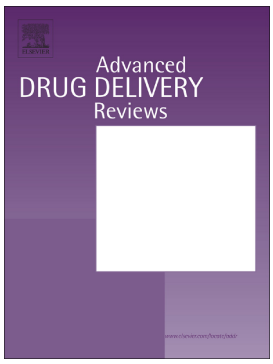

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Designing Peptide Nanoparticles for Efficient Brain Delivery

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Abstract

The targeted delivery of therapeutic compounds to the brain is arguably the most significant open problem in drug delivery today. Nanoparticles (NPs) based on peptides and designed using the emerging principles of molecular engineering show enormous promise in overcoming many of the barriers to brain delivery faced by NPs made of more traditional materials. However, shortcomings in our understanding of peptide self-assembly and blood-brain barrier (BBB) transport mechanisms pose significant obstacles to progress in this area. In this review, we discuss recent work in engineering peptide nanocarriers for the delivery of therapeutic compounds to the brain, from synthesis, to self-assembly, to *in vivo* studies, as well as discussing in detail the biological hurdles that a nanoparticle must overcome to reach the brain.

Keywords: Peptides; Self-Assembly; Drug Delivery; Blood-brain Barrier; Transcytosis; Glioma; Alzheimer's Disease; Parkinson's Disease

Abbreviations: *AChE,* inhibiting acetylcholinesterase; *AD*, Alzheimer's disease; *AFPS,* automated fast-flow peptide synthesis; *AGEs,* advanced glycation end products; *AMT*, Adsorptive-mediated transport; *ApoE,* apolipoprotein E; *ASO*, antisense oligonucleotides; *AVP*, arginine-vasopressin; *Aβ*, amyloid beta; *β*-Gal, *β*-galactosidase; *BACE1*, ß-secretase 1; *BAR*, Bin/Amphiphysin/Rvs; *BBB*, blood-brain barrier; *BBTB,* blood-brain tumour barrier; *BDNF*, brain-derived neurotrophic factor; *BECs,* brain endothelial cells; *BLA-NCA,* β-benzyl-L-aspartate NCA; *CBF*, cerebral blood flow; *CED*, convection-enhanced delivery; *CFPS*, Cell-free protein synthesis; *CME,* clathrin-mediated endocytosis; *CMT*, carrier-mediated transport; *CNS*, central nervous system; *CPP*, cell penetrating peptide; *CPT*, camptothecin; *cRGD*, cyclic arginine-glycine-aspartic acid; *Cryo-TEM*, cryogenic transmission electron microscopy; CSF, cerebrospinal fluid; *DACHPt*, (1,2-diaminocyclohexane)platinum(II); *DET*, diethylenetriamine; *DGL*, dendrigraft poly(Lys); *DHA*, dehydroascorbic acid; *DMT,* disease-modifying therapies; *DNA,* deoxyribonucleic acid; *dnMAML,*

dominant negative MAML; *DOX,* doxorubicin; *DSIP,* delta-sleep inducing peptide; *eGFP*, enhanced green fluorescence protein; *ELP,* elastin-like peptide; *ELR*, elastin-like recombinamer; ENCP, enveloped nanocomplex; *FA*, folic acid; *Fab*, fragment antigen-binding; *FDA*, food and drug administration; *FR*, folate receptor; *GABA*, γ-aminobutyric acid; *GBM*, glioblastoma; *GDNF*, glial cell line-derived neurotrophic factor; *GLUT1*, glucose transporter-1; *GMP*, good manufacturing practice; HMGB, high mobility group box 1 protein; *hTRAIL*, human tumour necrosis factor-related apoptosis-inducing ligand; *ING4*, inhibitor of growth 4; *ICV*, intracerebroventricular; ID, injected dose; IN, intranasal; *IP*, intraperitoneal; *IR*, insulin receptor; *IV*, intravenous; *LCST*, lower critical solution temperature; *LDLR*, low-density lipoprotein receptor; *LepR*, leptin receptor; *LHRH*, luteinising hormone-releasing hormone; *LNP*, LIM kinase 2 Nuclear translocation signal peptide; *LPL*, low-density lipoprotein; *LRP1*, low density lipoprotein receptor-related protein 1; *Lys^{P},* ethylene-glycol modified lysine; *Mfsd2a*, major facilitator superfamily domain-containing 2a; *mRNA*, messenger RNA; MW, molecular weight; *nAchR*, nicotinic acetylcholine receptor; *NCAs*, N-carboxyanhydrides; NEP, neprilysin protein; *NP*, nanoparticle; *NTA*, N-thiocarboxyanhydride; *NVU*, neurovascular unit; *OATP-2,* the organic anion transporting polypeptide-2; *PA*, peptide amphiphile; P([*N*-(5-aminopentyl]-α,β-aspartamide], P(Asp-AP); *PCL*, poly(e-caprolactone); *PD*, Parkinson's disease; *pDNA*, plasmid DNA; *PEG*, poly(ethylene glycol); *PEI,* polyethylenimine; *PIC*, polyion complex; *PICsome*, polyion complex polymersome; *PL*, poly(g-(4,5-dimethoxy-2-nitrobenzyl)-L-glutamate; *pORF*, plasmid open reading frame; *PPLG*, poly(g-propargyl L-glutamate; *PS-PAA*, polystyrene-b-poly(acrylic acid); *PTS,* peptide transporter systems; *RAGE*, receptor for advanced glycosylation end products; *RAP*, receptor-associated protein; *RAR*, retinoid acid receptor; *RLP,* resilin-like block; *RMT,* receptor-mediated transport; *RNAi,* RNA interference; *ROP,* ring-opening polymerisation; *ROS,* reactive oxygen species; *RVG29,* rabies virus glycoprotein peptide; *SAR,* structure-activity relationships; sc, single chain; *shRNA,* short hairpin RNA; *siRNA,* small interfering RNA; *SPPS,* solid phase peptide synthesis; *ß-Gal,* β-galactosidase, *TAT,* human immunodeficiency virus (HIV) transactivator of transcription protein; *Tf,* transferrin; *TfR*, transferrin receptor; *TJs*, tight junctions; *TMZ*, temozolomide; *TP*, therapeutic peptide; *Tyr-MIF-1*, tyrosine melanocyte-stimulating inhibitory factor-1; *VEGF*, vascular endothelial growth factor.

1. Introduction

To successfully deliver its cargo to a target tissue, a nanocarrier must navigate through the body while eliciting minimal immunogenic response and avoiding off-target delivery of cytotoxic compounds. In brain delivery, this challenge is often magnified by the need to achieve transport across the blood-brain barrier (BBB), perhaps the most tightly regulated biological barrier in the human body. Essential to success in this area are novel approaches and materials that combine complex functionality with biocompatibility. However, these two properties are often compromised as complex functions require access to untested chemistries that can be potentially toxic. Avoidance of toxicity is particularly critical in developing systems for the treatment of central nervous system (CNS) disorders, which adds an extra dimension to the toxicological profile: the neurological one. One way around this is to use the same chemical currency as nature and adapt, functionalise, and synthesise it to make intrinsically safe materials. Among these, polypeptides are the most versatile and indeed combine almost unlimited functionality with biocompatibility and biodegradation.

