1 value <10. The Cytometry machine was then run for each sample, and the median fluorescence noted. Data is expressed as the mean of the medians of triplicate cultures, ±SEM.
3. Results: Identification of the optimum AuNP ligand coating for BBB transcytosis

3.1. Aims of Chapter

The aim of this present study was to investigate different AuNP ligand coatings as the basis of a carrier for a therapeutic biomolecule. Specifically, the aims were to determine:

- Whether the AuNP ligand coatings are cytotoxic for brain endothelium
- Whether the AuNP can enter the brain endothelium and cross the endothelium
- A set of standard conditions for transfer assays
- Which ligand coating facilitates BBB transcytosis most efficiently
3.2. Introduction

Ligand coatings on AuNP are an important factor to consider when synthesising and utilising AuNPs as nanocarriers. They can be made with an array of various ligands, most prominently sugars, each having an impact on the ability to transverse biological barriers. Previous work from our group has shown that the ligand coating on the AuNP impacts the rate at which they transfer across a BBB model and which mode of transport they use; as illustrated in Figure 3.1 (Gromnicova et al., 2013, 2016).

![Figure 3.1 TEM images of Glucose and Insulin coated AuNP transferring across a model of the BBB](image)

Electron micrograph of a section through hCMEC/D3 cells growing on a transwell filter, 1 hr after application of insulin-coated or glucose 4nm gold nanoparticles to the apical surface. The cells were fixed and nanoparticles visualised by silver enhancement. Note the insulin AuNPs are sticking to the surface of the transwell and the pore. Insulin coated AuNPs appear to be moving across in large vesicles while glucose coated AuNPs seem to moving across the cytosol. Scale bar = 1µm.
3.3. Results

3.3.1. Transfer assay

To accurately evaluate which of the ligand coatings were most effective in transport across a model of the BBB, we modified a protocol developed by Sreekanthreddy et al (2016), Gromnicova et al. (2013). In brief a collagen type-1 based gel was produced in wells of a 24-well plate and was overlaid by a monolayer of hCMEC/D3 cells, schematically represented in Figure 3.2. These cells were allowed to proliferate until a confluent monolayer was achieved, after which they were recovered for analysis, along with the collagen gel. Collagenase digestion was used to first separate the hCMEC/D3 cells, which were peeled off, the gel was then further digested, and samples were labelled and stored in eppendorfs at 4°C before being prepared for ICP-Q-MS (which is further explained in section 3.3.2).

The cell media and wash represent the luminal content, the cell layer represents the intracellular content and the collagen gel layer represents the basal content. Rate of recovery, expressed as a percentage, was used to compare results from the different ligand coatings, by dividing the amount of Au in the layer by the total amount of Au in all three layers multiplied by 100.

![Figure 3.2 Diagram of the arrangement of components in a transwell used for transfer assay](image)

hCMEC/D3 cells were overlaid on a collagen gel and grown to confluency, after the experiment the layers were separated by collagen digestion, cells were peeled off before the gel was further digested. Image to the right shows the layer of hCMEC/D3 cells peeling off from the gel, this image is taken from above the well, looking down.
3.3.2. The ICP-Q-MS set up including the solubilization

The most sensitive way to measure the number of nanoparticles in a sample is using Inductively Coupled Plasma Quadrupole Mass Spectrometry (ICP-Q-MS) as gold concentration is directly related to the number of nanoparticles in the sample (Laborda et al., 2016)(Krzećuk, 2015).

The alkaline reagent tetramethylammonium hydroxide (TMAH) was used to solubilize AuNP samples for at least thirty minutes and then neutralised via exposure to hydrochloric acid (HCl) (Noireaux et al., 2019). This less aggressive approach was used for sample solubilization as other, harsher methods such as Aqua Regia or hydrofluoric acid are only necessary for tissue or fixed samples (Laborda, 2016).
3.4. Optimization of ICP-Q-MS

3.4.1. Detection range of Au in the ICP-Q-MS

In order to identify the limit of detection and linear range of this assay, a commercial Au standard solution was purchased from SIGMA® and solubilized using TMAH, as described in the (Methods & Materials). Limit of detection was determined using linear regression as this had a closer fit to the data points as determined by the R² value of 0.9998. The assay did not reach a saturation limit, but we did not expect to measure values over 500ng/ml. The detection range was set at 5-500 ng/ml.

![Graph showing detection limit of Au in ICP-Q-MS](image)

**Figure 3.3 Detection limit of Au in ICP-Q-MS**
Detection range of Au in the ICP-Q-MS, comparing concentration of Au against the detected Au found by the ICP-Q-MS. R² = 0.9998. Error bars are ±SEM, some are shorter than height of the symbol, dotted lines are 95% confidence bands of the linear detection range.
3.4.2. Standards vs AuNPs

The Au solution we were using as a standard was already in single atomic form, while our AuNP were gold clusters and therefore may have behaved differently from the Au standards in the solubilization step and processing by the ICP-Q-MS. The aim of this experiment was to determine whether atomic gold and solubilized AuNPs generated a different signal in ICP-Q-MS. AuNP with a glucose-C2 ligand coating were compared to equivalent concentrations of the Au standard solution. Although the detected level of AuNP began to drift above that expected at concentrations of 250 ng/mL and above when compared to the standard solution, this was deemed acceptable for downstream experiments.

![Figure 3.4 Comparison of Au Standard with AuNP in ICP-Q-MS](image)

Figure 3.4 Comparison of Au Standard with AuNP in ICP-Q-MS

AuNP and the Au standard solution were both solubilized with TMAH and made to the correct concentration before being run in triplicate in the ICP-Q-MS. Error bars are ±SEM but some are shorter than height of the symbol, dotted lines are 95% confidence bands of the linear detection range.

The deviation seen as the sample size increases could be due to the increase in volume of Au affecting the solubilization step, or the ability of the ICP-Q-MS to accurately measure the sample.
3.4.3. Correcting the deterioration of sensitivity as number of samples tested increased

Initial ICP-Q-MS runs showed some decrease in sensitivity as the run progressed, Figure 3.5 shows the Au standards at the start of the run compared to the same standards tested at the end of run. Standards at the start were as expected while the same at the end were less accurate.

Figure 3.5 Comparison of Au Standard run at the start or the end of experiment

Au standard run at the start of a test in the ICP-Q-MS showed results similar to the expected value, however the same standard run at the end of the test showed far more variability. Error bars are ±SEM but some are shorter than height of the symbol

Using increased washes and a different wash solution (20 g/l 1-butanol, 0.5 g/l EDTA (acid) 0.5 g/l triton-x100 and 10 g/l NH₄OH made up in ultra-pure H₂O) between testing of samples, with all future runs and periodical standards to monitor any fluctuations in the ICP-Q-MS we were able to produce a sensitive, reproducible method for detecting AuNP concentrations (Figure 3.6).
Figure 3.6 Comparison of Au Standard Run throughout an ICP-Q-MS test
Using a stock solution of Au Standard at 100ng/ml and 0ng/ml were run periodically throughout a test in the ICP-Q-MS. Dotted line represents the exact predicted value while the highlighted area is ±5 ng/ml of this expected value.
3.4.4. Effect of Matrix (i.e. media/gel/cells) on ICP-Q-MS

ICP-Q-MS is prone to interferences caused by sample matrix components and therefore we tested whether collagen digested layers in the transfer assay could affect our results. Using a blank transfer assay, the cell and gel layer were collagenase digested and spiked with AuNP before being digested with TMAH and run in the ICP-Q-MS. Digested cells appeared to have the biggest impact on the expected result in ICP-Q-MS (Figure 3.7). ANOVA of samples at 200 and 100 ng/ml of Au showed no significant difference between the matrices and at 500ng/ml there was a significant difference between the media +wash and the cell layer and the cell layer and the collagen gel layer.

Figure 3.7 Effect of matrix on ICP-Q-MS at different concentration ranges
ICP-Q-MS is prone to interferences caused by sample matrix components, a blank transfer assay was prepared for ICP-Q-MS and spiked with AuNP of known concentration. A high (500ng/ml), medium (200ng/ml) and low (100ng/ml) AuNP were used to show how the effect of matrix differs due to concentration of AuNP used in sample.
Red line is the expected value with the red highlighted area is ± 5% of the expected value
Error bars ±SEM *=P<0.05
3.4.5. TMAH is a more effective and consistent reagent than aqua regia for solubilization of AuNP samples

Figure 3.7 shows that matrix can have an effect on the expected results from the ICP-Q-MS, with media +Wash and the cells showing a significant deviation at high concentrations of Au. This could be caused by incomplete solubilization of the sample by TMAH. Aqua Regia is a more aggressive form of solubilization of material and has been used to prepare samples before running in ICP-Q-MS. Aqua Regia is concentrated nitric acid and hydrochloric acid in a molar ratio of 1:3 and can be used cold or boiling to digest material. Figure 3.8 shows that both cold and boiling Aqua Regia results were more variable and less consistent than the samples digested by TMAH.

Figure 3.8 Effect of solubilization method on reproducibility of results
Solubilization of spiked transfer assay samples by TMAH, Aqua regia, cold or boiling. TMAH solubilized samples were within ±5% of the expected value (red area) in each of the matrixes while Aqua Regia solubilization, cold or boiling had more variable results. 3 biological replicates with 3 technical replicates.
3.4.6. Sample storage temperature and freeze-thaw cycles influence the detected AuNP concentration of media +wash and gel

There was some suggestion that sample results can fluctuate over time due to freeze/thaw cycling under storage. To test this hypothesis, the transfer assay was repeated with just 1 AuNP (NP12) and after solubilization of gels, samples were stored at different temperature in eppendorfs, and after thawing spun down to avoid any loss of sample. The results are shown in Figure 3.9. One-way ANOVA showed no significant difference between the gold detected in the cell samples but there was a significant difference in gold detected in the media +wash and the gel layer. The results show that the storage of samples can have some effect on the value of Au concentration being measured by the ICP-Q-MS, this is most noticeable in the Media +Wash samples. Since all data produced for the transfer assay were from samples frozen at least once, the most interesting results are the difference between 1x and 5x Freeze/thaw cycle sample results. It appears to affect the results from media +wash samples more than cell or gel layer samples.

Figure 3.9 Effect of Storage on Transfer assay ICP-Q-MS data
Temperature and freeze/thaw cycle affected the ICP-Q-MS quantification of Au in Media +Wash and the Gel Layer. One-way ANOVA showed no significant difference between the storage method of the cell layer samples. Error bars are ±SEM but some are shorter than the height of the symbol, *=P<0.05, *=P<0.01, *=P<0.001. 3 biological replicates with 3 technical replicates
3.4.7. Ligand coating can cause AuNP to stick to plastic

AuNP have been known to be ‘sticky’, and they bind to charged surfaces which are used in cell culture plates. Using a silver enhancement method which binds to the AuNP making them more visible, we were able to determine if and where the AuNPs were sticking to the plastics, Figure 3.10.

Figure 3.10 Silver enhancement of plastic that was in contact with AuNPs

i) Silver enhancement of the transwells which housed the gels for the transfer assay, a-d. are transwells which were exposed to different AuNP during the process and have different degrees of colour change a. NP5 b. NP6 c. NP10 d. NP2 e. is a transwell with no silver enhancement solution added and f. is a silver enhanced transwell which contained no gel

ii) Silver enhancement of the transwells which housed the gels for the transfer assay a. is a well which contained a gel but was not exposed to AuNP b. is a transwell which housed a gel that was exposed to NP10. The well was scratched to show the colour is produced from the silver binding to AuNP attached to the walls of the transwell

iii) Silver enhancement of the Eppendorf tubes that the sample were stored in between running samples in ICP-Q-MS a. is from a gel layer sample from a gel exposed to NP10 b. is a blank c. is from a gel layer sample from a gel exposed to NP1.

All pictures were taken 15 minutes after being exposed to silver enhancement solution.
From this data we can conclude that;

- The detectable range of Au and AuNP was satisfactory for experimentation (5-250ng/ml)
- Using wash solution (20 g/l 1-butanol, 0.5 g/l EDTA (acid) 0.5 g/l triton-x100 and 10 g/l NH₄OH made up in ultra-pure H₂O) during running of samples in ICP-Q-MS increased the reliability of measurements
- The effect of the matrix was minimal
- TMAH was a more reliable and reproducible solubilization reagent than cold or boiling Aqua Regia
- Storage conditions can have a small impact on the reproducibility of results
3.5. Characteristic of AuNP ligand coating

AuNPs of different coatings were provided by Midatech Pharma, along with characteristic data (Table 3.1). This data can be used to compare the AuNPs and their ability to cross our model of the BBB which can help us understand if there are any trends, in the size of NP, length of ligand etc.

It is important to note that these comparisons have multiple variables and some can only act as a guide to which trend may be occurring.

The Description of ligand section provides the chemical nomenclature for the ligands that are attached to the AuNP, in brief the sugar molecules (gluco=glucose, galacto=galactose etc) have been altered so they can be attached onto the surface of the AuNP. Isomers of the sugar are denoted (α or β) and the number preceding the name of the sugar reflects the length of ligand tail which attaches the modified sugar to the AuNP surface (5-, 11- etc).

The Gold concentration shows the amount of gold found in the suspension (by Aqua Regia solubilization assay) which reflects the number of nanoparticles in a given sample and is important for comparison when running samples in ICP-Q-MS.

The Nuclear magnetic resonance (NMR) data shows the ratio of ligand to Au and the expected value is 0.3M/0.1M.

Particle size determined by DLS shows the hydrodynamic size of the AuNPs in the given samples while core diameter size by TEM shows the size of the gold core of the AuNP not the total AuNP and ligand size.

Zeta potential is a measurement of the electrical charge of particles in a given suspension, these measurements can often be inconsistent and this needs to be considered when evaluating the data.

pH value is important to consider, especially if there is any indication that it is affecting the viability of cells, to which it is exposed.

Date of synthesis is important as although these AuNP are remarkably stable, they can deteriorate over time and can also allow us to determine if there is any batch to batch variability in AuNP with the same composition, synthesized on different dates.
Table 3.1 Characteristic and number of AuNP ligand coating

<table>
<thead>
<tr>
<th>AuNP’s No.</th>
<th>Description of ligand</th>
<th>Gold concentration [μmol/ml]</th>
<th>NP$^1$ Concentration [μmol/ml]</th>
<th>NMR</th>
<th>Particle Size by DLS [nm]</th>
<th>Core Diameter [TEM] Size [nm]</th>
<th>Number Counted</th>
<th>Zeta Potential (mV)</th>
<th>pH</th>
<th>Date of synthesis</th>
<th>Midtech’s Code</th>
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<tbody>
<tr>
<td>1</td>
<td>Thioethyl β-D-glucopyranoside@Au</td>
<td>1516</td>
<td>0.077</td>
<td>0.3M/0.1M</td>
<td>6.168</td>
<td>3.373</td>
<td>752</td>
<td>-15.4 ± 6.02</td>
<td>6.7</td>
<td>23/06/14</td>
<td>ID0828</td>
</tr>
<tr>
<td>2</td>
<td>5-Thiophenethyl β-D-glucopyranoside@Au</td>
<td>4418</td>
<td>0.224</td>
<td>0.3M/0.1M</td>
<td>5.952</td>
<td>1.601</td>
<td>1337</td>
<td>0.548 ± 10.5</td>
<td>7.4</td>
<td>23/06/14</td>
<td>ID0829</td>
</tr>
<tr>
<td>3</td>
<td>12-Thiododecanyl β-D-glucopyranoside@Au</td>
<td>472</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>4</td>
<td>Thioethyl α-D-galactopyranoside@Au</td>
<td>4168</td>
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<td>0.3M/0.1M</td>
<td>4.021</td>
<td>2.469</td>
<td>760</td>
<td>-28.6 ± 7.01</td>
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<td>25/06/14</td>
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<td>Thioethyl β-D-galactopyranoside@Au</td>
<td>4031</td>
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<td>0.3M/0.1M</td>
<td>3.952</td>
<td>1.769</td>
<td>1136</td>
<td>-22.1 ± 7.27</td>
<td>5.2</td>
<td>25/06/14</td>
<td>ID0836</td>
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<tr>
<td>6</td>
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<td>4362</td>
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<td>2.271</td>
<td>2.08</td>
<td>851</td>
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<td>Thioethyl 2-acetamido-2-deoxy- β-D-glucopyranoside@Au</td>
<td>4081</td>
<td>0.207</td>
<td>0.3M/0.1M</td>
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<td>1.853</td>
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<td>8</td>
<td>Thioethyl β-D-glucuronopyranoside@Au</td>
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<td>7.9</td>
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<td>GalactoseC2 + PEGamline@Au</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<td>11</td>
<td>[Pt(IV-suc)]17Al24-[a-GalC2]21@Au</td>
<td>3978</td>
<td>0.202</td>
<td>0.3M/0.1M</td>
<td>7.351/ 2.882</td>
<td>1.824</td>
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<td>5780</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>13</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<td>N/A</td>
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<td>Thioethyl β-D-glucopyranoside@Au</td>
<td>4100</td>
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<tr>
<td>15</td>
<td>Glutathione@Au</td>
<td>6660</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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For ease of comparison AuNP ligand coating will be referred to AuNP 1-15 rather than their full description. Table 3.1 shows the full description as well as key characteristics of the AuNP ligand coating.
3.6. Effect of AuNP coatings on cellular respiration

Before we could assess the effectiveness of the ligand coating of AuNPs to transport into and across cells, we needed to first establish that they were not cytotoxic. Assays which measure the effect a stimulus has on cellular respiration are a good indication if they are cytotoxic. An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed on cells cultured in 96 well plates, with 4 replicates per treatment, and 3 biological replicates. The principle of the assay is that the viable cells can metabolize a yellow substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its purple salt formazan, which is determined by measuring absorbance. AuNPs were applied to the confluent hCMEC/D3 cells at varying concentrations, from 8 to 50µg/ml. **Figure 3.11** shows the percentage survival of hCMEC/D3 cell, with only NP11 ligand coating showing a clear decrease in cell viability at 50µg/ml of AuNP.
Figure 3.11 Effect of coating on AuNP to brain endothelial cells (hCEMC/D3) cellular respiration. Percentage survival of hCEMC/D3 exposed to AuNPs coated with different ligands by MTT assay. Red area represents ±10% of 100% of survival rate, our acceptable range. Data shown as mean (Error bars ±SEM) of 3 independent experiments with 4 technical replicates of each condition in each experiment. Negative control was untreated cells and positive control was 30ug/ml Digitonin (30min treatment, average survival =13.85%, not shown). One-way ANOVA was performed for each graph, NP-2, 7, 11 were the only ones to show any significant difference. NP-2, 7, 11 results were compared using unpaired T-tests and the results are shown. * = P<0.05, ** =P<0.01. NP11 was the only NP to show increased toxicity as dose increased and therefore was removed from any future experiments.
3.6.1. Comparison of ligand coating effect on relative percentage recovery

Using the method already described for the transfer assay and the optimization steps for the solubilization and running of samples for the transfer assay in the ICP-Q-MS, the relative recovery of each of the AuNP ligand coatings in the Media +wash, Cell and gel layers, was compared (Figure 3.12).
Figure 3.12 Recovery of Au (%) from the transfer assay of different AuNPs Percentage recovery rate of gold in each compartment of the transfer assay for AuNP with different ligand coatings. Error Bars ±SEM
3.6.2. Au Recovery (Endothelial/collagen) compared to NP core diameter by TEM

The size of AuNP can have a large impact on their ability to transcytosis across brain endothelial cells (Xie et al., 2017). TEM is a standard method for determining the core size (diameter) of AuNP. **Figure 3.13** shows the relationship between the size of AuNP and the Recovery rate (%) of the Au in the Media +Wash layer, Cell layer and Gel layer for NPs 1-9. (There was insufficient data for NP10-15). There was no clear relationship between the size of the NPs (TEM) tested and their Recovery rate in Media +wash, cell and gel layer. Note: Size is not the only variable being compared, as all these AuNPs have different coatings and variables, **Fig 3.13** is examine if there is a trend.

![TEM size vs Cell layer Recovery](image1)

**TEM size vs Cell layer Recovery**

![TEM size vs Gel layer Recovery](image2)

**TEM size vs Gel layer Recovery**

Figure 3.13 Correlation of AuNP diameter size (TEM) with Recovery (%) in Cell and Gel Layer. Line of linear regression with 95% Confidence limits, Cell layer $R^2=0.09526$ and Gel layer $R^2=0.4006$. 

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Dynamic Light Scatter (DLS) can estimate the range of size and percentage distribution of NPs in a sample more quickly than TEM. TEM allows for the accurate measurement of NPs in a dried ‘pure’ state, while DLS can allow for the measurement of the hydrodynamic diameter of the AuNPs as well as any molecules attached or absorbed onto their surface. Comparison of the size of the NPs, measure by DLS and their rate of transfer to the cell and gel layers, confirmed the results in Figure 3.13. There was no significant correlation between size and recovery rate to the cell layer (R2 = 0.156) or to the gel layer (R2 = 0.0849).
3.6.4. Au Recovery (%) compared to AuNP Zeta Potential

Positive Zeta Potential can be important for drug delivery since it can facilitate effective adhesion to an epithelial surface (Honary and Zahir, 2013). Figure 3.14 shows the relationship between the size of AuNP and the Recovery (%) of the Au in the Cell layer and Gel layers for NPs 1-9. There was a slight trend between the charge of the NPs tested and their recovery (%) in the Cell and Gel layers. Note: Clearly zeta potential is not the only variable being compared, as all these AuNPs have different coatings, Fig 3.14 examines if there is a trend. Measurement was not possible for all the samples so only NP2,4-9 are shown.

Figure 3.14 Correlation of Zeta potential with Recovery (%) in Cell and Gel Layer
Line of linear regression with 95% Confidence limits –Cell layer $R^2=0.3362$, Gel layer $R^2=0.2197$
3.6.5. Recovery (%) compared to batch to batch variability

AuNP synthesis has had issues with batch to batch variability which can cause differences in characteristics of the AuNP and their coatings and consequentially their interactions with cells (Nadia Tzelepi, Thesis 2019, The Open University). Of the range of ligand coatings on the AuNPs NP10 and NP13 are both α-Galactose-C2/Pegamine (50:50 ratio) coated AuNP and NP1, 12 and 14 are β-Glucose-C2 coated AuNP. Figure 3.15a and Figure 3.15b showed no significant difference between batches and their recovery found in each of the samples, Media +wash, cell, and gel layers.

![Graph](image1)

**Figure 3.15a** α-Galactose-C2/Pegamine (50:50 ratio) batch to batch variability

NP10 and NP13 are both AuNPs coated with α-Galactose-C2/Pegamine (50:50 ratio). They were produced at different times, and therefore could have batch to batch variability, however two-way ANOVA showed no significant different between the batches. Error bars ±SEM

**Figure 3.15b** β-Glucose-C2 coated AuNP batch to batch variability

NP1, NP12 and NP14 are both AuNPs coated with β-Glucose-C2 and were produced at different times, however two-way ANOVA showed no significant different between the batches. Error bars ±SEM
3.6.6. Effect of ligand length on transcytosis

The length of ligand could affect the size of AuNPs and may affect the ability of the AuNPs to cross our BBB model. NP1 and NP2 both have β-glucose molecules as ligands however NP1 has a C2-linkage and NP2 has a C5-linkage, which should increase its diameter. However NP1 diameter is 6.2nm (DLS) and 3.4nm (TEM), NP2 is 5.9nm (DLS) and 1.6nm (TEM), which is counter to what we would expect. (Note that DLS measures the total diameter of the NP, while TEM measures the gold core.) Ligands are attached as the AuNPs are formed in a 1 step synthesis process and the longer ligand appears to have caused a reduction in core size. NP2 is significantly less effective at crossing and entering the hCMEC/D3 cells than NP1, Figure 3.16. We assume that the coating density is the same for both ligands, consequently the ratio of ligand:Au is higher for NP2 although it is smaller. This may correlate with decreased endocytosis and transcytosis and suggests that even with a chemically identical ligand (β-glucose) exposed on the outside of the NPs, the ratio of Au:ligand (and possibly the density of the NP) affects the rate of endocytosis and transcytosis. Note: Clearly ligand length is not the only variable being compared, as these AuNPs have different coatings, Fig 3.16 examines if there is a trend.

Figure 3.16 Recovery (%) of transcytosis of β-Glucose-C2 and -C5 coated AuNPs
NP1 and NP2 AuNP’s are both coated with β-Glucose but NP1 has a C2 linkage and NP2 has a C5 linkage. The percentage recovery of transfer Au to the cell and gel layers was significantly different for the two NPs.

Error bars ±SEM **** P=<0.0001
3.6.7. Au recovery rate in the cell layer compared to gel layer

To evaluate which ligand coating facilitates BBB transcytosis most efficiently, the relative Au recovery rate between gel and cell layers was compared. The levels in the cells indicate the rate of endocytosis, while levels in the gel represent the rate of transcytosis. AuNP ligand coating that had high levels in the gel layer with lower levels in the cell layer could be more efficient for delivery of cargo across the BBB and into the CNS; AuNPs found at relatively higher levels in the cells imply that the NPs are retained or delayed in a compartment of the endothelial cell after endocytosis. **Figure 3.17** shows that NPs 12 and 14 (β-D-glucopyranoside) have high transcytosis, whereas NPs 10 and 13 (α-Galactose-C2/ PEGamine 50:50) have highest uptake into the cells. Only two coatings were taken forward to focus future experiments.

![Figure 3.17 Percentage recovery of Au found in gel layer vs cell layer from the transfer assay of ligand coating on AuNP](image)

NP10 and NP13 coating are highest on average (both in gel and cell layer), NP12 and NP14 coating are highest in the Gel layer and lower in the cell layer. Error bars are ±SEM but some are shorter than height of the symbol.
3.7. Conclusion

From this set of experiments we have developed an efficient method to assess the effect that ligand coatings can have on the ability of AuNPs to transfer across a BBB model. This means we can rapidly screen numerous coatings and compare their characteristics.

From our results it appears that size, Zeta potential and batch to batch variability had no consistent effect on the ability of the AuNPs to enter the endothelial cells and to transfer across and into the hydrogel layer. However the length of ligand did have an effect, both on the synthesis and therefore the characteristics of the AuNPs and also on their ability to transfer across the cells and into the gel.

From this set of AuNPs we chose three to take forward into later studies: α-Galactose-C2/PEGamine coated AuNP (NP10) which were high in both layers; β-Glucose-C2 coated AuNP (NP14), which had the highest ratio in the gel:cell layer; and α-Galactose-C2 coated AuNP (NP4) as a comparison since these NPs had been used in previous studies of endocytosis by hCMEC/D3 cells.
4. Chapter 4: Design and production of the therapeutic cargo (BDNF-H9C)

4.1. Introduction

BDNF is a small cytokine (dimer structure is 27.2kDa) that produces a biological effect at low concentrations (1± 0.52 pg/ml in healthy individuals’ CSF) (Suliman, Hemmings and Seedat, 2013). BDNF has been proposed as a suitable treatment for a number of neurological disorders including Alzheimer’s, Parkinson’s and Huntington’s disease (Bathina and Das, 2015; Chen et al., 2017; Giuffrida, Copani and Rizzarelli, 2018) and was therefore an attractive target for a therapeutic cargo.