Synthetic polypeptide materials have largely proven their suitability in drug and gene delivery with overwhelming examples in the literature and a number of successful products in clinical trials or

market stage [1]. Although still less exploited in the treatment of CNS disorders, a growing body of work shows that nanomaterials based on amino acid building blocks have significant promise in brain delivery. Evidence of this is the first polymeric drug reaching the market, Copaxone® (glatiramer acetate from Teva Pharmaceutical Industries Ltd.). This random copolymer, which consists of L-alanine, L-lysine, L-glutamic acid and L-tyrosine, is Food and Drug Administration (FDA)-approved for the treatment of relapsing–remitting multiple sclerosis and has been among the top 10 selling drugs worldwide [2–6]. Another promising candidate is Opaxio™ (paclitaxel polyglumex from CTI Biopharma), a P(Glu)-paclitaxel conjugate which is in phase III clinical trials for the treatment of several different cancers. Of note, Opaxio™ was designated as an orphan drug for glioblastoma (GBM) treatment in combination with radiotherapy [7–9].

In this review, we focus on the design of peptide nanoparticles for the delivery of therapeutics into the brain, focussing chiefly on delivery *via* the BBB. We summarise representative examples of polypeptide-based nanocarriers in the preclinical stage applied in the delivery of therapeutics and imaging agents to the brain. We then identify the different strategies followed to cross the main barrier protecting the brain (the BBB), as well as identifying alternative methods to achieve delivery of NPs to the brain. We first discuss the molecular engineering of nanocarriers, including synthetic pathways by which one can make and functionalise peptides (**Section 2)**, and the supramolecular rules to control peptide self-assembly (**Section 3**). In the latter section, we argue that traditional, top-down design approaches may not be the best approach to producing engineered peptide nanostructures. Instead, evolutionary approaches, both *in* silico and experimental, might prove more fruitful. The majority of the review focuses on the BBB structure and the mechanisms of transport that facilitate the entry of peptides and proteins across the BBB and into the brain (**Section 4**). Given the challenges involved in engineering NPs that can cross the BBB, we then look at alternative pathways for brain delivery, including localised, convection-enhanced, and intranasal administration (**Section 5**). Finally, we discuss the alterations to the BBB that occur due to pathological conditions such as stroke, which both pose challenges and provide opportunities in engineering NPs for brain delivery **(Section 6**).

2 Synthetic Pathways to Produce and Functionalise Peptide Nanoparticles for Brain Delivery

Peptide NPs are typically formed from the self-assembly of either polypeptide-bearing amphiphilic block-copolymers or peptide amphiphiles (PAs) [10]. Although not always required, peptide NPs often benefit from being functionalised with ligands that target specific receptors expressed, for example, at the surface of brain endothelial cells (BECs) or the nasal epithelium [11]. This strategy introduces additional complexity in terms of design and chemical control that complicates the

manufacturing process, especially in order to achieve BBB-crossing following non-invasive intravenous (IV) administration. However, using targeting ligands often leads to enhanced biological performance, which makes this additional complexity worthwhile [12]. As such, targeting ligands such as glucose [13], peptides [14,15] or proteins [16], are often linked at the NP surface by conjugation, before or after NP self-assembly [17,18]. Here, we discuss the main pathways for polypeptide synthesis (**Section 2.1**) and ligand conjugations (**Section 2.2**), to produce and functionalise polypeptide nanocarriers for brain delivery. The biological purpose and outputs of the examples depicted within this section are then addressed in detail in Section 4.

2.1 Synthesis of polypeptides

Polypeptides can be synthesised using both biological and chemical methods (**Figure 1**). Biological, or recombinant, protein production is based on exploiting the expression of a peptide by biological hosts *via* a recombinant DNA template. The host cells can be prokaryotic or eukaryotic, depending on the nature of the peptide, the desired post-translational modifications, and the value of the produced compound [19]. Recombinant synthesis is most often used in pharmaceutical and protein synthesis applications, however it is increasingly popular in self-assembly studies [20–25] (Figure 1A). The recombinant technique stands out with advantages such as high control over the sequence and molecular weight (MW) of the polypeptide [26]. It also has significant environmental benefits, such as moderate energy consumption and the use of water as a solvent. However, before polypeptide synthesis, a time-consuming process of genetic construction, optimisation, and troubleshooting is required. Furthermore, toxicity of the produced peptide to the host can lead to low or even zero yields, particularly in peptides that are short or tend to self-assemble [27]. The purification and extraction of the target peptides from the host cells can also be laborious and expensive requiring complex chromatographic techniques. All these aspects can be quite limiting when combined with the stringent regulatory requirements for the mandatory good manufacturing practice (GMP) for clinical translation. Cell-free protein synthesis (CFPS) is a newer approach that bypasses a number of the challenges and limitations of recombinant synthesis methods [28]. In this technique, the protein synthesis machinery from either eukaryotic and prokaryotic cells are extracted and used in solution, avoiding the requirement to support cell viability and growth [29]. While cell-free methods have been used in research for over half a century [30], comparatively recent improvements have made affordable gram-scale synthesis viable in the lab and 100-liter-scale production viable in industry [31,32]. Furthermore, the compatibility of CFPS with combinatorial approaches makes it ideal for evolutionary and self-optimizing studies of peptide self-assembly [32,33], which we discuss in Section 3.3. Due to these benefits, along with its ease of use [34], cell-

free synthesis is an increasingly popular alternative in a number of research areas [35,36]. However, to our knowledge, CFPS has been almost entirely overlooked by the peptide NP community.

For both historical and practical reasons, the organic synthesis of peptides and polypeptides is the more popular approach in self-assembly studies. Peptide bonds can be formed relatively easily but the challenging aspect is to control both sequence and MW. Two main methods are well established: solid phase peptide synthesis (SPPS) and ring-opening polymerisation (ROP) of N-carboxyanhydrides (NCAs). In SPPS, polypeptides are produced by repeating cycles of deprotection and coupling reactions of N-protected amino acid derivatives on resin beads as the solid supports (Figure 1B). Using this method, polypeptides with arbitrary amino acid sequences can be produced with a high accuracy [37]. However, because of the inefficiency of the coupling reaction and the presence of side reactions, chains longer than 50 amino acids cannot be reliably produced. One way around this limitation is to further couple the relatively short peptides produced by SPPS using chemical ligation, producing artificial proteins with longer chains [38]. Significant progress in producing longer artificial proteins over a hundred amino acids long was demonstrated very recently by Pentelute *et al.,* who reported an automated fast flow peptide synthesis (AFPS) technique. By applying flow chemistry to SPPS, they achieved comparatively rapid synthesis of long-chain polypeptides (up to 164 units within 6.5 h) [39]. Although it represents significant progress, this novel flow chemistry approach still has a number of limitations common to SPPS, including low yield, long preparation time, and laborious purification steps.

For the production of larger quantities or longer peptides, the living ROP of NCAs is currently the most widely reported method for synthetic polypeptides, but can only be used to produce chains made of single or a few randomly distributed amino acid species [40,41]. Generally, the polymerisation is initiated by nucleophiles or bases and chain growth is propagated by the ring opening of NCA monomers and the release of carbon dioxide under inert air (Figure 1C). ROP of NCAs stands out with advantages including high yield, controllable MW, short production cycle, and scalable synthesis, as well as the possibility to construct new architectures (i.e. polymer brush, star and dendrimer) and introduce unnatural amino acids (i.e. N-substituted amino acids for polypeptoid backbones) [40,42]. Efficient catalysts and methodologies have been developed to produce controlled/living ROP of NCA, such as nickel and cobalt complexes by Deming *et al.* [43], or the use of high-vacuum technique by Hadjichristidis *et al*. [44]. The major drawback of NCAs comes from their instability to heat and moisture, which limits their storage time and poses a limitation for industrial applications in terms of scalability and reproducibility. Although alternative monomer precursors such as N-(phenyloxycarbonyl) amino acids [45] or NCA analogues such as N-thiocarboxyanhydride (NTA) [46] have been proposed to substitute NCAs, advances in NCA storage

conditions and the robustness and universality of the ROP of NCA makes it the more frequently employed method both in academia and industry. Most of the polypeptide-based nanocarriers described within this review, apart from elastin-like peptides (ELPs, described in detail in sections 3.1 and 4.3), are produced using this method. Examples of this are poly(ethylene glycol) (PEG)-NH_2 initiated polypeptides produced by Kataoka *et al.* [14,47] (described in detail in section 4.2.5) or the poly(L-lysine)-grafted polyethylenimine (PEI) (PEI-*g*-P(Lys)) produced by Chen *et al.* using the dendritic PEI as initiator [48] (described in detail in section 4.2.3).