To attach proteins to AuNP requires modification of the protein. Previous attempts to attach delicate proteins similar to BDNF to the outside of AuNP relied on colloidal (naked) AuNPs simply mixing with the protein. Proteins can ‘stick’ to the nanoparticle by processes such as thiol or amine binding. However, this does not confer an orientation of the protein and active sites can be blocked or the protein surface altered, leaving the protein inactive (Jazayeri et al., 2016)(Tsai et al., 2013). To increase the active conformity of the protein attached to the AuNP and increased uniformity between nanoparticles, a ligand exchange reaction to attach the BDNF to the AuNP was proposed. The most common method would require an available free thiol group on the BDNF, which, when attached, would not affect the binding site or functional activity of the BDNF. To achieve this, recombinant human BDNF with a C-terminal-H9C (9x Histidine followed by a cysteine tag) (BDNF-H9C) was produced, with the free thiol group in the cysteine acting as the donor/acceptor for the ligand exchange reaction. This should attach the BDNF-H9C to the AuNP without effecting the 3D structure of the BDNF (and the cysteine knot at the centre of its structure) thereby retaining its biological activity.
4.2. Aims of the chapter

1. Development of assays for BDNF quantitation and activity
2. Production of BDNF-H9C plasmid
3. Measurement of BDNF-H9C produced from transfected cells by ELISA and TrK-B CHO assay
4. Optimisation of production by modification of transfection conditions
4.3. Results
In this section the measurement and production of recombinant BDNF for attachment onto AuNPs is discussed, including optimization of production and purification.

4.3.1. Mutagenesis of BDNF plasmid
To produce recombinant hBDNF (hrBDNF) for attachment to AuNP a cDNA ORF Clone, Human C-His Tag was obtained from Sino-Biological® shown in Figure 4.1a. The C-terminus contains a chain of ten histidine residues commonly used as a tag for purification (SinoBiological, no date). Primers were designed to alter the last histidine residue in the HisTag (CAC) to a terminal cysteine residue (TGC) shown in figure 4.1c. The schematic for this design is shown in figure 4.1d. Mutagenesis was achieved by using a QuikChange Site-Directed Mutagenesis Kit from Agilent Technologies® to mutate the plasmid to produce a modified recombinant BDNF which is referred to as BDNF-H9C.
Figure 4.1 Plasmid and primer design and use in the mutagenesis of hrBDNF

a) Schematic layout of a pCMV3-His plasmid which is the base plasmid of the Human BDNF C-His tagged plasmid, purchased from Sino-biologicals®. It has Hygromycin and Kanamycin as selective antibiotic resistance for growth in mammalian and E.coli cells respectively.

b) Highlights sections of the plasmid section important for the stage of mutagenesis including the sequences for primers (T7 and BGH) to be used for sequencing of the correct mutagenesis of the plasmid.

c) Primer sequences were designed to alter the last histidine in the His-tag of the plasmid to a cysteine (CAC → TGC).

d) Basic steps in the mutagenesis of the plasmid, from preparation, temperature cycling, digestion and transformation.

Modified from figures and information from Sino-biologicals and Quikchange Site-Directed Mutagenesis kit.
4.3.2. Confirmation of mutagenesis

The hrBDNF-H9C and hrBDNF plasmids were transformed into *E. coli* and grown overnight on kanamycin LB-agar plates to which transformed bacteria are resistant. A total of 3 clones from each were selected and the plasmids sequenced using T7 (forward) and BGH (reverse) primer binding regions that spanned the ORF of the hrBDNF. To confirm the sequence, a primer was designed to bind inside the enhancer region of the plasmid shown in figure 4.2a. with the sequence and chromatogram in figure 4.2b and c showing the terminal cysteine residue. Initial attempts to sequence the mutated clones (3) of the plasmid proved unsuccessful, with the resulting sequences not fully demonstrating the full sequence of the BDNF region, linker and tag. New primers were designed to bind inside the enhancer region of the plasmid (figure 4.2a) from this we were able to have a ‘clean’ sequence (figure 4.2b and 4.2c) which showed the mutagenesis had been successful in all 3 clones selected for expansion from the Kanamycin agar plates and that we could continue with transfecting our modified plasmid into cells to produce BDNF-H9C.

![Primer code and sequence](image)

<table>
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<th>Primer code</th>
<th>Primer sequence</th>
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<td>Conor P2</td>
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</table>

![Chromatogram](image)

Figure 4.2 Sequencing of mutated plasmid BDNF-H9C
a) Sequence of designed primers used to sequence fully the mutated region of the BDNF-(H9C)  
b) The complete sequencing of BDNF-(H9C); Red region is part of the enhancer sequence, blue region is pro-protein sequence, green is BDNF, Grey is linker sequence, yellow is the 9 histidine residue for tag and pink is the cysteine that was mutagenetically produced from a histidine.  
c) Chromatogram showing a ‘clean’ sequence with clearly defined bases
4.4. BDNF ELISA

A BDNF ELISA (enzyme-linked immunosorbent assay) was developed to accurately measure the concentration of BDNF-H9C and whether the antigenic structure was intact. This assay used a sandwich ELISA format. Briefly, a capture antibody (Anti-hBDNF, Clone 37129, Catalog no. MAB848, R and D systems) that bound to human BDNF was coated to wells of a 96 well plate and supernatant from transfected cells was added. A biotinylated detection antibody (Biotinylated anti-hBDNF, Clone 37141, Catalog no. BAM64, R and D systems) was added that bound to the BDNF in the sample. Following this, streptavidin-peroxidase was added followed by chromogen and absorbance was read at 450 nm. This process is depicted in figure 4.3.

Figure 4.3 Protocol for quantification of BDNF in sandwich ELISA
4.4.1. Detection of hrBDNF in ELISA

To identify the limit of detection and linear range of this assay, commercial hrBDNF was used as the antigen. Using the ELISA protocol in figure 4.3 The limit of detection was determined using observed values that fit a linear regression as this had a closer fit to the data points as determined by the $R^2$ value. The saturation limit of the assay started at 320 ng/mL ($R^2$ value = 0.9735) shown in figure 4.4.

![Figure 4.4 Detection of hrBDNF in the sandwich BDNF ELISA](image)

BDNF sandwich ELISA with concentration of hrBDNF against the absorbance values at 450nm wavelength

a) Detection range of hrBDNF in the ELISA comparing concentration of hrBDNF sample with absorbance value produced. The limit of detection was consistently found to be 0.5ng/ml and saturation at 320ng/ml. $R^2$ value = 0.9735
4.4.2. Effect of Sample Matrix in detection limits

All previous experiments with the BDNF ELISA used hrBDNF diluted in HBSS -Ca/Mg. The medium used for harvesting transfected cells in future experiments could affect the detection of the hrBDNF. Therefore, the aim of this experiment was to determine if the media used could affect the accuracy at which we could detect the level of hrBDNF in our samples. RPMI was the base media used for the cell-lines used for transfection, Used RPMI media refers to cells that were mock transfected (exact same procedure as the real transfection but with no cDNA) and grown.

Note: RPMI fresh and used media values were subtracted from media values spiked with hrBDNF.

![Graph](image)

**Figure 4.5 Comparison of hrBDNF in different media in the BDNF ELISA**
hrBDNF concentration in different media applied to ELISA. error bars are ±SEM

From **Figure 4.5** it appears we could accurately measure hrBDNF in our ELISA and the media had little effect once a blank was used, therefore if the BDNF-H9C was produced, and bound to anti-BDNF antibodies in a similar way to hrBDNF then we would be able to measure the quantity produced.
4.5. Trk-B CHO assay

4.5.1. Trk-B CHO assay principals

The BDNF ELISA can determine whether BDNF-H9C can bind to antibodies designed for the wild type mature hrBDNF but does not confirm that BDNF-H9C will have a similar biological function to the wild-type mature form. In vivo, mature BDNF binds to the Trk-B receptor with high affinity (Binder and Scharfman, 2004)(Giuffrida, Copani and Rizzarelli, 2018). Although NT-3 and NT-4 have been shown also to bind to the Trk-B receptor, the wild type cells (CHO-K1) do not produce any of these cytokines naturally, and any found in media (i.e. blank controls) were subtracted from the samples values (Autry and Monteggia, 2012).

TrkB-NFAT-bla CHO-K1 cells contain a β-lactamase reporter gene under the control of the NFAT response element that has been stably integrated into CHO-K1 cells along with the TrkB receptor. Upon stimulation with BDNF or NT-3 and NT-4, TrkB-NFAT-bla-CHO-k1 cells express β-lactamase.

The binding of BDNF to the TrkB receptor leads to Trk dimerization and autophosphorylation. The phosphorylated receptor then recruits and increases the phosphorylation of phospholipase which leads to activation of PI3K, ERK and the PKC pathway which in turn results in the activation of nuclear factor of activated T-cells (NFAT) leading to β-lactamase expression (Wang et al., 2008). If β-lactamase is not produced, and therefore cells have not been stimulated by BDNF, CCF2 is not

![Figure 4.6 Representation of the Trk-B CHO assay](image)

BDNF binds to the Trk-B receptor which leads to NFAT signalling and the production of beta-lactamase, which converts the CCF2 that has been added to the system to a blue product.
cleaved and cells remain green. Under CCF2 cleavage cells will appear blue. CCF2-AM is the lipophilic, esterified form of CCF2 and readily enters the cell. Cleavage of the CCF2-AM to CCF2 by endogenous cytoplasmic esterases rapidly converts this molecule into the negatively-charged substrate, which is then retained in the cytosol.

Figure 4.7 FRET system inside the Trk-B CHO Assay
In the absence of B-lactamase activity, excitation of the coumarin (at 409nm) in the intact molecule, results in FRET to the fluorescein, which emits a green fluorescence signal (at 520nm). In the presence of B-lactamase, enzymatic cleavage of CCF2 spatially separates the two dyes and disrupts FRET, so that excitation of the coumarin (at 409nm) now produces a blue fluorescence signal (450nm)
4.5.2. Detection and saturation limit of rBDNF in Trk-B CHO assay

The detection limit of the assay, where it begins to intersect with zero hrBDNF was approximately 0.1ng/ml of hrBDNF. From this we used 0.5ng/ml as our lowest detectable hrBDNF value. The point of saturation was >10ng/ml of hrBDNF. From this we used 10ng/ml as our upper detectable limit. Figure 4.8a shows the standard curve of recombinant hBDNF. Figures 4.8b and c shows representative images of the Trk-B CHO cells without and with stimulation with BDNF.

![Graph showing detection and saturation limit of hrBDNF](image)

**Figure 4.8 Detection limit of hrBDNF in Trk-B CHO Assay**

a) Detection and saturation limit of hrBDNF in Trk-B CHO Assay, red line is the response ratio of a sample containing no hrBDNF

b) The composite green/blue image of Trk-B CHO cells exposed to 0.1ng/ml of hrBDNF

c) The composite green/blue image of Trk-B CHO cells exposed to 10ng/ml of hrBDNF

The statistical analysis is interpolation with third order polynomial cubic, error bars are ±SEM.
4.5.3. Linear detection limit of rBDNF in Trk-B CHO assay

It is important to find the linear detection range for hrBDNF in the Trk-B CHO Assay so as to accurately measure the amount of biologically active material in a sample. It appears that the linear detection limit of the assay is between 1 and 10 ng/ml of hrBDNF. Although the response ratio can be altered by incubation time with hrBDNF, rarely was it needed to exceed the linear portion of the assay.

Figure 4.9 Linear detection of hrBDNF in the Trk-B CHO assay
The linear detection of hrBDNF in Trk-B CHO assay was 1-10ng. Statistical analysis is an interpolation with third order polynomial cubic, error bars are ±SEM
4.5.4. Culture plate effect on detection limit of the Trk-B CHO assay

This protocol was adapted from (SinoBiological) from a 384 well-plate to a 96 well-plate. Using the surface area of the wells, the number of cells was increased proportionally for the assay. However, a lower density of cell seeding was also used to see if it affected the response ratio produced, as the company suggest that if the cells are over grown it can reduce their sensitivity to BDNF. **Figure 4.10** shows a standard curve for Trk-B CHO assay for cells seeded at both 30,000 cells/well and 20,000 cells/well. There were no significant differences in limit of detection. Subsequent experiments used a seeding density of 30,000 cells/well.

![Graph showing response ratio vs. ng/ml of hrBDNF for 30,000 and 20,000 cells/well.]

**Figure 4.10** Comparison of seeding density and its effect on the response ratio of the Trk-B CHO assay

Trk-B Assay was run with a range of hrBDNF, but with different initial seeding density at -16 hours.
4.5.5. Detection of hrBDNF detected by ELISA and biological activity correlate

To determine whether the concentration of hrBDNF and the biological activity were correlated the same concentration of hrBDNF was used in the ELISA and Trk-B CHO assay in parallel. Figure 4.11 shows the correlation between BDNF concentration and biological activity of hrBDNF between 0-15 ng/ml. This shows that biological activity is directly linked to concentration determined by ELISA.

Figure 4.11 Comparison of hrBDNF detection in the BDNF ELISA and Trk-B CHO assays
The hrBDNF was made to known concentration ranging from 0-15ng/ml and run in ELISA and Trk-B assay at the same time. Error bars are ±SEM
4.5.6. Trk-B CHO cell assay in 3D model

The Trk-B assay was effective at quantifying the amount of biologically active hrBDNF and BDNF-H9C in suspension. To confirm this method for calculating BDNF activation in the 3D model for AuNPs crossing the BBB, we planned to use a monolayer of hCMEC/D3 cells and a collagen matrix seeded with Trk-B CHO cells. This was developed while the production of BDNF-H9C was being optimized. Figure 4.12 shows a schematic of this 3D model which includes Trk-B CHO cells grown in a collagen-based hydrogel with a monolayer of hCMEC/D3 seeded on top.