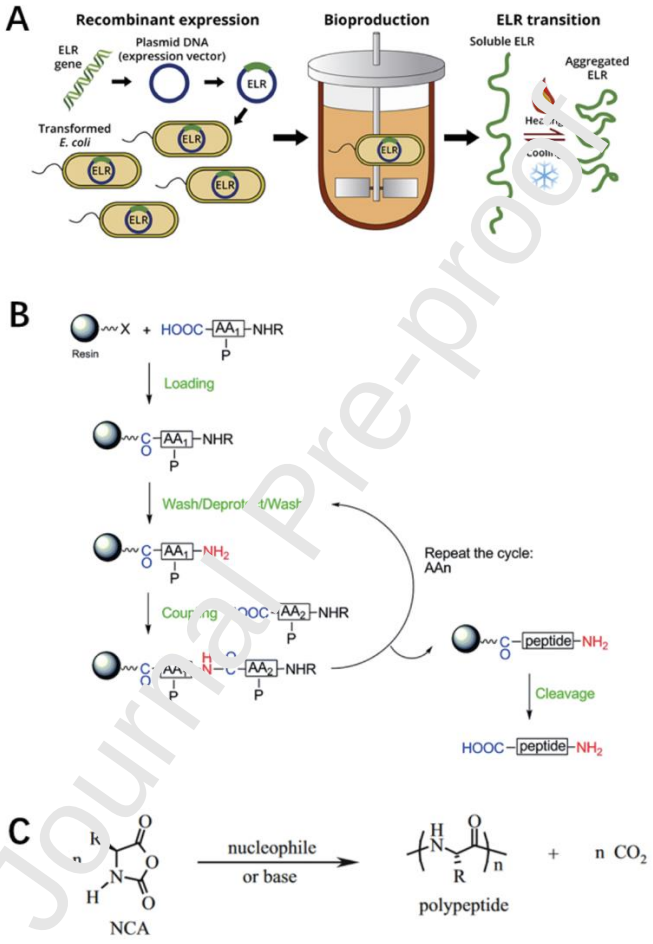

Figure 1. Recombinant DNA and organic synthesis approaches to peptide production. A) Schematic production of elastin-like peptide (represented as ELR (elastin-like recombinamer) in the scheme) by the recombinant DNA method. Reproduced with permission [24]. Copyright 2019 Elsevier. B) General scheme of solid-phase peptide synthesis (SPPS). Reproduced with permission [37]. Copyright 2011 RSC Publishing. C) Ring-opening polymerisation (ROP) of N-carboxyanhydride (NCA). Reproduced with permission [40]. Copyright 2011 Springer Nature.

2.2 Functionalisation of Peptides for BBB Targeting

One of the main non-invasive strategies to allow NPs to reach the brain consists of the introduction of specific targeting ligands that can interact with carrier proteins or receptors at the surface of BECs

to facilitate brain entry [49]. The ligands are conjugated at the NP surface, and this conjugation can be carried out either before (pre-formulation conjugation) or after (post-formulation conjugation) the formation of the NPs [17]. Both pre- and post-conjugation approaches have been applied to decorate polypeptide-based NPs for brain delivery. A fuller exposition of the effects of particular targeting ligands upon delivery efficacy is presented in Section 4.

In the pre-formulation conjugation approach, two methods can be employed to synthesise the polymer-ligand conjugates: "grafting-to" and "grafting-from". In the "grafting-to" method, the targeting ligands are conjugated to polymers by specific reactions with the end group or pendant side groups as a post-polymerisation modification [50]. A number of different reactions have been used for this strategy, including the nucleophilic attack of amino groups to N-hydroxysuccinimide esters (amine-NHS ester chemistry) [51,52], thiol-ene coupling of cysteine residues with alkene-containing groups such as maleimides (thiol-maleimide chemistry) [14,15,48], and "click" reaction of azide with alkyne to form a 5-membered triazole ring ("click" chemistry) [53,54]. With relatively low requirements on the substrates and reaction conditions, the "grafting-to" method has been widely employed for the conjugation of a plethora of ligands to polypeptides. As an example, Kataoka *et al.* introduced cyclic Arg-Gly-Asp peptide (cRGD, an integrin binding sequence overexpressed in tumour neovasculature) to PEG-*b*-P(Glu) micelles [14] bearing the anticancer agent (1,2-diaminocyclohexane)platinum(II) (DACHPt) [55,56] . Surface-tuneable micelles were first prepared by mixing methoxy-end-capped copolymers (MeO-PEG-*b*-P(Glu)) with maleimido-endcapped copolymers (Mal-PEG-*b*-P(Glu)) at a controlled ratio. Cysteine-containing cRGD ligand was then attached onto the micellar surface by thiol-maleimide chemistry (**Figure 2**), yielding DACHPt micelles with a range of 5-40% cRGD ligands. The biological performance of these micelles in fully described in section 4.2.5.

In contrast to "grafting-to", the "grafting-from" method is based on the introduction of the targeting moiety at the very first step of polymer synthesis either in the initiator or the monomer. Using this approach, Kataoka *et al.* synthesised a glucosylated polypeptide copolymer by sequential ROPs of ethylene oxide and β-benzyl-L-aspartate NCA (BLA-NCA), as initiated from a protected glucofuranoside (ligand-initiator) [13,57]. With further deprotections, the glucose-conjugated polymer Glucose(6)-PEG-*b*-poly(α,β-aspartic acid) (Gluc(6)-PEG-*b*-P(Asp)) was obtained and its assembly and *in vivo* biological outputs are described in section 4.1. Compared to "grafting-to", where the purity of the conjugated product relies on the efficiency of the conjugation reaction and a following quantification to determine the conjugation rate is necessary, the "grafting-from" method can guarantee the production of nearly 100% conjugated polymers, provided that the ROP reaction is well-controlled. However, multiple protection-deprotection and group substitution reactions were

involved in the whole procedure, which restricts the "grafting-from" method from wide-spread application in the preparation of ligand-polypeptide conjugates.

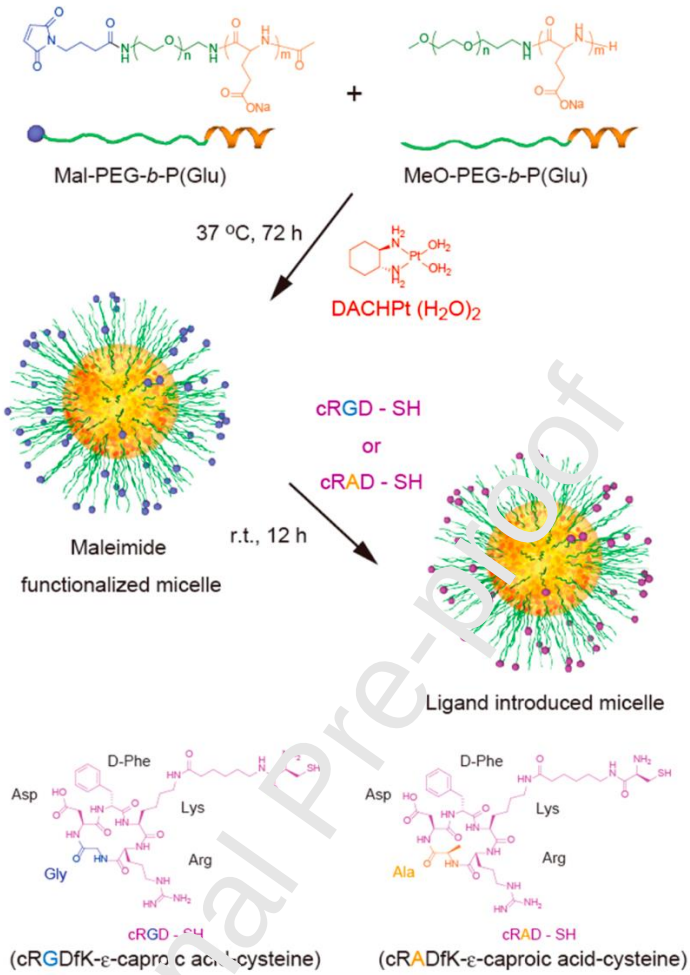

Figure 2. Introduction of cRGD ligand to PEG-b-P(Glu)/DACHPt micelles with the post-formulation conjugation strategy. Reproduced with permission [14]. Copyright 2013 American Chemical Society.

3 Peptide Self-Assembly

Controllably producing peptide nanostructures requires us to predict secondary and tertiary structure from amino acid sequence and the subsequent self-assembly behaviour of the polypeptide. The first of these is a major open problem in molecular biology [58], while the second is a major open problem in physical chemistry [59]. As such, most peptide self-assembly work still relies on rules of thumb developed for polymer self-assembly. However, the rich array of interactions between peptides, combined with the chirality, order, and rigidity associated with peptide secondary structure, mean these simple rules of thumb almost always fail to describe peptide self-assembly [60]. An extensive summary of progress in describing the fundamental aspects in this area is provided in a recent review by Raymond and Nilsson [61]; we focus here on work of particular relevance to drug delivery to the brain.

3.1 The Hydrophobic Effect in Peptide Self-Assembly

Amphiphilic molecules aggregate to reduce the interaction of a hydrophobic moiety with the surrounding aqueous phase. The structure that an amphiphile will self-assemble into can be estimated using the packing factor, $p = \frac{v}{a_0 l}$, which assigns an area, a_0, to the hydrophilic moiety and a length, l, and volume, v, to the hydrophobic moiety [62–64]. This can be seen as the ratio of two areas: a_0, and $\frac{v}{l}$; any difference in these two areas inherently leads to some curvature in the structure. This simple model is most applicable to low MW amphiphiles [65–67]. A similar trend can be seen in amphiphilic diblock copolymers where f, the mass of the hydrophilic block divided by the total mass of polymer, plays the largest single role in determining the morphology of the self-assembled structure [68]. The relation is even more qualitative but, roughly, when $f < 0.33$, vesicles are formed, when $0.33 < f < 0.55$ cylindrical micelles are formed, and for $f > 0.55$ spherical micelles are most often seen [63,69]. Kinetic factors and the greater conformational complexity of polymers also play a major role in determining system morphology [70,71]. Despite these limitations, varying f is still the most common method for targeting two of the most popular structures for drug delivery applications: micelles and vesicles (**Figure 3**).

Examples of vesicles made of amphiphilic block copolypeptides in the literature are rather scarce [72–81]. A major issue is that the anisotropic hydrogen bond networks between peptide backbones make the high, isotropic curvatures of micelles and vesicles energetically unfavourable. Manipulation of secondary structure, either by disruption of inter-peptide hydrogen bonding in β-sheets, or by using blocks that form α-helices (and hence use hydrogen bonds intramolecularly, to stabilise the helix), is a powerful approach to manipulate peptide self-assembly [79,82,83]. The Deming group have been particularly successful in applying the second approach to produce peptide vesicles [84–86]. Bellomo *et al.* produced giant vesicles using diblock copolypeptides based on ethylene glycol-modified lysine (P(Lys^P), poly(N_ϵ-2-(2-(2-methoxyethoxy)ethoxy)acetyl-L-lysine) and leucine [87]. The pegylated lysine adopted an α-helical conformation at physiological pH [88]. Forming the copolypeptide with one (or both) of the blocks comprising racemic amino acids disrupted helical structure, allowing the helicity of the polypeptide to be systematically varied. Dispersing highly helical P(Lys^P)$_{100}$-*b*-P(Leu)$_{20}$ in water resulted in the formation of micron-sized vesicles and a few, larger, sheet-like structures (Figure 3A). It is of note that for P(Lys^P)$_{100}$-*b*-P(Leu)$_{20}$, $f = 0.85$; in simpler amphiphilic diblocks this block size ratio would be expected to produce micelles, but here strong interactions between the peptide backbone drive vesicle formation for remarkably small hydrophobic segments. Holowka *et al.* studied the self-assembly of unmodified P(Lys)$_x$-*b*-P(Leu)$_y$ and P(Glu)$_{60}$-*b*-(Leu)$_{20}$ diblock copolypeptides, where x = 20-80 and y = 10-30

($f = 0.53 - 0.85$, which might be expected to produced highly curved structures) [89]. Systematically varying f and the degree to which either block was racemic yielded a range of structures including sheets, fibers, and irregular aggregates, though no clear trends emerged from the data. Again, this demonstrates the breakdown of simple geometric rules used to describe the self-assembly of simpler, amphiphilic block copolymers.