Figure 4.12 Proposed 3D assay to test the ability of a nanocarrier to transfer across hCMEC/D3 monolayer and activate Trk-B

Trk-B cells are to be seeded into a collagen-based hydrogel which could support them, and after which hCMEC/D3 cell are seeded on top and grown to produce a monolayer with good BBB properties. Once the BBB properties have been established the nanocarrier’s ability to transfer across the hCMEC/D3 layer can be tested with immunofluorescence for Trk-B activity and ICP-Q-MS to quantify gold.
4.5.6.1. Trk-B CHO cells growth in Collagen Hydrogel

Initial experiments were done to demonstrate that Trk-B cells could survive in collagen-based hydrogels. Two different seeding densities were used to see if cell number plays a role in survival in a collagen-gel as cell-cell interactions play a large role in cell survival and signalling. Figure 4.13 shows cell proliferation of a high (5,000,000 cells/ml) and low (2,500,000 cells/ml) seeding density over 10 days. Cell number was normalised to percentage of seeded with 100% referring to the number initially seeded. Time point 0 refers to the time after the gel solidified. To count the cells the collagen gel was digested, and the cells counted in a haemocytometer.

![Figure 4.13 Percentage survival of Trk-B CHO cells compare to initial seeding in a collagen-based hydrogel over 9 days](image)

Two different seeding densities of 2.5 and 5.0 x10^6 cell/ml of gel were tested to see their effect on the ability of Trk-B CHO cells to survive in a collagen-based gel. After the gel solidified it was digested and cells were counted to discover the percentage of initial seeding cells lost in culture. 2-way ANOVA found no significant difference between column factor i.e. initial seeding density (P value= 0.1436)
4.5.6.2. Trk-B CHO assay activity in collagen hydrogel

After it was found that the Trk-B CHO cells could survive in the collagen gel, it was investigated if they could be activated and measured in a similar manner to the monolayer assay. Trk-B CHO cells were seeded either dispersed in the collagen gel or in a monolayer, beneath the collagen gel or alone in media and exposed to the same concentration of rBDNF. **Figure 4.14a** shows a schematic of the different Trk-B CHO seeding conditions. Fluorescence was measured by a plate reader to see if they were activated and confirmed by fluorescence microscopy.

**Figure 4.14b** shows that cells grown with the collagen gel - whether dispersed within it or underneath the gel, showed no fluorescence response after treatment with hrBDNF indicating Trk-B CHO cells do not respond to hrBDNF in the same way as when grown in media or the gel was hindering in some the way the ability to measure the response produced. Since Trk-B activation cannot be measured directly in the 3D model, the 3D model was completed without the integrated Trk-B assay and the BDNF-H9C conjugated AuNP would be captured from the post hCMEC/D3 portion of the 3D culture. This represents the amount of AuNP that have crossed the BBB model. The AuNP from this section would be isolated and added directly to the Trk-B CHO activity assay as performed in **Figure 4.7**.
Figure 4.14 The response ratio of hrBDNF to Trk-B CHO cells in/under/without collagen gel

Trk-B CHO cells were grown either in, under or without the collagen-based hydrogel, they were then exposed to Trk-B CHO assay reagents and run on a plate reader with the relative fluorescence wavelengths. Error bars are ±SEM.
4.5.7. Transfection design for production of BDNF-H9C

Following the design of assays to detect quantity and biological activity of BDNF, a larger quantity of the BDNF-H9C protein was needed to attach to the AuNPs.

4.5.7.1. Mock transfection vs rBDNF plasmid

Firstly, using the original hrBDNF-H10 plasmid COS-7 cells were transfected using lipofectamine 2000 and hrBDNF levels were compared to a mock transfected cell supernatant. The hrBDNF plasmid transfected cells were able to produce hrBDNF, but only in small quantities, between 3-7 ng/mL in cell culture media.

![Graph showing comparison of hrBDNF produced in the supernatant of either mock transfection or control hrBDNF transfected COS-7 cells. Using 3 separate clones of the control plasmid. Error bars are ±SEM.](Image)
4.5.8. **BDNF-H9C protein is produced at lower quantities than hrBDNF**

The concentration of hrBDNF was then compared to the hrBDNF-H9C. Again, COS-7 cells were transfected using lipofectamine 2000 and compared it to a mock transfected cell supernatant. The hrBDNF-H9C was produced in even lower quantities than hrBDNF with around 2 ng/mL per mL of cell culture medium (Figure 4.16).

![Figure 4.16 Comparison of BDNF produced (tested by ELISA) in the supernatant of either control hrBDNF plasmid or BDNF-H9C plasmid transfection of COS-7 cells Using 3 technical replicates of supernatant collected from 3 separate transfections of 3 separate clones of each plasmid. Error bars are ±SEM](image-url)
4.5.9. Transfection optimization

Every mammalian cell type has a characteristic set of requirements for optimal introduction of foreign DNA; there is a tremendous degree of variability in the transfection that work even among cell types that are very similar to one another (Rose, 2003). To increase the amount of hrBDNF-H9C being produced the transfection protocol was optimised.

There are a number of different methods of transfection which will yield different transfection efficiencies in different cell types including calcium phosphate, DEAE-dextrin, electroporation and liposome-mediated transfection.
4.5.9.1. Cell type affects transfection efficiency

When producing protein there are a number of options possible when deciding which cell type to use. While bacterial and insect cell lines are useful there can be differences in the post translational modifications which are needed for some proteins. BDNF has a delicate 3D structure with post translational modifications in its synthesis (for example the proBDNF 32-kDa precursor is N-glycosylated and glycosulfated on a site, within the pro-domain) so a mammalian cell line was used for production (Mowla et al., 2001). Transfection was tested in three mammalian cell lines, Cos-7, CHO-K1 and HEK293. These mammalian cells have all been used to produce human protein to variable degrees of success (Lalonde and Durocher, 2017). Figure 4.17 shows the concentration of BDNF-H9C per mL of culture media in the 3 different cells lines after 48, 72 and 96 h post transfection. CHO-K1 cell line produced significantly more than the others at 48, 72 and 96 hours post transfection. From hence forth all transfection and production of BDNF-H9C was on CHO-K1 cells.

![Figure 4.17 Concentration of BDNF-H9C produced 48, 72 and 96 hours post transfection using CHO-K1, Cos-7 and HEK293 cell type](image)

At 48 hours post transfection, transfected CHO-K1 cells produced significantly more BDNF-H9C than the Cos-7 line (P =0.0091). At 72 hours post transfection, transfected CHO-K1 cells produced significantly more BDNF-H9C than the Cos-7 and the HEK293 cell lines (P <0.001 for both). At 96 hours post transfection, transfected CHO-K1 cell produced significantly more BDNF-H9C than the Cos-7 line (P <0.0001) and the HEK293 line (P =0.0004). The Hek293 cell line also produced significantly more than the COS-7 cell line (P = 0.0250). Using 3 technical replicates of supernatant collected from 3 separate transfections of 3 separate clones. Error bar is ±SEM P values calculated using paired t-tests.
4.5.9.2. Transfection reagent

There are numerous transfection reagents available with different modes of action. Lipofectamine is a cationic lipid reagent that has been used frequently since the late 90’s and JetPEI is a linear polyethlenimine derivative. Using CHO-K1 cells and a fixed amount of plasmid DNA, seeding density and time point of harvest (72hrs). Figure 4.18 shows that lipofectamine used at 1 µl/well had the greatest production of BDNF-H9C.

![Figure 4.18](image_url)

**Figure 4.18** Comparison of transfection reagents on BDNF-H9C production. CHO-K1 cells were seeded at 100,000 cells/well after 12 hrs they were exposed to either 1, 2, 4µ/well of either lipofectamine2000 or JetPEI and media was harvested 72hr after being transfected. Error bar is ±SEM
4.5.9.3. Transfection reagent concentration

To further optimise the use of Lipofectamine 2000, using a fixed seeding density (100,000 cells/well) the concentration of Lipofectamine 2000 and time point post transfection were investigated. Results showed no significant difference between the concentrations of lipofectamine 2000 used at the same time point.

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![Graph showing comparison of concentration of lipofectamine 2000 and time point of harvesting media on BDNF-H9C found in the supernatant. CHO-K1 cells were seeded at 100,000 cells/well, transfected with 1, 2, 4ul of lipofectamine 2000 and media was harvested from 24-120hours post transfection. There was no significant difference related to the concentrations of lipofectamine used. Error bar is ±SEM.](image-url)
4.5.9.4. **cDNA concentration**

Another impactor on the ability to successfully transfect and therefore produce BDNF-H9C is the cDNA concentration when transfecting the cells. CHO-K1 cells were seeded at 100,000 cells/well and media was harvested from 24-120 hours post transfection and exposed to either 0.5, 1, 2μg/well of cDNA with a fixed amount of lipofectamine 2000 (4μl/well). There was no significant difference between concentrations of cDNA used.
4.5.9.5. Seeding density

From the previous experiments we have tested a selection of different seeding densities before transfection. However, to fully understand the ideal transfection parameters, it was investigated how different seeding densities with a fixed concentration of cDNA and transfection reagents affected the production of BDNF-H9C. Figure 4.20 shows the BDNF-H9C production over 6 days at varying cell densities. There was no significant difference in BDNF-H9C produced by the different seeding densities except for day 5 and 6, at 50,000 and 100,000 cells/well which were significantly higher than 25,000 and 200,000 cells/well. Cells that are too confluent when seeding and are in the stationary phase at time of transfection, i.e. not actively dividing, take up foreign nucleic acid to a lesser extent than dividing cells. On the other hand too few cells can results in poor growth after transfection due to lack of cell to cell contact. (Peng et al., 2017; Thermofisher, 2019c)

**Figure 4.20 Transfection optimization: Seeding density**
Comparison of the seeding of cells in a 12-well plate before transfecting and the subsequent BDNF-H9C production over the following 6 days there was no significant difference produced by the seeding density until at day 5, 50,000 and 100,000 is significantly higher than 25,000 and 200,000 (P = 0.0075) and at day 6, 50,000 and 100,000 is significantly higher than 25,000 and 200,000 (P = 0.0405) using unpaired t-test. Error bars =±SEM
4.5.9.6. **Time point of media harvest**

Based on the work described above, an initial seeding of 100,000 cell/well, 1 µg/well plasmid DNA and 4 µl/well Lipofectamine to transfect CHO-K1 cells, was chosen and the media was harvested daily. **Figure 4.21** shows that after day 5 the concentration of BDNF-H9C plateaux at 10 ng/mL in cell culture media, this is likely due to over confluent cells and limit of nutrients in the media.

![Graph showing BDNF-H9C production over days after transfection](image)

**Figure 4.21 Comparison of time points of media harvest**

Cells were seeded at optimum transfection conditions to produce BDNF-H9C and media was harvested once per sample of cells, at the given day after transfection. Statistical analysis shows third order polynomial curve errors and 95% confidence intervals, red line is when more than 50% of cell were dead/dying determined by Trypan blue staining. Error bars =±SEM
4.5.9.7. Media

CHO-k1 are a robust cell line and can be grown in a number of different media with successful growth. CD CHO media is an engineered media for the use of CHO cells for expression of recombinant proteins in suspension culture (Thermofisher, 2019d). Ham’s F-12 with +2 mM Glutamine + 10% FBS is a recommended media (Thermofisher, 2019d). CHO-K1 cell and their derivatives lack an enzyme required for proline synthesis, and therefore require addition of 0.1-0.2 mM proline into their media which is already included in Ham’s F-12 and RPMI-1640. RPMI-1640 was shown to give higher Insulin like growth factor-1 (IGF-1) protein levels as compared to Ham’s F12 medium in a CHO-k1 expression system (Mohmad-Saberi et al., 2013).

Figure 4.22 Comparison of how media affected the production of BDNF-H9C against time point of harvest.
Cells were seeded at optimum transfection conditions to produce BDNF-H9C and media was harvested once per sample of cells, at the given day after transfection. Error bars =±SEM
4.5.9.8. Antibiotics

Antibiotics are known to impact the ability of cells to be transfected and thereafter affect the ability of the cell to produce recombinant protein. The antibiotics penicillin and streptomycin prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria (Ryu et al., 2017) However, antibiotics can stress cells and limit the amount of protein produced. To test the effect of antibiotics on BDNF-H9C production, cells were transfected and then fed with either antibiotic containing or antibiotic free media. Fig 4.23 shows the production of BDNF-H9C over 4-6 days post transfection. There was no significant different between using antibiotics in the production of BDNF-H9C.

Figure 4.23 Effect of antibiotics on the production of BDNF-H9C
CHO-K1 cells were transfected at their optimal conditions and RPMI media with or without penicillin and streptomycin was used to feed the cells. Media was harvested 4, 5- and 6-days post transfection to be tested for BDNF-H9C content. Two Way ANOVA showed no significant difference between +/- Antibiotics.
4.5.10. Optimal conditions for transient transfection

From Figure 4.17 to Figure 4.23 The optimal condition for transient transfection in a 12 well culture plate is

- Cell type: CHO-K1 cells
- Transfection reagent: Lipofectamine 2000
- Concentration of Lipofectamine 2000: 1μl/well is sufficient
- cDNA concentration: 0.5μg/well
- Seeding density: 50,000 cells/well
- Harvest time point: 5 days
- Culture media: RPMI-1640
- Use of Antibiotic: Yes
4.6. Stable transfection of BDNF-H9C producing CHO-K1 cells

Although optimization of transfection has increased the yield of BDNF-H9C it had not reached a level which would be useful for the ligand exchange reaction needed to effectively coat the AuNP. To overcome this a stable cell line was produced containing the BDNF-H9C plasmid. A stable transfection is one in which plasmid DNA is integrated into the genome of the host cell.

4.6.1. Transient vs stable transfection

Transient transfection for the production of protein is a commonly used process; and can yield useful quantities for experimental purposes. However, by passing the cell after transfection the production is usually stopped or halted. Figure 4.24 shows that by passage 5, CHO-K1 cells stopped producing BDNF-H9C. Therefore, a transient transfection would not be an effective way to produce BDNF-H9C.