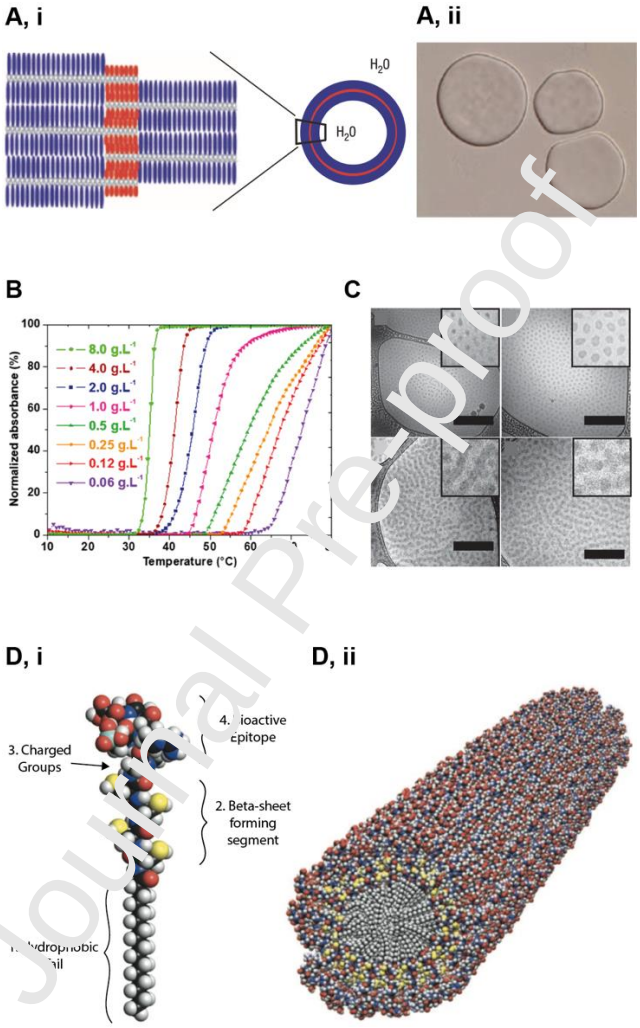

Figure 3. Peptide self-assembly driven by the hydrophobic effect. A, i) Helical diblock copolypeptides of $P(Lys^P)_{100}$-*b*-$P(Leu)_{20}$ assemble into A, ii) giant vesicles due to reduced intermolecular hydrogen bonding between helices. Reproduced with permission. Copyright 2004 Springer Nature. B) Absorbance as a function of temperature for a suspension of ELP-based polypeptides. Reproduced with permission [90]. Copyright 2019 American Chemical Society. C) TEM micrographs of self-assembled ELP-RLP structures undergoing a transition from spherical to non-spherical micelles as $\boldsymbol{f}$ is changed. Reproduced with permission [25]. Copyright 2017 American Chemical Society. D) Schematic showing i) the structure of a single peptide amphiphile and ii) its self-assembly into long nanofibers. From Hartgerink *et al.* [91]. Reproduced with permission from AAAS.

Studying block copolypeptides in which one block is synthetic yields more 'traditional' self-assembly behaviour, including familiar morphological transitions from micelles to worms to vesicles as the relative size of the hydrophilic group decreases [92–99]. The synthetic block can simplify the interactions between the polymers, add functionality from a well-understood polymer, and act as a macroinitiator for ROP of NCAs. Quadir *et al.* successfully reported the production of pH-sensitive vesicles using PEG-*b*-poly(g-propargyl L-glutamate) block copolymers and their use in delivering doxorubicin (DOX) to breast adenocarcinoma cells [100]. Chécot *et al*. compared varying lengths of both poly(butadiene)-*b*-poly(Glu) and poly(isoprene)-*b*-poly(Lys) and found that the copolymers that were comprised of <70 mol% peptide formed pH-sensitive vesicles [97]. They also found that changes in conformation of both polypeptides, induced by pH alterations, led to an increase in NP size [99]. Vesicle and micelle formation can also be seen when the *hydrophobic* block is synthetic [101]. Li *et al.* studied the self-assembly of polystyrene-*b*-poly(γ-propoargyl-L-glutamate-g-ethylene oxide) in organic solvent as a function of water concentration [102]. At low water concentrations, micelles were obtained. As water concentration was increased, morphological transitions to worms and vesicles were seen, reminiscent of the early work of Eisenberg *et al.* on polystyrene-*b*-poly(acrylic acid) (PS-PAA) polymers [103]. Several groups have used poly(butadiene)-*b*-P(Lys) copolymers to produce vesicles that exploit the ionisation and change in secondary structure of lysine at low pH to induce vesicle swelling [104,105]. Gebhardt *et al*. found that stable vesicles of a pH switchable diameter could be formed from PB-*b*-P(Lys) with the amino acid in a helical conformation at low- and intermediate pH and in a β-sheet conformation at high pH [104,106]. The latter observation of the polypeptides forming vesicles in a β-sheet conformation is particularly remarkable and may be the product of the deeply non-ergodic nature of these self-assembled structures.

Polypeptides with more complex amino acid sequences can yield more controlled self-assembly behaviour than 'simpler' diblock copolypeptides [27,90,107]. ELPs, initially described by Urry and co-workers [108,109], are based on the pentamer sequence VPGXG, where X represents a guest residue and can be any amino acid except proline. Weitzhandler *et al.* used recombinant synthesis to produce copolypeptides with an elastin-like block of $[XGVPG]_x$-Y and a resilin-like block (RLP) of G-$(QYPSDGRG)_y$N, where x = 20-80 and y = 40-160 (Figure 3B, Ref. [25]). Systematic variation of the guest peptide with Ser, alternating Ala and Gly, or Val, (from most to least hydrophilic) in the ELP allowed the hydrophobicity of the ELP to be systematically varied. ELPs exhibit a lower critical solution temperature (LCST) in water, meaning that they become insoluble with increasing temperature and, hence, self-assemble (Figure 3B). RLPs on the other hand exhibit an upper critical solution temperature, meaning that they become soluble with increasing temperature. This

produces a system in which the tunable geometry and solubility of the two blocks compete against one another to determine self-assembly conditions and assembled morphology. Studying the structures formed in response to a temperature increase allowed for the isolation of structures in which the ELP formed the hydrophobic block. Increasing the size of the corona-forming block in these systems led to a transition from non-spherical micelles to spherical micelles, similar to more traditional amphiphilic block copolymer systems (Figure 3C) [25,110]. Deviations from traditional behaviour were seen elsewhere, in which the tuning the hydrophobicity of an oligopeptide attached to an extremely large AGVPG ELP led to a micelle-to-worm transition, despite a negligible change in f [111]. As will be discussed in Section 4.3, ELP-based micelles have been more successfully translated into applications than the structures discussed so far.

Perhaps the standout examples of molecular engineering in synthetic peptide self-assembly are peptide amphiphiles (PAs, Figure 3D). At the simplest level, these molecules comprise an oligopeptide modified with a hydrophobic, lipid or lipid-like tail, thus combining the structural features of amphiphilic surfactants with the functions of bioactive peptides [112–114]. In a landmark work, Hartgerink *et al.* developed a PA that self-assembles into nanofibers based on a molecular design comprising 4 domains: (1) a hydrophobic alkyl tail, (2) charged amino acids to increase solubility, (3) a β-sheet forming peptide sequence, and (4) small bioactive sequences (Figure 3D, i) [91]. A reduction in pH was found to drive the self-assembly of the PAs into nanofibers with a uniform diameter of ≈7 nm, while an increase in pH led to dissolution of the fibers. Extensive variations on the design of Hartgerink *et al.* can be found in the literature. Generally, a PA's backbone is composed of 8-30 amino acids with a hydrophilic block, formed by polar amino acids, and a hydrophobic block with apolar amino acids or grafted alkyl, acyl, or aryl lipidic tails [115–117]. In some cases, PAs include a linker segment in between these two blocks, such as 2 kDa PEG or glycine, and a bioactive sequence that recognises targets in cells or tissues [113]. The interplay between hydrophobic, electrostatic, π-π interactions, and hydrogen bonding drives the self-assembly of PAs into wide array of well-defined nanostructures, including, micelles, vesicles, nanotubes, nanofibers, and nanosheets (Figure 3D, ii) [112,113]. As will be seen throughout Section 4, these structures show great promise in treating a number of brain disorders, however the rules describing the structures and given PA will self-assemble into remain poorly understood.

3.2 Polyion Complexation as a Pathway to Controlled Polypeptide Self-Assembly

The overwhelming majority of the peptide NPs successfully applied to brain delivery are peptide polyplexes. These structures are formed by complexation of oppositely charged polypeptides and are bound together by both electrostatics and counterion entropy [118].

Therapeutics can then be encapsulated within these NPs *via* electrostatics, preferential partitioning, or chemical attachment. Indeed therapeutics can even form the building blocks of polyplexes, such as in the pegylated dendrigraft poly(Lys) (DGL-PEG) NPs of the Jiang group that appear throughout the Section 4 [119]. Making polyplex NPs from diblock copolymers, in which one block is a polypeptide and another block is an uncharged synthetic block such as PEG, adds geometrical constraints that can produce spheroidal and hollow spheroidal structures similar to those produced by simple amphiphiles. The extensive body of work from the Kataoka group on the self-assembly of negatively charged PEG-*b*-P(Asp) and positively charged PEG-P(Asp-XX), where XX refers to a cationic modification to the aspartic acid block, is particularly significant here **(Figure 4**). Koide *et al.* first reported that these pairs of oppositely charged block copolymers undergo controlled self-assembly into hollow vesicular structures referred to as polyion complex polymersomes (PICsomes) [120]. Anraku *et al.* later found that, remarkably, the size of the self-assembled vesicles varied linearly with polymer concentration [121]. Wibowo *et al.* reported that the geometry of the PICsomes depended on both the temperature of self-assembly and the relative size of the PEG block [122], however no quantitative relations between these factors could be established. As with other polyplex NPs the materials from which PICsomes are made can be readily varied, such as the siRNA and PEG-*b*-P([*N*-(5-aminopentyl]-α,β-aspartamide] (PEG-*b*-P(Asp-AP)) PICsomes of Kim *et al* (Figure 4B) [123]. Micelle-like peptide polyplexes are also often reported [57]; however, this name is possibly a misnomer as the core of these structures is hydrophilic and it is not clear whether the polymer blocks are as highly segregated as one would expect in a true micelle. Semantics aside, these peptide polyplex 'micelles' can encapsulate a range of drugs and the structures themselves are highly amenable to modification. This tunability makes them ideal for fine-grained studies of the effects of functional group density upon NP interactions with biological tissues [57]. As we will see in Section 4, peptide polyplex NPs have demonstrated efficacy in brain delivery and benefit from being extremely simple to produce. However, a lack of understanding of the underlying physical chemistry that governs their self-assembly means that systematically engineering polyplex NPs is still a process of guess work.

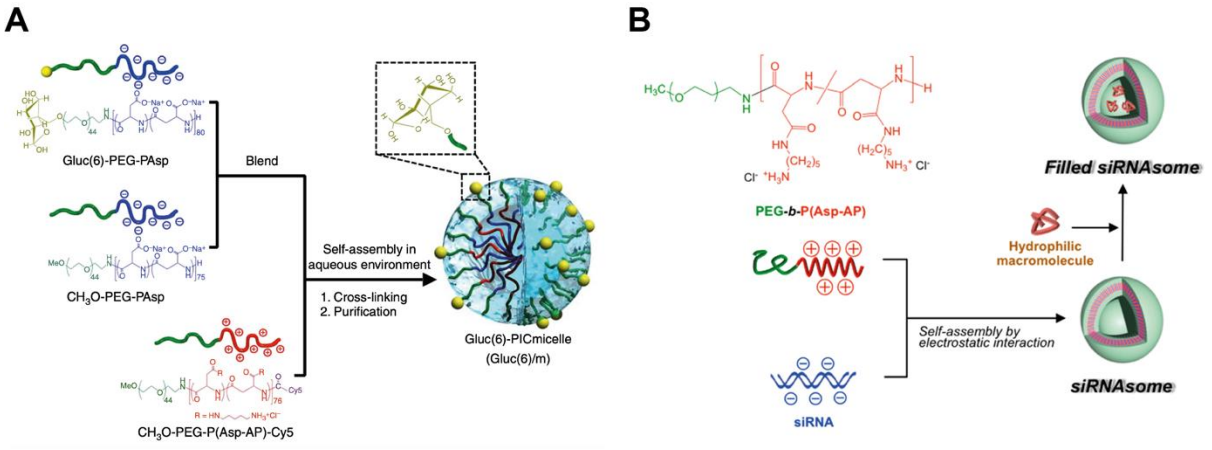

Figure 4. Functional structures from peptide complexation. A) Schematic showing a PIC "micelle" formed by the complexation of oppositely charged aspartic acid-based polypeptides. Reproduced under the terms of the CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/) [57]. Copyright 2017 the Authors. B) Schematic showing a vesicle-like PICsome formed from the complexation of siRNA and positively charged PEG-*b*-P(Asp-AP). Reproduced with permission [123]. Copyright 2019 American Chemical Society.

3.3 Rational Design is Possibly Not the Best Approach to Engineer Peptide Self-Assembly

The majority of efforts to apply a reductionist approach to peptide self-assembly fail. Indeed, multiple recent commentaries have argued that evolutionary methods, both *in silico* and experimental, may be more effective ways to study the self-assembly of complex molecules (**Figure 5**) [124,125]. Simulations-aided materials discovery is the oldest and most obvious of these approaches. Frederix *et al.* used coarse-grained molecular dynamics simulations to map out the self-assembly behaviour of all possible di- and tripeptides formed from the 20 proteinogenic peptides (Figure 5A) [126,127]. Pleasingly, good agreement with experiment was observed, and only a weak correlation was observed between the hydrophobicity of a tripeptide and its propensity to self-assemble. This second finding underscores the limitations in considering only the hydrophobic effect when designing peptide NPs. Sasselli *et al.* and Scott *et al.* studied the self-assembly of Fmoc-modified peptides and the production of tripeptide emulsifiers using a similar approach [128,129]. Smadbeck *et al.* used fully atomistic molecular dynamics to predict the self-assembly of acetylated tripeptides [130]. The effects of point mutations in tripeptides could then be studied systematically, allowing promising candidates for experimental studies to be identified. Given the far greater degree of detail in the simulations, they were naturally performed on smaller ensembles, for smaller times, and covered a less extensive range of parameter space than those of Frederix *et al.* However, running fully atomistic simulations allowed for a remarkably detailed study of bonding schemes

between peptides, as well as detailed investigation of the effects of peptide terminus acetylation, which is widely observed to impact peptide assembly [131,132].