![Figure 4.24 Comparison of concentration of BDNF-H9C produced after each passage with a transient transfection protocol](image)

CHO-K1 cells were transfected at their optimal conditions and passaged 1:4 after reaching confluency and seeded into a fresh plate. Error bars =±SEM
4.6.2. BDNF-H9C levels affected by Hygromycin B selection

To produce a stable transfection, the plasmid contains a hygromycin resistance gene that allows for hygromycin selection of transfected cells. The recommended concentration of hygromycin B for antibiotic selection for CHO-K1 cells is 300-600µg/ml (Popp et al., 2018). To determine if this is ideal, cells which have been previously transfected were exposed to different concentrations of Hygromycin B. Figure 4.25 shows that CHO-K1 producing BDNF-H9C after 3 days post selection decreased the BDNF-H9C produced and then 12 days post selection the 500 and 800 µg/ml exposed cells were back to producing larger quantities of BDNF-H9C.

Figure 4.25 Comparison of concentration of selection antibiotic used to produce a stable transfection and production of BDNF-H9C

Before selection there was no significant difference between the growing cells, 3 days post selection (addition of hygromycin containing media) and 12 days post selection and the samples from cell with high level of hygromycin (800 and 500) were significantly higher than the low level of hygromycin. Statistical analysis showed there was a significant difference between Hygromycin concentration and BDNF-H9C produced by two-way ANOVA and then an unpaired t-test to compare the concentrations from each time point. Error bars ±SEM
4.6.3. Time point of harvesting media from CHO-K1-BDNF-H9C

After CHO-K1 cells producing BDNF-H9C had been selected with Hygromycin B, it was critical to find the ideal time point to harvest media from these cells. After reaching confluency media was harvested and tested for BDNF-H9C by BDNF ELISA. **Figure 4.26** shows that day 7 had the highest quantity of BDNF-H9C, however there was increased cell debris and decrease in the number of healthy cells observed. After media harvesting the cells were trypsinized spun down and resuspended in fresh media and were grown successfully again.

![Figure 4.26 Time point of harvest of media from CHO-K1-BDNF-H9C](image)

*Harvesting media from confluent transfected and selected CHO-K1 cells that were adapted to producing BDNF-H9C. Error bars =±SEM*
4.6.4. CHO-K1-BDNF-H9C adaptation to suspension

Even with a stable transfection which can produce BDNF-H9C after multiple passages, the level of production was still low. Another option to increase protein production is to grow cells as a suspension culture. If they can grow as a suspension cell-line then we could increase the number of cells per ml of media used and then use a bioreactor, which could increase yield dramatically.

Adaptation to suspension was tried three different times, and although the cells were able to survive in suspension they stopped producing BDNF-H9C.

4.6.5. Production of BDNF-H9C in a monoclonal population of our producing cell.

The stably transfected cells used to produce BDNF-H9C comes from a mixed population of cells. To increase production, a high producing monoclonal cell line was investigated. Cells were isolated into a 96 well plate. Wells with the highest production of BDNF-H9C were passaged into larger wells and eventually flasks and compared to the mixed or polyclonal population. There was no significant difference between these two populations.
4.6.6. Optimal conditions for stable transfection

From Figure 4.17 to 4.26 the optimal conditions for stable transfection in a 12 well culture plate is

- Hygromycin selection concentration: 500µg/ml
- Harvest point after reach confluency: 7 days
- Suspension or adherent adaptation: adherent
- Monoclonal selection increases production: No

However, even after these trials the highest level of BDNF-H9C produced was < 20 ng/mL in the cell culture media. The initial aim was to produce approx. 100µg of BDNF-H9C for use in exchange reaction onto the surface of the AuNP, and further studies *in vitro*. At the efficiency we managed to produce it would require 10 litres of harvested supernatant media from our cell line, which was produced, concentrated and purified as follows.
4.7. Purification of BDNF-H9C

To purify BDNF-H9C, the histidine tag was used for nickel column purification. Briefly, harvested media samples were collected and centrifuged to remove cell debris. The media was circulated through a nickel column for a minimum of 5 cycles at 4 °C. Contaminants were washed from the column using a low concentration of imidazole and finally the BDNF-H9C was eluted using a high level of imidazole. The elution solution was dialysed into a storage buffer using a dialysis membrane with a 10kDa Molecular weight cut off and the sample concentrated by centrifugation to 0.5 mL in HBSS.

4.7.1. Purification steps

To purify the BDNF-H9C we used Nickel columns, dialysis and centrifugation concentrators. The BDNF-H9C has 9x Histidine amino acids at its C-terminus which aid in the purification from supernatant media and only 6x Histidines are needed for a protein to adhere to a Nickel column. Supernatants from the producing cells were stored at 4°C, spun at 18,900 RCF then the samples were passed through a 0.2µM Nalgene filter to remove any cell debris. Samples were run through a Nickel Column at 5ml/min for 24hrs, after which protein was eluted using an 250mM Imidazole soln with 5x column volume (5mls) per 500mls of supernatant media used.

The concentration process retained >90% of produced BDNF-H9C. Figure 4.27 was a pilot study of the process (using 6ml of the original supernatant sample media (which had a total of 42ng of BDNF-H9C) and producing approx. 2.3ml of a concentrated elution solution (total 31.2ng of BDNF-H9C), 1ml of each of the 3 elutions (each elution was 1 column volume, i.e 5ml) was removed for quantification (total 7.8ng of BDNF-H9C was not in the final concentrated elution solution) therefore 39/42 ng total equal 92.8% yield.
Figure 4.27 Comparison of BDNF-H9C concentration during different stages of the elution from the nickel column
Concentration of BDNF-H9C detected in different stages of the elution from the nickel column. Error bars =±SEM
4.7.2. Western Blot

The concentrated, eluted BDNF-H9C sample was treated with an equal volume of 2x reduced SDS sample buffer and run on a 10% SDS PAGE gel and blotted onto nitrocellulose. Total protein was visualised with Sypro-Ruby; Western blotting with ECL was used to identify BDNF bands.

**Figure 4.28**, Sypro-Ruby showed that most of the protein in the eluted BDNF-H9C sample was a protein of approximately 70kDa which is probably albumin from the FBS. The Western blot showed a single band of 13.5 kDa in rBDNF, and two major bands in the eluted BDNF-H9C sample of 13.5kDa and 14.5kDa. (the expected molecular weight for BDNF-H9C). The two bands around 25KDa would appear to correspond to truncated forms of proBDNF (Mowla et al 2001). **Figure 4.28** demonstrates that the sample was contaminated with other proteins, most likely albumin and was not sufficiently pure or in high enough concentration to be used in the exchange reaction.

Our engineered plasmid was sent to a specialist company (Icosagen®) which have a history of producing BDNF from CHO-k1 cells, in the hope they could produce BDNF-H9C in significant quantities for future experiments. However, they were unable to produce high enough quantities to be usable.

This confirms that there wasn’t a technical error with the production and rather something related to the expression of the plasmid in these cells.
Figure 4.28 Sypro-Ruby and ECL western blot of rBDNF and BDNF-H9C
4.8. hrBDNF vs BDNF-H9C in Trk-B CHO assay

Using samples harvested from producing cells, and mock transfection cell media as the negative control, BDNF-H9C was examined both by BDNF ELISA and Trk-B CHO assay and compared to relative levels of hrBDNF. The BDNF-H9C samples followed a linear level up to 12.5ng/ml of sample. Figure 4.29 shows that the BDNF-H9C being produced binds to the BDNF ELISA and produced a similar response in biological activity to the hrBDNF.

Figure 4.29 Comparison of BDNF-H9C samples in BDNF ELISA vs Trk-B CHO
Error bars =±SEM
4.9. Conclusion

BDNF is reported as a possible therapeutic cytokine which could be used to treat numerous neurodegenerative disorders however, it does not cross the BBB in significant quantities by itself. The aim of this chapter was to produce a modified version of BDNF (BDNF-H9C) that was biologically active and in sufficient quantities to attach onto AuNPs.

In this chapter I found that I was able to produce a mutagenically altered plasmid for BDNF-H9C and optimized the transfection and production of a stable cell line to produce BDNF-H9C protein. This BDNF-H9C was biologically active but was produced in insufficient quantities and purity to be used in exchange reaction for attachment onto AuNPs.

Chapter 6 will discuss alternative ways to produce sufficient pure functional protein to attach to the nanoparticles including BDNF mimetic peptides.
5. Chapter 5: Targeting the Brain Endothelium

5.1. Introduction

The Transferrin receptor (TfR) is highly expressed on the brain endothelial cells and, due to these cells containing efflux pumps and other mechanisms which inhibit the crossing of therapeutic agents, TfR has been increasingly used as a target for crossing the BBB. However there has been the ‘unfulfilled promise’ of antibodies against transferrin receptor such as the OX26 antibody, which was shown to bind to the capillaries in rat brains but not the vasculature of other organs. It was however shown to have a maximum of 0.44% of the injected dose of OX26 detectable in brains with capillaries removed (i.e antibody that had crossed the BBB and entered the brain parenchyma) (Paterson and Webster, 2016). Interestingly, Yu et al have shown that the affinity of the antibody was affecting its ability to transverse the BBB, they investigated a panel of low affinity antibodies at a therapeutic level and showed that the level of antibody detectable in the brain after 24 hrs was inversely correlated with the antibody’s affinity for the transferrin receptor. The higher affinity antibodies seemed to be retained in the capillaries, not being released on the abluminal side and ending up in lysosomes (Yu et al., 2011).
5.1.1. Peptides for TfR targeting

Peptides as targeting molecules have numerous advantages including being relatively easy and cheap to produce, small size, flexible (regarding their sequence and conjugation possibilities), and can be highly specific (Le Joncour and Laakkonen, 2017). Peptides compared to antibodies benefit from low-immunogenicity, fast blood clearance, higher levels of diffusion through tissues and excellent tolerance in patients. The relatively short half-life of peptides can be negated by the presence of a cyclical structure with a cysteine bond as well as blocking of the C- and/or N- terminus by attachment to another structure such as a nano-carrier (Le Joncour and Laakkonen, 2017). The addition of peg-amine on quantum dots coated with targeting peptides decreased the amount of non-specific uptake by the liver and spleen but also did not increase the uptake in the target tissue, suggesting that the receptor mediated targeting may have been compromised by the other ligand. (Akerman et al., 2002).

Ideally, targeting of the TfR with a peptide attached to a nanocarrier would not directly compete with Tf as this could lead to decreased targeting efficiency as well as possible negative physiological consequences. From the literature as well as work from a previous student in our group (Mo Daas) we compiled a list of peptides which have been previously shown to bind to TfR (Table 5.1).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Source</th>
<th>Structure</th>
<th>Isolation</th>
<th>Attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep-1</td>
<td>FITC-GAWSIDCSMNYCLYIEG (18)</td>
<td>Mo Daas Thesis (Medimmune)</td>
<td>Cyclic</td>
<td>TFR protein</td>
<td>Pegamine/peptide</td>
</tr>
<tr>
<td>Pep-2</td>
<td>FITC-GAIHHPQGDSVSCWR (18)</td>
<td>&quot; &quot; &quot;</td>
<td>Cyclic</td>
<td>Control</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Pep-8</td>
<td>FITC-GASYIVTRWVGCMY (18)</td>
<td>&quot; &quot; &quot;</td>
<td>Cyclic</td>
<td>TFR protein</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Pep-9</td>
<td>FITC-GAARDLLETWYGFTCWNV (18)</td>
<td>&quot; &quot; &quot;</td>
<td>Cyclic</td>
<td>TFR protein</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Pep-10</td>
<td>FITC-GALHECYYWVGDDCF (18)</td>
<td>&quot; &quot; &quot;</td>
<td>Cyclic</td>
<td>TFR protein</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Pep-R1</td>
<td>FITC-GACENWWGDYTCGAGAG (16)</td>
<td>Ruoslahti et al. Nature 1996</td>
<td>Cyclic</td>
<td>In vivo phage</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Pep-R2</td>
<td>FITC-GACLLSSRLDACGAGAG (16)</td>
<td>&quot; &quot; &quot;</td>
<td>Cyclic</td>
<td>In vivo phage</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>Pep-L</td>
<td>THRPPMWSPWVPPCK-FITC (14)</td>
<td>Lee et al. Eur. J.Biochem 2001</td>
<td>Linear (1 internal C)</td>
<td>TFR in vitro</td>
<td>Galactose/-SS-</td>
</tr>
</tbody>
</table>

Table 5.1. Table of transferrin receptor targeting peptides with their sequence, source, basic structure, isolation and possible attachment type onto AuNPs.

Purple letters are the amino acids associated with targeting from the literature. Green C is the cysteine of Pep-L used to attach onto AuNPs via exchange reaction. Red C's are the cysteines which bind together to form the cyclic structure. () number inside bracket is number of amino acids in peptide chain. Underlined amino acids form the loop.
5.1.1.1. Pep-1 to -10

Peptides -1 to -10 were identified during Mo Daas’ research project and have a cyclic structure, isolated by binding onto TfR (Figure 5.1a). The cyclical structure of Pep-1 to 10 is formed by the disulphide bridge created from the binding of the free thiols in the cysteines in their sequence (Figure 5.1b). This bond is critical for the 3D structure of these peptides and therefore critical for their binding to human TfR. These bound cysteines do limit the possible mechanism for attachment onto the surface of the AuNP and it requires either an electrostatic interaction (non-covalent) or a covalent interaction which leaves the TFR-binding loop unaltered and exposed.

Figure 5.1 Absorbance produced by binding of Daas peptide sequences to Mouse, Rat and Human TfR against a control Antigen.
(a) Binding of peptides to the transferrin receptor of mouse, rat and human cerebral microvascular endothelial cells by means of a phage ELISA screening of peptides already flagged as “hits” from previous screens. Pep-1 and Pep-2 were highlighted for future experiments. Pep-1 and pep-10 were highlighted as being the most promising to continue study. (b) Schematic of one of the cyclic peptide structures including the disulphide bond formed by the cysteines and the attached FITC used for visualization in binding experiments. Figure adapted from Daas et al. unpublished.
5.1.1.2. Pep-R1 and R2

In 1996 Pasqualini & Ruoslahti injected phage libraries intravenously into mice and subsequently rescued the phages from individual organs, and were able to recover the bound phages from the brain (Pasqualini and Ruoslahti, 1996). When tested as isolated phages (R1-CLSSRLDAC, R2-CENWWGDVC and R3-WRCVLREGPAGGCWAFNRHRL) each targeted the brain several fold more effectively than the kidney (Pasqualini and Ruoslahti, 1996; Arap, Pasqualini and Ruoslahti, 2014).
5.1.1. Pep-L

Lee et al used a biopanning process which isolated phages that bound to and internalized with human transferrin receptor. These phages contained the sequence THRPMWSPVWP (T12) and was able to bind in a dose dependent manner, while not competing with transferrin (Lee et al., 2001). We modified the T12 sequence and developed Pep-L, a 14 amino acid peptide sequence, which uses T12 as its targeting sequence (THRPMWSPVWP) with an attached cysteine for ligand exchange onto AuNPs using its free thiol and a lysine as a spacer and attachment for FITC, or a serine, in the non-FITC version (Figure 5.2).

Figure 5.2 Schematic of Pep-L sequence with and without FITC attachment. Pep-L has been synthesised with a free Cysteine which can be used in the thiol exchange reaction for attachment onto AuNP, and either a Lysine and FITC molecule for ease of identification or Serine.
5.2. Aim of the Chapter

1. Identification of the peptide sequence which binds preferentially to hCMEC/D3 cells.

2. Identify optimal conditions for binding to hCMEC/D3 cells.

3. Identify optimal conditions for attachment of chosen peptide onto AuNP.

4. Does addition of the chosen peptide onto AuNP increase binding and/or transfer across the BBB model?
5.3. Results

5.3.1. Comparison of fluorescence of peptides with FITC against concentration

The relationship between peptide concentration and fluorescence was determined to ensure that the fluorescence readout from the following FACS experiments allowed for interpretation of relative levels of uptake. To calculate this, each peptide was loaded in triplicate at varying dilution across a range from 10,000ng/ml to 1ng/ml into a 96-well plate and run through a fluorimeter (Figure 5.3). The data was then used to standardize the concentrations of each peptide required for equal fluorescence with the FITC-dextran. Control, used at a concentration of 24.8µg/ml, as shown in Table 5.2.

![Fluorescence units vs. peptide concentration](image)

**Table 5.2 Comparison of peptide fluorescence units and the calculated concentration used in future experiments.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fluorescence Units</th>
<th>Fl Peptide/Fl Dextran</th>
<th>Concentration used (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep-R1</td>
<td>40723</td>
<td>1.42</td>
<td>10.0</td>
</tr>
<tr>
<td>Pep-R2</td>
<td>71265</td>
<td>2.48</td>
<td>5.7</td>
</tr>
<tr>
<td>Pep-1</td>
<td>24026</td>
<td>0.84</td>
<td>16.8</td>
</tr>
<tr>
<td>Pep-2</td>
<td>33800</td>
<td>1.18</td>
<td>12.0</td>
</tr>
<tr>
<td>Pep-10</td>
<td>14497</td>
<td>0.5</td>
<td>28.1</td>
</tr>
<tr>
<td>Pep-L</td>
<td>19221</td>
<td>0.67</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Figure 5.3 Comparison of peptide concentration and their fluorescence.
The fluorescence units measured for each peptide at varying concentrations from 10,000ng/ml to 1ng/ml in a 96-well plate run in triplicate in two repeat experiments. Error bars are ±SEM.

Table 5.2 Comparison of peptide fluorescence units and the calculated concentration used in future experiments.
The fluorescence units measured each peptide at a concentration of 1µg/ml were determined and a ratio of relative fluorescence to FITC-dextran at the same concentration was calculated. From this, the relative concentrations of each peptide with equal fluorescence were then calculated when FITC-dextran was used at 24.8 µg/ml.
5.3.2. Time course of peptide uptake on hCMEC/D3 cells

Before relative uptake could be compared across all peptides, the time course of uptake was determined to ensure that the 3-hour incubation time suggested for the uptake experiments was adequate. hCMEC/D3 cells were incubated with Pep-R1, -R2, -1, -2, -10 and -L from 0hrs to 3hrs before harvest for FACS. The time course of peptide uptake is shown in Figure 5.4. It demonstrates progressive uptake over the entire period.

Figure 5.4 Comparison of peptide binding compared to exposure time.
The median fluorescence detected in hCMEC/D3s analysed by FACS when incubated with each peptide for defined lengths of time. Error bars are ±SEM but are too small to be seen.
5.3.3. Comparison of peptides binding to hCMEC/D3 cells

To determine the level of peptide uptake in hCMEC/D3s, cultures were incubated for 3 hours with each of the FITC-labelled peptides before the cells were washed, harvested and analysed through flow cytometry. FITC-labelled dextran was used as positive control to demonstrate the basal rate of endocytosis of the cells. Results obtained from the FACS experiments using all 6 peptides plus FITC-labelled dextran demonstrated that peptides -1, -10 and -L resulted in a significantly higher level of uptake in hCMEC/D3 compared to FITC-dextran (Figure 5.5).

![Figure 5.5 Comparison of Peptide binding to hCMEC/D3 cells.](image)

The median fluorescence detected in hCMEC/D3 cells analysed through FACS when incubated with each peptide for 3 hours. (**** = p<0.0001,* = p<0.05. n=3). Data shown is the mean of the median fluorescence of the 3 biological replicates (with at least duplicate technical replicates of each biological replicate). Values were compared to the mean of the median fluorescence of FITC-dextran. Error bars are ±SEM.

From this information it appears that Pep-L was able to bind more efficiently than the other peptides, and so the following experiments used a version of the Pep-L peptide.
5.3.4. Inhibition of Pep-L binding to hCMEC/D3 cells by FBS/BSA/Transferrin

Initial binding experiments with peptides used HBSS to dilute the peptides while exposed to the hCMEC/D3 cells. It was thought necessary to investigate whether transferrin or other serum proteins could affect the ability of Pep-L to bind to hCMEC/D3 cells. A range of concentrations of FBS/BSA/Transferrin which reflect their blood and media concentrations were used (Table 5.3).

<table>
<thead>
<tr>
<th></th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS (%)</td>
<td>20</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>BSA (mg/ml)</td>
<td>8</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Transferrin (mg/ml)</td>
<td>1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 5.3 Concentration of FBS, BSA and Transferrin used in the inhibition assay.

Figure 5.6 Inhibition of Pep-L binding to hCMEC/D3 cells by FBS, BSA and Transferrin. (a) Effect of FBS concentration on Pep-L binding to hCMEC/D3 cells. (b) Effect of BSA concentration on Pep-L binding to hCMEC/D3 cells. (c) Effect of Transferrin concentration on Pep-L binding to hCMEC/D3 cells. Error bars are ±SEM. * P<0.05; ** P<0.01; P<0.001 using an unpaired t-test.
FBS, BSA and Transferrin were all able to affect the ability of Pep-L to bind to hCMEC/D3 cells to some degree (Figure 5.6).

The BSA solution is a highly pure suspension of albumin, which is commonly used as a blocking agent, the BSA could be non-specifically binding to some of the TfR, thereby blocking the site at which Pep-L binds or it could bind the free peptide resulting in less available peptide for TfR binding. BSA contains a number of disulphide bridges which could interact with the free cysteine in the Pep-L, and/or BSA is known to have strong interactions with anionic molecules and the FITC attached to the Pep-L is negatively charged in these conditions (Yin et al., 2015). This may explain the inhibition of Pep-L binding caused by pure BSA.

The failure of high levels of transferrin to block Pep-L binding to the cells, implies that Pep-L does not bind to the same part of the receptor as transferrin does, i.e. transferrin does not compete with Pep-L for binding to the receptor (Mu et al., 2017). Theoretically transferrin in the media, may lead to an increase in the number of TfR, allowing more Pep-L to bind, this may reach a saturation limit, which could explain the apparent levelling off between 0.25 and 1mg/ml

FBS is a complex mixture of proteins, growth factors and molecules including albumin and transferrin, the increase in binding we have shown with increase in FBS concentration could be due to a combination of the factors seen with BSA and transferrin separately. Alternatively growth factors and nutrients in FBS can increase cellular growth leading to upregulation of the TfR.

To examine what impact BSA, transferrin and FBS was having on the binding of Pep-L, the next experiment was to compare their effect on the number of TfRs on the cell surface of the hCMEC/D3 cells (Figure 5.7).
5.3.5. Altering TfR on hCMEC/D3 cells

Figure 5.6 shows FBS, BSA and transferrin can reduce the binding of Pep-L to hCEMC/D3 cells. Using a cell surface ELISA protocol, we exposed confluent hCMEC/D3 cells to a large range of FBS, BSA and transferrin concentrations for 24hrs, to investigate if the reduction in binding is caused by reduction in the level of TfR on the cells.

Figure 5.7 Alteration in surface level of Transferrin Receptor by exposure to FBS, BSA and Transferrin. a) Expression of TfR on hCMEC/D3 cells measured by ELISA after exposure to FBS (a), BSA (b) or transferrin (c) for 24hrs. Error bars are ±SEM. Values were compared to media alone using an unpaired t-test. * =P<0.05, ** =P<0.01.

Figure 5.7 suggests a partial explanation to the results from figure 5.6. BSA reduces Tfr levels but not to the extent that could explain the reduction in Pep-L binding seen in figure 5.7, which leaves open the possibility that BSA directly binds to and reduces the available free peptide. With regards to FBS, 2-20% FBS solution lead to a significantly lower level of TfR on the cell surface. This suggests that whereas the BSA in the FBS could reduce Pep-L uptake the unexpected increase in Pep-L uptake caused by FBS (Figure 5.7a) is not due to an increase in surface Tfr (Figure 5.7a) but due to other factors in the FBS, causing increased endocytosis of the receptor and associated peptide.
5.3.6. Attachment and characterisation of Pep-L to AuNP

Pep-L was our chosen peptide to continue our study and the next step was to attach it to AuNPs. Some optimization would be necessary including identification of the optimum AuNP ligand coating for BBB transcytosis and exchange, and what ratio of Pep-L to AuNP would be best for the exchange reaction.

5.3.6.1. Pep-L (+/-FITC) in an oxidised or reduced state

Pep-L was synthesized with and without FITC, for the use in different experiments (the chemistry of the FITC molecule may impact the exchange reaction and binding). Pep-L (+/- FITC) was suspended in DMSO at 10mg/ml concentration, in 20µl aliquots and was stored at -80°C.

Ligands and peptides with a free thiol can be rapidly oxidized, especially in these low volumes (high surface area to volume ratio), which would affect the ability of the thiol to be used to exchange onto the surface of the AuNP (Figure 5.8). The process to reduce the peptides, if oxidized, could be detrimental to the AuNP (for example TCEP, used in the following experiment would also strip the AuNP of its ligand coating and could dramatically affect the stability of the AuNP.

![Figure 5.8 Representation of the oxidised and reduced states of Pep-L](image)

In the reduced state Pep-L cysteine has the -SH group free for thiol exchange reaction. In an oxidized state 2 Pep-L can dimerise via a disulphide bridge and would not be freely available for the exchange reaction onto the surface of the AuNP. This can be reverted by a reducing agent such as TCEP.
PEP-L elutes at 5.53min whether treated with TCEP or not and PEP-L-FITC elutes at 6.05min whether it is treated or not. Figure 5.9 demonstrates that Pep-L +/-FITC was not oxidized and therefore we could continue to use them in further experiments.
5.3.6.2. Fractionation of AuNP and Pep-L coated AuNP

An initial pilot study using the exchange reaction to attach Pep-L+FITC on to α-Galactose-C2/PEGamine (50:50) AuNP was undertaken.

The reaction mixture was separated using Fast Protein Liquid Chromatography (FPLC) using a superdex-200 column, that can analyse and purify mixtures of proteins (10-500kDa) or nanoparticles on the basis of size.

Using FPLC to separate the reaction mixture of AuNPs with bound and unbound Pep-L+FITC:

**Figure 5.10a** chromatogram shows two peaks of nanoparticles, with the left peak coming out in fractions 23-26 and the right peak coming out in fraction 30-32. The NPs in fractions 23-26 are larger than the NPs in fractions 30-32 and are potentially NP-peptide conjugates. These fractions were then analysed for fluorescence (FITC, 485nm excitation), which initially showed no difference in the levels of fluorescence between the two peaks (**Figure 5.10b** lower trace). TCEP was then added to the fractions which strips the FITC-peptide bound to the AuNP (fluorescence-quenched by proximity to the gold) and makes it free to fluoresce. When the fractions were run in the same fluorescence plate assay after TCEP treatment, fractions 23-26 showed higher levels of fluorescence than the other fractions (**Figure 5.10b** upper trace). This indicates that the left peak in **figure 5.11a** corresponds to AuNPs which have Pep-L+FITC bound to it, which has increased their size compared to the base AuNP (right peak), and they can be clearly separated from each other.
Figure 5.10 Demonstration of Pep-L+FITC attachment to α-Galactose-C2/PEGamine (50:50) AuNP

a) FPLC graph is the volume and corresponding time point at which the reaction mix passed through our column

b) The time point fraction before TCEP showing no change in Fluorescence units, addition of TCEP strips the ligand coating of the AuNPs and show that samples 23-27 correspond to fractions which had Pep-L+FITC attached AuNP

c) Shows the Fluorescence units from the time point fractions of free Pep-L+FITC
5.3.6.3. Dynamic Light Scattering

During the exchange reaction AuNPs can aggregate. These aggregates not only increase the size but also alter the properties which make them suitable as nanocarriers illustrated in Figure 5.11.

Because of this we can use Dynamic Light Scattering (DLS) which can determine the size distribution profile of small particles in suspension, which means we can accurately calculate the size and distribution of AuNPs in a sample, and if they have aggregated. Figure 5.12 is an example of the read out produced from the DLS.