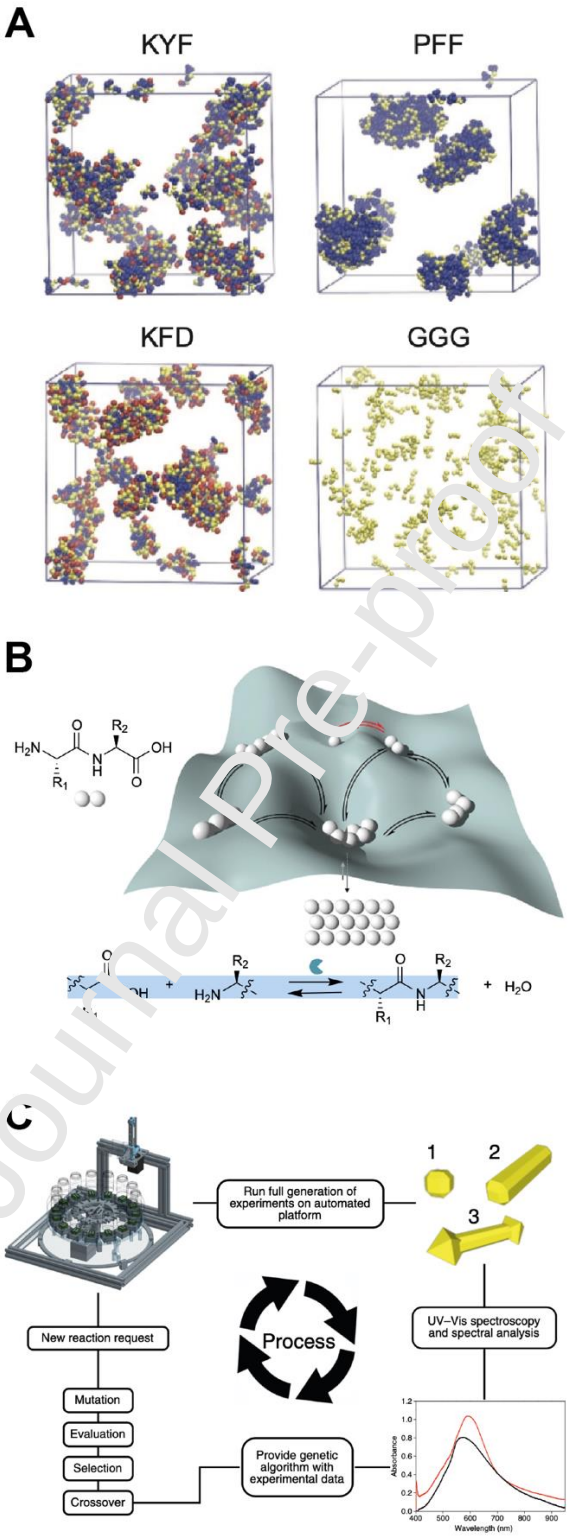

Figure 5. Evolutionary and computational approaches to peptide design. A) Appropriate coarse-graining and force-field parameterisation allows for the systematic study of the self-assembly behaviour of a broad palette of short oligopeptides. Reproduced with permission [127]. Copyright 2014 Springer Nature. B) Studying a solution containing a mixture of dipeptides in the presence of a relatively non-specific protease allows the system to probe its self-assembly free energy landscape

without external intervention. Reproduced with permission [133]. Copyright 2016 Springer Nature. C) Home-made experimental setups driven by a genetic algorithm allow for the optimisation of nanoparticle synthesis on the timescale of days without human intervention. Reproduced under the terms of the CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/) [134]. Copyright 2020 the Authors.

Self-assembly studies of large ensembles of longer peptides are beyond the reach of current simulations approaches and will likely remain so for some time; experimental approaches suffer from no such limitation. A dynamic combinatorial library approach, in which peptide combinations can be tested *in situ* and promising candidates either amplified or isolated are a powerful method for studying large numbers of permutations of peptides [135–137]. Pappas *et al.* applied these methods to study the self-assembly of unprotected molecules up to 8 residues long (Figure 5B) [133]. Mixtures of Leu, Phe, Trp, Ser, and Asp dipeptides were investigated in the presence of thermolysin, a relatively non-specific protease that does not cleave terminal peptide bonds. As such, dipeptides were stable in the presence of thermolysin, while oligopeptides were continuously formed and broken apart. Self-assembly into more thermodynamically stable structures retarded thermolysin-catalysed hydrolysis, meaning that oligopeptides with a greater propensity for self-assembly were inherently amplified with this technique. The system is naturally sensitive to initial conditions; different combinations of input dipeptides resulted in convergence upon different final structures. Diphenylalanine was found to evolve from a free-flowing tubular dipeptide assembly into a gelled dispersion of short rods comprising 80% F_6 after 15 days. By contrast, ditryptophan exhibit limited molecular transformation, with only around 20% of W_2 converting into larger oligomers. Combinations of FS and FD converged up to a single, highly amplified FDFSFDFS oligomer, reminiscent of the alternating sequences of charged/polar and hydrophobic motifs associated with β-sheet formation [138,139].

The method of Pappas *et al.* only allows peptide self-assembly to be optimised towards a single goal: thermodynamic stability. It is not obvious how one would target a specific function or structure this way, nor are the most useful structures necessarily the most thermodynamically stable. Autonomous exploration of parameter space using a genetic algorithm allows self-assembly to be engineered towards an *arbitrary* goal. Furthermore, to our knowledge, it is an entirely neglected area within peptide NP self-assembly, although it has long been used in related disciplines [125,140–143]. To illustrate the usefulness of this technique, we turn to work from the inorganic NP community. Salley *et al.* used an autonomous experimental platform made entirely from commercially or freely available parts to study gold NP synthesis (Figure 5C) [134]. With each iteration, a genetic algorithm sought reaction conditions that maximised the value of a user-defined fitness function. Within 10 generations, often significantly fewer, the setup had converged upon a

synthesis method for rods and spheres of low polydispersity and was even capable of being set an arbitrary goal, yielding novel, faceted structures. The versatility of this method is such that it could be readily and affordably applied to peptide NP self-assembly. Peptide NPs could then be optimised for any number of goals such as particle shape, size or polydispersity, target-receptor binding, or all of the above.

4 Transport to and Across the BBB

Negotiating the biological barriers that regulate transport of molecules through the body and into the brain is one of the biggest open problems in drug delivery today. The CNS functions are maintained by the meticulous coordination of the activity of multiple cells within a neurovascular unit (NVU), including vascular cells (endothelial cells, pericytes, and smooth muscle cells), glia (astrocytes, oligodendrocytes, and microglia) and neurons [144–146]. Within the NVU, the continuous non-fenestrated endothelium lining on the brain vasculature forms the BBB, which acts as a barrier that separates blood and brain compartments, and strictly regulates blood-to-brain and brain-to-blood transport of molecules [145]. BECs are tightly connected by tight junction proteins, such as claudin-3, -5, and -12, occludin, and ZO-1, -2, and -3, which impair exchange of molecules through the paracellular route (i.e., transport through the intercellular space between cells) between blood and brain. BECs are sheathed by other cells of the NVU, including pericytes and vascular smooth cells, and astrocyte end feet [147,148]. Pericytes shelter 60-70% of the basal endothelial surface, while astrocyte end feet reach up to ≈99% of the surface, overlapping pericytes and contributing to the barrier properties of the BECs [147–149]. In contrast to the peripheral endothelium, BECs exhibit a low rate of transcytosis regulated by specific proteins [150,151] which, together with the sealed cell-to-cell contacts of the endothelium, restrict the entry of blood-derived molecules into the brain. Apart from small lipophilic molecules (< 400 Da) with a polar surface area (< 60-70 Å) and weak hydrogen-bonding potential (< 6 hydrogen bonds), which may cross the brain endothelium by diffusion [49], other molecules require specialised carriers or receptors in the apical surface of BECs to facilitate their transport across the BBB. In the following section, we describe carrier-, receptor- and adsorptive-mediated transport across the BBB (**Figure 6**), specifically for shuttling of peptides and their use by the community to transport peptide-based NPs.