Due to initial difficulties with aggregation of sample, we tested a range of ratios of Pep-L (±FITC) in the exchange reaction with two different base AuNP coating, spun filtered the solution and tested the samples in the DLS. The conjugation process seemed to significantly increase the size of the NPs and caused aggregation, the majority of this aggregation was reversed by making the dilution solution (ddH2O) to a 1xPBS solution. This suggests the interaction is electrostatic.
From this result we decided to use a low ratio Pep-L+FITC (1:1.5), a low ratio Pep-L (1:1.5) and High ratio Pep-L 1:10) to further study the effects of attachment on the AuNP (with base coating β-Glucose-C2). The low ratio samples produced the most reliable and reproducible diameter size, with negligible aggregation, allowing us to make a direct comparison between Pep-L with and without FITC. A higher ratio of 1:10 was chosen as it was stable and reproducible and will allow for a comparison between a high and low ratio Pep-L.

Figure 5.12 Example of the read out produced from a sample after the exchange reaction from the DLS (Malvern instruments).

After the exchange reaction, the solution was spin-filtered and then measured in the DLS. The sample above is shown to be a mixture of 3 sizes, 26.98, 334.8 and 5307 nm in diameter with the respective percentage volume of the sample, 79.1, 14 and 7.
Figure 5.13 Size and percentage volume of sample produced due to a range of ratio of Pep-L (±FITC) with β-Glucose coated AuNP in 1xPBS.
Blue area is an agreed upon acceptable range of size for the ligand AuNPs (2-7nm).
5.3.6.4.  Cell viability assay of Pep-L coated AuNP

We needed to establish that after the addition of targeting peptides to the base AuNPs (β-Glucose-C2 ligand coating), it did not affect the viability of hCMEC/D3 cells. We used AlamarBlue® a fluorescent assay to determine the viability of cells exposed to a range of concentrations (8-50μg/ml) of the chosen Pep-L coated AuNP. At 24hrs there was no reduction in cell viability from any of the Pep-L coatings compared to the control, β-GlucoseC2 only coated AuNP. None of the NP-Pep-L combinations were significantly cytotoxic in concentration ranges 8-50μg/ml, as measured using the Alamar blue assay.
5.3.6.5. Electrophoresis of Pep-L coated AuNP

To demonstrate that the Pep-L (±FITC) were covalently attached to the AuNP, they were characterised by gel electrophoresis. NPs run on agarose gel electrophoresis migrate depending on the charge of their coating. Pep-L (±FITC) will affect how the NP runs. Pep-L on its own has a net positive charge at this pH, however with the FITC attached it has a net negative charge, therefore AuNPs with Pep-L should migrate towards the negative, while AuNPs with Pep-L+FITC attached should migrate towards the positive. Figure 5.14 a. shows the gel after running, illustrating the brown colouring of the NPs, it appears that the different versions of peptide coating onto the NP is affecting their migration. They all appear to stay around the well, which suggest they are near neutral, but the high ratio Pep-L:NP (10:1) appears to be migrating to the negative, as we expected. Figure 5.14 b. Black/white contrast image showing that all the samples have streaking towards the positive, but again the high ratio Pep-L:NP seems to show a significant amount moving towards the negative. Figure 5.14 c. Ex.495nm/ Em. 519nm is the excitation and emission to see FITC. It shows that the FITC on the FITC-Pep-L coated AuNP is not being fully quenched and is also moving toward to the positive. Figure 5.14 shows that: 1, the sample with the high exchange ratio, had more Pep-L attached and migrated further than the low ratio sample: 2, Pep-L +FITC coated AuNP shows fluorescence at a position displaced from the free Pep-L +FITC, which implies that the AuNP have the Pep-L +FITC attached.
<table>
<thead>
<tr>
<th></th>
<th>Running control</th>
<th>1ug of FITC-Pep-L</th>
<th>Np only</th>
<th>Np + FITC-Pep-L (1:1.5)</th>
<th>Np + Pep-L (1:1)</th>
<th>Np + Pep-L (1:10)</th>
<th>Running control</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Picture</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>b. Black/white contrast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. FITC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex.495 nm/Em.519 nm</td>
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</tr>
</tbody>
</table>

Figure 5.14 Gel Electrophoresis of Pep-L (±FITC) AuNP
a) Colour Image of gel Electrophoresis
b) Black and White contrast of gel Electrophoresis
c) Fluorescence Ex.495nm/Em.519nm image of gel Electrophoresis
5.3.7. Transport of Pep-L coated AuNP across model of the BBB

Using the transfer assay previously developed for comparing nanoparticle ability to transfer across a model of the BBB; ICP-Q-MS was used to quantify the Au in the endothelial cell layer or in the collagen layer, after 3 hours of incubation with Pep-L (±FITC) coated NPs, (Figure 5.15).

In the endothelial layer, nanoparticles which were coated with Pep-L+FITC and the low ratio Pep-L coated NP (1:1.5 exchange ratio) showed significantly greater uptake than the base AuNPs.

In the collagen layer the AuNPs which were coated with Pep-L+FITC were also significantly higher (P =0.0399) than the base NPs.

The results show that the addition of Pep-L (±FITC) increased the amount of Au that was detected in the endothelial layer compared to the base AuNP. The AuNP which had Pep-L+FITC attached also had significantly higher levels of Au in the collagen layer however, the higher ratio Pep-L AuNP did not have significantly higher levels in either the endothelial or the collagen layer but there is a trend towards this. This suggests that there is a specific ideal ratio to the amount of Pep-L (±FITC) attached to AuNP to increase transfer across the BBB. High affinity antibodies have been reported to have difficulties, moving across the BBB, getting stuck in the cells and not being released from the receptor, a similar interaction could be occurring with the high ratio Pep-L AuNPs. The high ratio could bind multiple receptors at once, reducing the rate of NP release and decreasing AuNP transcytosis. The low ratio should only be binding 1 receptor and is therefore able to unbind from the receptor and transcytosis across the cell is therefore easier. This is a hypothesis and could be tested using silver enhancement of TEM images of the different coatings on hCMEC/D3 cells, to see where the AuNPs are localised in the cells.

Pep-L+FITC coated nanoparticles, having a higher level in the collagen layer suggests that the addition of the FITC, is increasing the ability of the AuNP to transfer across the cell layer. This could be due to the net negative charged produced by the FITC, or some other biological interaction we are unaware of.
Figure 5.15 Transport of peptide and β-Glucose coated AuNP across a 3-D BBB model.

a) Gold (Au) found in the endothelial cell layer after the 3hr incubation with NPs. b) ng of Au found in the collagen layer after the 3hr incubation with NPs. Error bars are ±SEM, *= p<0.05, ***= p<0.001. Statistics are paired t-tests against the control (β-Glucose only coated NP). Results are based on duplicate technical replicates and 6 biological replicates.
5.4. Conclusion

From this chapter we were able to identify the peptide sequence which binds preferentially to hCMEC/D3 cells over the others, Pep-L. We identified conditions for binding to hCMEC/D3 cells, which was a 3-hours exposure, and that BSA, FBS and transferrin can impact the binding of Pep-L to hCMEC/D3 cells. BSA and FBS interaction appears to be related to their effect on the amount of TfR on the cell surface. We identified optimal conditions for attachment of Pep-L (±FITC) onto AuNP, including ratio of peptide to AuNP and solvent. Addition of Pep-L (±FITC) onto AuNP from a 1:1.5 ratio exchange reaction, resulted in statistically higher levels of Au in the endothelial than the control, base AuNP. The Pep-L+FITC AuNP (1:1.5) also had statistically higher levels of Au in the collagen layer, which means it transferred across the BBB at a great rate than the base AuNP.
6. Conclusion and Discussion

6.1. Ligand coating

Our initial aim was to find the optimal ligand coating on the AuNPs and from the range of ligand coatings we were supplied by Midatech®, we were able to identify the optimal coatings for detectable levels transferring across the endothelial cells. The coating consisting of 50:50 α-Galactose-C2/PEGamine coated AuNP proved to be the most transferred. The β-Glucose-C2 coating was also of interest as it had a high ratio of Au in the gel compared to the endothelial layer. These two coatings along with the α-Galactose-C2 coated AuNP (which acted as a comparable control coating) were the base AuNP for all future experiments.

When comparing the transfer rate of the different ligand coatings, the different properties were studied to identify the most important factor. The properties recorded were the length of the ligand, physical size, Zeta potential and batch to batch variability. These factors all had a distinct effect on transfer across the BBB, however, the size range which we were comparing was small.

The literature indicates that if we used AuNPs of larger size it would have a significant impact on the NPs’ ability to transfer across the BBB (Betzer, 2017). Hoshyar et al. demonstrated that with AuNPs of 10nm, approximately 0.2% of the injected dose was found in the rodents’ brain after 24hrs. For larger AuNPs (50 and 250 nm), the levels in the brain were very low, and a high proportion was taken up by phagocytic cells in the liver and spleen (Hoshyar et al., 2016).

The length of the ligand seemed to affect both the synthesis and the ability of the coated AuNP to cross the BBB model. While the base coatings are modified sugars and have been found to increase the transfer across the BBB, experimentation to find the ideal ligand coating, would seem to be a fruitful area for future research. While we have demonstrated that in our simple model, the type of ligand coating was a significant factor in rate of transfer across the cells, the literature would support the view that the ligand coating will have a huge impact on the ability of the AuNPs to cross barriers in vivo, their biocompatibility in biological fluids (blood, urine, spinal fluid etc) and removal after delivery of the therapeutic cargo (Shlar et al., 2014; Male, Gromnicova and McQuaid, 2016; Martínez-Ballesta et al., 2018).

The next logical step to progress this project would be identifying and combining BBB targeting ligands (such as Pep-L) that could increase the amount of AuNPs that can cross the BBB. Pep-L was designed to hijack the transport system used for the transport of iron-loaded transferrin by targeting the TfR, Table 6.1 demonstrates some of the positive results targeting the TfR that other groups have had.
The method of attaching these ligands can influence the nanocarrier design; ligands can be attached to the AuNP via an exchange reaction which leads to a covalent bond or by non-covalent electrostatic interaction. The exchange reaction results in ligand attachment which is more stable which we opted for, designing our ligands and cargo to have a free thiol group which can be easily exchanged onto the surface of the AuNP by simply mixing (Chapter 1. Ligand exchange).

However there are other methods for attachment which may be more effective in the nanocarrier system, i.e a non-covalent attachment may allow the ligand and/or therapeutic cargo to be released more easily in vivo (Ding et al., 2014; Shlar et al., 2014).

The results demonstrate that Pep-L increases the amount of Au that crosses our BBB model (Chapter 5). However, whether this remains true in vivo needs to be confirmed. Using published methods (Gromnicova et al., 2016), the Pep-L coatings mentioned in Figure 5.15 can be compared and the amount of Au measured in the brain of rats following intracarotid injection.
6.2. Therapeutic cargo

6.2.1. BDNF-H9C

BDNF was selected as a therapeutic molecule, which if it could cross the BBB in significant quantities could be useful in the treatment of a number of neurological disorders (Bryant et al., 2009). Before we developed a method to modify the BDNF for attachment in a nanocarrier system, we developed the BDNF ELISA for quick quantification of BDNF produced in a supernatant, and a Trk-B CHO assay which accurately measures the biological response of the BDNF produced. The ELISA and Bio-assay for BDNF match both wild type and BDNF-H9C. The Bio-assay should be suitable for future evaluation of BDNF mimetics, (it is possible for a peptide mimetic to work in a biological assay, but not an ELISA and conversely a slightly altered form of BDNF could work in ELISA but possibly not in the bioassay).

From this study we found that we were able to produce BDNF-H9C, which remained as biologically active as wild type BDNF, but were unable to produce enough BDNF-H9C, of high enough purity, for the necessary chemical and biological experiments we had initially proposed. Icosagen® is a biotechnology company with a history of producing challenging recombinant proteins in mammalian cells and they also have a track record for producing high quality BDNF. Icosagen® found that Furin, an enzyme involved in the processing of BDNF, is inefficient in CHO cells. Hence, the Icosagen® expression system includes an overexpressing Furin-CHO cell line (Chen, Zhang and Deng, 2015). However, when supplied with the BDNF-H9C plasmid to use in their expression system they also found they were unable to produce over 20ng/mls (too low in quantity to continue) and found that the BDNF-H9C protein was difficult to isolate from their purification process.

6.2.2. Increase BDNF-H9C production

Commercial BDNF is conventionally produced from microbial cultures, which often leads to low fidelity protein product (often due to incorrect glycosylation due to microbial production), in the form of large insoluble aggregates, because of the inability of the host to correctly form the characteristic cysteine-knot fold of BDNF (Angart et al., 2017). This is corrected by isolation and refolding which is expensive and time consuming and this BDNF product can still have an attenuated biological activity (Hackel et al., 2014). Hackel et al, engineered BDNF through two successive rounds of direct evolution from which they identified a BDNF mutant that can be expressed in yeast, and showed a 5-fold improvement in expression and 4-fold increase in specific Trk-B binding activity compared to wild-type BDNF (Hackel et al., 2014). This mutant form could be used as the basis for the BDNF-H9C modification, which not only could increase the amount of BDNF-H9C produced but also the biological activity of the BDNF-H9C compared to wild-type BDNF.

Proteins such as BDNF have huge therapeutic potential, but have been limited due to issues in production, ability to transverse cellular barriers, and stability. These biological agents are very
active at low concentration and if designed correctly should be biologically active when attached to the carrier. This means that relatively little is required to cross the BBB and act in the extracellular environment.

6.2.3. BDNF Mimetics

One possible way to overcome this problem is using molecules which mimic the biological function of BDNF such as peptides, small molecules and drugs.

6.2.3.1. Peptides

In comparison to proteins with delicate 3D structures, engineered peptides are easy to synthesize, have high target specificity and selectivity, and low toxicity (Gonsalvez et al., 2018). The standard drawback of therapeutic peptides, stability and half-life, could be overcome by their attachment to AuNPs.

A number of peptide mimetics of BDNF have recently been developed. Cyclo-dPAKKR (dPro-Ala-Lys-Lys-Arg) is a peptide that structurally mimics the region of BDNF that binds p75NTR, which promotes myelination of NGF dependent neurons, TrK-B expression in vitro, and significantly increases the number of myelinated axons in vivo (Xiao et al., 2013). The linear form of dPAKKR exerts no effect upon either myelin protein expression or the formation of myelinated axonal segments (Xiao et al., 2013; McLean et al., 2017). The 3D structure is critical to its binding to the receptor so altering the sequence for attaching onto AuNP, especially via an exchange reaction would need optimization.

TDP6 is a conformationally constrained tricyclic dimeric mimetic of the loop 2 region of BDNF that acts as putative TrK-B agonist. It was shown to significantly enhance TrK-B signalling in primary oligodendrocytes and increases oligodendrocyte-mediated myelination in vitro, compared to its conformationally unconstrained version DP6 (Table 6.1)(Xiao et al., 2014; Gonsalvez et al., 2018).

<table>
<thead>
<tr>
<th>Tricyclic dimer (TDP6)</th>
<th>Tricyclic dimer peptide 6 (TDP6):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Contains two monomeric monocyclic peptides dimerized by a bridging amide (E=K) and an internal disulphide (C=C) bond.</td>
</tr>
<tr>
<td></td>
<td>• The monomers are conformationally constrained by 2 bridging disulphide bonds between terminal cysteine residues (C=C).</td>
</tr>
<tr>
<td></td>
<td>• These bonds form the tricyclic structure.</td>
</tr>
<tr>
<td></td>
<td>• The overall structure of this peptide closely resembles the dimeric loop 2 region of the native BDNF dimer.</td>
</tr>
<tr>
<td>Control dimer (DP6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Consists of two monomeric linear peptides joined by a bridging amide bond (E-K).</td>
</tr>
<tr>
<td></td>
<td>• The terminal and internal cysteine residues are modified to serine, and thus unable to form disulphide bonds. As a result, this peptide lacks a constrained structure.</td>
</tr>
</tbody>
</table>

Table 6.1 Demonstration of the structural difference between TDP6 and DP6. Modified from Xiao et al. 2014.

Betrofin-3 and Betrofin-4, (RGIDKRHWNSQ and SYVRLTMDSSKRGWR, respectively) are BDNF peptide mimetics derived from solvent-exposed loops in BDNF. Both Betrofin-3 and -4 showed binding to TrK-B and p75NTR, were biologically active, and stimulated neurite outgrowth and
promoted survival when applied to primary cerebellar granule neurons, which was inhibited by BDNF in a competition assay (Pedersen et al., 2009). Betrofin-3 was attached to the C-terminal of RADA16 (synthetic amphiphilic peptide with RADARADARADARADA sequence), to form a functional self-assembling peptide hydrogel scaffold, which promoted the outgrowth of axons and dendrites in a rat model of Traumatic Brain Injury (Shi et al., 2016).

Betrofin-3 does not contain a cysteine (which is important for the exchange reaction) in its structure, and has been shown to still be biologically active when 1 or 2 of the amino acids are removed from either end of the sequence and it is still biologically effective when attached to a polypeptide (RADA16) (Pedersen et al., 2009; Shi et al., 2016).

6.2.3.2. Small molecules

The compound, 7,8-dihydroxyflavone (7,8-DNF) was found in a large screen of chemical libraries on cell-based Trk-B receptor-dependent survival assays (Liu, Chan and Ye, 2016). 7,8-DNF is a Trk-B agonist that mimics BDNF, it promotes neuronal survival and regeneration in models of traumatic brain injury (Cerquone Perpetuini, Mathoux and Kennedy, 2019). 7,8-DNF specifically binds to the extracellular domain of Trk-B with high affinity and compared to BDNF it has a longer half-life (134 minutes in plasma versus 10 minutes in plasma), and is considerably smaller (254Da compared to 27kDa) (Liu, Chan and Ye, 2016).

LM22A-4 activates TrkB receptor along with AKT and ERK1/2 in cultured hippocampal neurons. The effectiveness of the LM22A-4 has been tested in models of stroke, spinal cord injury, epilepsy, and neurodegenerative diseases such as Rett syndrome, Fragile X syndrome and Huntington’s disease. In a mouse model of stroke, Han et al. showed that the administration of the LM22A-4 three days after ischemia resulted in phosphorylation of downstream targets, AKT and ERK1/2, and improved limb swing speed and gait after stroke (Travaglia et al., 2016).

Compared to the peptides (Betrofin-3 and -4), where we have a history of logical progression from modifying, attaching onto the AuNP, comparison and characterisation after attachment, and biological assessment (Chapter 5) the small molecules would need more work. The small molecules would most likely have to be designed with a linker sequence like the modified sugar ligands used in the initial synthesis step. This would then have to be accessible to still be biologically active and this depends on which method is used for attachment (exchange or during the formation of the AuNP).
<table>
<thead>
<tr>
<th>TfR Targeting mechanism</th>
<th>Biological effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic Iron-mimicking peptide as RMT (CRTIGPSVC)</td>
<td>HSV-Thymidine Kinase gene specifically delivered to mouse brain tumours through a non-canonical allosteric binding mechanism to TfR.</td>
<td>(Sidman et al., 2010)</td>
</tr>
<tr>
<td>Liposomes decorated with Tf-Poly-L-arginine loaded with imaging agents or β-gal expressing plasmid</td>
<td>4% of injected dose of imaging agents reached the brain 24h after i.v. injection. Greater β-gal compared to injection of naked DNA.</td>
<td>(Sharma et al., 2013)</td>
</tr>
<tr>
<td>PEGylated liposomes decorated with anti-TfR antibody (8D3) loaded with plasmid encoding β-glucoronidase</td>
<td>At 48h post i.v. injection 10-fold higher β-glucoronidase activity observed in brain, liver and spleen in mouse model of mucopolysaccharidosis VII.</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td>cTfRMAb (chimeric anti-mouse TfR monoclonal antibody) complexed with tumour necrosis factor receptor (TNFR); cTfRMAb-TNFR.</td>
<td>Mice model of Parkinson’s disease (PD) i.v. treated for 3 weeks showed 130% increase in striatal tyrosine hydroxylase (TH) and improvements in behavioural testing.</td>
<td>(Zhou et al., 2011a)</td>
</tr>
<tr>
<td>PEGylated chitosan nanoparticles decorated with anti-TfRMAb (R17-217): CS-PEG-BIO/SA-TfRMAb.</td>
<td>Decreased infarct volume, neurological deficit, and ischemia-induced caspase-3 activity in mice model of stroke i.v. injected with CS-PEG-BIO/SA-TfRMAb.</td>
<td>(Karatas et al., 2009)</td>
</tr>
<tr>
<td>cTfRMAB complexed with erythropoietin (EPO): cTfRMAB-EPO. cTfRMAb complexed with glial-derived neurotrophic factor (GDNF): cTfRMAB-GDNF.</td>
<td>Mouse model of PD i.v. treated for 3 weeks showed &gt;300% and &gt;250% increase in striatal TH respectively and improvements in behavioural testing.</td>
<td>(Zhou et al., 2011b)</td>
</tr>
<tr>
<td>cTfRMAb complexed with single chain Fv (ScFv) antibody: cTfRMAb-ScFv.</td>
<td>Bi-functional binding to TfR and Aβ, accumulation in mouse brain &gt;3%ID/g. Mouse model of Alzheimer’s disease (AD) showed 40-60% reduction in Aβ fibrils.</td>
<td>(Cabello et al., 2010; Sumbria et al., 2013)</td>
</tr>
<tr>
<td>Monovalent binding anti-TfR antibody. “Brain Shuttle” antibody for AD.</td>
<td>Enhanced RMT compared to bivalent Ab. Increased destruction of β-Amyloid plaques in mouse model of AD. Changes in binding mode attenuated peripheral effects.</td>
<td>(Niewoehner et al., 2014; Weber et al., 2018)</td>
</tr>
<tr>
<td>High (anti-TfR/BACE1) and low (anti-TfR/BACE1) affinity bispecific antibodies anti TfR and β-amyloid cleaving enzyme-1 (BACE1).</td>
<td>In WT mice i.v. injected, high-affinity binding to TfR caused a dose-dependent reduction of brain TfR levels and lysosomal degradation of TfR.</td>
<td>(Bien-Ly et al., 2014)</td>
</tr>
<tr>
<td>Variants of the 8D3 anti-TfR with reduced affinity fused with II-1 receptor antagonist II-1RA: IgG1M-IL-1RA.</td>
<td>Male C57B/1 mice i.v. injected with IgG1M-IL-1RA showed 22 to 69-fold greater brain content of lower affinity variants vs 8D3. Reverse of mechanical hyperalgesia also observed.</td>
<td>(Webster et al., 2017b)</td>
</tr>
<tr>
<td>Human Tf fused to iduronate 2-sulfatase (IDS):JR-141.</td>
<td>Immunoreactivity of JR-141 found in brain in TFRC-KI/ids-KO mice.</td>
<td>(Sonoda et al., 2018)</td>
</tr>
<tr>
<td>Liposomes decorated with anti-TfR loaded with GDNF-expressing plasmids.</td>
<td>Rat model of PD i.v. treated showed 77% increase in TH activity and neurobehavioral improvements.</td>
<td>(Zhang and Pardridge, 2009)</td>
</tr>
</tbody>
</table>

Table 6.2: Transferrin receptor mediating RMT cargo delivery through the BBB. Evidences of efficient RMT utilizing the Transferrin Receptor are summarized. Edited from Pulgar 2018.
6.2.4. Alternative molecules for treatment of Neurodegenerative disorders

BDNF has a lot of therapeutic potential for the treatment of neurodegenerative disorders but there are a wealth of other possible proteins and molecules which could be attached to our nanocarrier system.

Ciliary Neurotrophic Factor (CNTF) is a 200 amino acid residue protein, whose neuroprotective effects are well established (Gonsalvez et al., 2018). CNTF has been shown to promote adult hippocampal and subventricular zone neurogenesis and differentiation of neural stem cells and is expressed in astrocytes. CNTF administration has been shown to alleviate cognitive impairment and to stabilize synaptic protein levels in a transgenic mouse model of AD. CNTF, like BDNF, has limited BBB permeability, poor plasma stability, unsuitable pharmacokinetics, unwanted systemic effects and in a clinical trial had limited bioavailability and multiple adverse effects (Kazim and Iqbal, 2016).

Peptide-6 is a peptide mimetic of CNTF (Ac-VGDGGLFEKKL-NH2), which corresponds to a biologically active region (amino acid residues 146–156) of human CNTF and was identified using epitope mapping.

![Figure 6.1 Design and structures of CNTF small-molecule mimetics and their mechanism of action to enhance formation of NPCs and mature neurons.](image)

**Figure 6.1** Design and structures of CNTF small-molecule mimetics and their mechanism of action to enhance formation of NPCs and mature neurons.

a) Protein Database rendering of one 4-helix bundle of truncated human CNTF (Residues 2–187), generated from CNTF. The structures of P6 and P021 are also shown. From the neurogenic 11-mer, Ac-VGDGGLFEKKL-NH2 (P6), a truncated, still neurogenic pentamer, with an adamantylatedglycine group (redoval), Ac-DGGLAG-NH2 (P021) was designed.

b) P6 and P021 enhance neural progenitor cells (NPCs) formation and maturation and integration of new-born neurons by competitively inhibiting the LIF signalling and increasing BDNF expression respectively. Modified from S.Kazim 2016.

P6 has a plasma half-life of over 6hrs (as compared to approximately 3mins of recombinant CNTF), is BBB permeable, increases BDNF mRNA levels, thus increasing the survival, maturation, and integration of new-born cells (Gonsalvez et al., 2018). Preclinical studies employing P6 showed neurogenic and neurotrophic effects in animal models of sporadic AD, familial AD, Down’s syndrome, autism, and traumatic brain injury. (S. Kazim, 2016)
Peptide 6, or its shorter form Peptide 021 (which is the minimal active region of p6) has similar neurogenic and neurotrophic effects as P6, with increased pharmokinetics (longer stability and increased activity) of P6. These peptides could be modified to be attached to the surface of the AuNPs in a similar exchange reaction to Pep-L.

6.2.5. Future work

AuNPs allow for the attachment of multiple molecules which can be used as therapeutic cargo for multiple diseases. This thesis has described an effective method for the comparison of AuNP coatings and their ability to impact on the transfer across a BBB model. In Chapter 4, we have shown that the modification of BDNF, is possible to produce a biologically active molecules, but it is impractical for future work. In Chapter 5 we have shown how peptides can be altered to still have biological effects and be attached onto AuNP by the exchange reaction. Once attached these peptides altered the properties of the AuNP, in this case increasing its ability to transverse across a model of the BBB. Using the same principals, we can alter peptides, such as Betrofin-3 or Peptide-021, to attach on to the AuNP and test if 1: it can still activate Trk-B receptors similarly to BDNF (by the Trk-B CHO cell line assay) and 2: if its attachment affects the ability of the AuNP to transverse the endothelium in vitro and cross the BBB in vivo.

6.2.5.1. Protein corona

The physiological environment is likely to have a large impact on AuNPs and how their coatings can affect their ability to cross the BBB both in vitro and in vivo. Blood in an in vivo environment and serum in cell culture media in vitro, allow the interaction of proteins which can adhere to the AuNP surface forming a protein corona (Kelly et al., 2015; Lara et al., 2017).

The corona can impact not only the route which the AuNP takes in the body, but also its ability to cross different biological barriers. The corona may shield targeting ligands bound to the AuNP, change the charge of the AuNP or have an effect on clearance from the system.

From results shown in Figure 5.6 and discussed in that section, changes in FBS (the chief contributor to proteins which could interact on the AuNPs protein corona in media) concentration did impact the AuNP behaviour, how exactly ligand coating will affect the protein corona in vitro, is difficult to predict and would definitely be an avenue for future investigation (Lara et al., 2017).
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