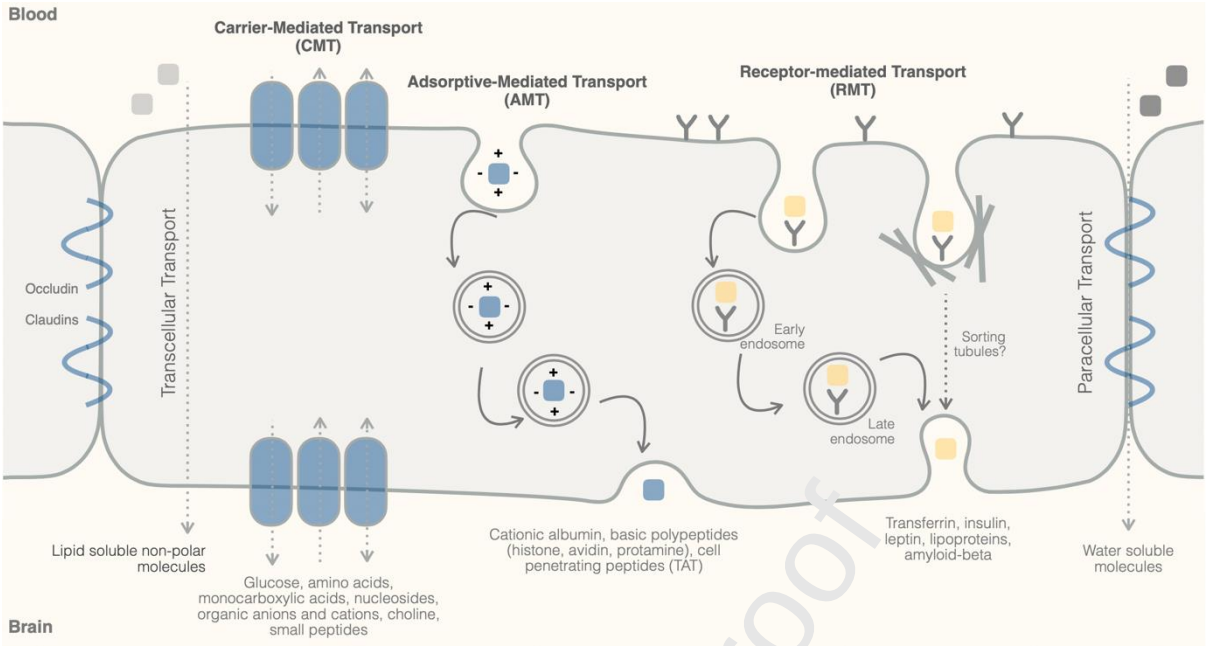

Figure 6. Schematic representation of the mechanisms of transport across the BBB. Small lipid-soluble molecules (MW < 400 kDa) passively diffuse through the BECs (transcellular transport). Water-soluble molecules are transported through the intercellular space between the BECs (paracellular transport). Carrier proteins on the apical membrane surface of BECs facilitate the transport of specific substrates, such as glucose and small peptides (carrier-mediated transport, CMT). Adsorptive-mediated transport (AMT) appears to be triggered by interaction of polycationic peptides with anionic components at the surface of the BECs. Receptor-mediated transport (RMT) facilitates the transport of a variety of macromolecules (such as, peptides and proteins) by binding to a specific receptor, followed by intracellular trafficking via endosomal and, possibly, tubular sorting, and exocytosis.

4.1 Carrier-mediated Transport

Carrier-mediated transport (CMT) refers to transport mediated by a membrane carrier protein. CMT mediates transcellular transport of carbohydrates (glucose), neutral, basic, and acidic amino acids, monocarboxylic acids (lactate, pyruvate, ketone bodies), nucleosides (adenosine, guanosine, uridine), fatty acids, organic anions and cations, amines, choline, and vitamins [145,146]. BECs express membrane carrier proteins at their apical and/or basal surface to mediate transport of small peptides from blood-to-brain and/or brain-to-blood. Mounting evidence suggests that small neuroactive peptides (< 10 amino acids), including arginine-vasopressin (AVP) [152], tyrosine melanocyte-stimulating inhibitory factor-1 (Tyr-MIF-1) [153], enkephalin [154,155], delta-sleep inducing peptide (DSIP) [156], peptide T analogue [157], and luteinising hormone-releasing hormone (LHRH) [158] permeate across the BBB through distinct saturable peptide transporter systems (PTS). PTS-1 and -2 facilitate an efflux of Tyr-MIF-1/enkephalins and AVP from brain to blood, respectively. PTS-3 mediates unidirectional transport of peptide T analogue from blood to brain [157,159], while PTS-4 transports LHRH bidirectionally [158]. Although the transport of these small neuropeptides

was extensively investigated in the 1980s, a comprehensive study is still missing to fully characterise PTS at the BECs. Apart from PTS, the organic anion transporting polypeptide-2 (OATP-2; *SLC21A3*) is strongly expressed at the BBB in humans, and findings suggested that OATP-2 mediates transport of opioid peptides, such as [D-penicillamine-2,5] enkephalin and deltorphin (II), across the BBB [160]. Glutathione is a tripeptide (CEG) and, similar to other small peptides, a saturable and specific transporter has been identified for transport across BECs [161,162]. Among these carrier proteins expressed at BECs, glutathione has been explored for drug delivery into the brain (G-Technology®, a PEGylated liposomal formulation for delivery of systemically administered therapeutics, 2BBB, Leiden, Netherlands). Although not fully characterised, the glutathione transporter is the main endogenous carrier protein considered for CMT of peptides across BECs [163]. Apart from these carriers, which mediate the transport of small peptides, other carrier proteins have been widely studied to allow the BBB crossing by means of ligand-installed peptide-based NPs, including GLUT1, choline and nicotinic acetylcholine receptor (nAchR) (**Table 1**).

Table 1. Carrier proteins at the brain endothelium initiating carrier-mediated transport.

Carrier Protein	Substrates	Direction
PTS 1-5	Arginine-vasopressin, tyrosine melanocyte-stimulating inhibitory factor-1, enkephalin, peptide-T, delta sleep-inducing peptide, luteinising hormone-releasing hormone	Blood-to-Brain or Brain-to-Blood
OATP-A	[D-penicillamine-2,5] enkephalin, deltophin II	-
Glutathione SH	Glutathione SH	Blood-to-Brain
GLUT1	Glucose	Blood-to-Brain
Choline	Choline	Blood-to-Brain
nAchR	Rabies virus glycoprotein	-

In CMT, one of the most exploited transporters is GLUT1, which mediates the transport of glucose. Recently, Kataoka *et al.* reported the use of glucose-bearing NPs to cross the BBB by means of glycaemic modulation, in which fasting was used as an external trigger to provoke rapid glycaemic increase to then trigger GLUT1 to undergo transcytosis [57]. In this work, PIC micelles self-assembled from the oppositely charged block copolymers PEG-P(Asp) and PEG-poly([5-aminopentyl]-α,β-aspartamide) (≈ 30 nm diameter) were functionalised with varying densities of glucose (% glucose = 10, 25 and 50) by introducing different amounts of Gluc(6)-PEG-P(Asp) in the self-assembly.

Interestingly, glucose density within the NPs played a key role in directing NPs towards different brain cells. NPs bearing 25% glucose were mostly found in neurons and microglia while NPs containing the highest density (50%) accumulated within the brain capillary walls. In a more recent study, the authors delivered antisense oligonucleotides (ASO) to the brain following this on-off switch glycaemic control strategy [12]. PEG micelles of ≈ 45 nm in diameter consisting of positively charged PEG-P(Lys) were modified with 3-mercaptopropyl amidine, 2-thiolaneimine, and negatively charged ASO. Different glucose amounts, ranging from 0 to 103 ligands per particle, were produced by using Gluc(6)-PEG as initiator for the ROP of Lys(TFA)-NCA. PIC micelles exhibited half-lives of circulation in blood of > 80 minutes regardless of glucose density. However, NPs bearing 52 units of glucose showed by far the highest brain accumulation after IV injection (6.9 ± 1.9% injected dose (ID)/g of brain, 17-fold increase compared to the naked NPs) with a wide distribution within the brain, and the highest amount observed in the cerebral cortex (26 ± 7% of accumulation amount within the brain) (**Figure 7**). These results underscore the importance of both binding avidity and affinity and correlate well with recently reported models [. 64]. The optimised 52 units glucose-bearing PIC micelles were employed to deliver a model ASO and showed efficient and consistent knockdown in various brain regions. Additionally pH- and reduction-sensitive GLUT-1 targeting PIC micelles constructed from PEG-P(Lys) modified with 3-(2-pyridyldithio)propionate were also recently employed by the Kataoka group to deliver the amyloid-beta (Aβ) aggregation inhibitor 3D6-fragment antigen-binding (Fab) [13]. These PIC micelles achieved 41-fold increased brain accumulation compared to the free Fab and improved Aβ aggregation inhibition when multiple doses were applied to an Alzheimer's disease (AD) mouse model.

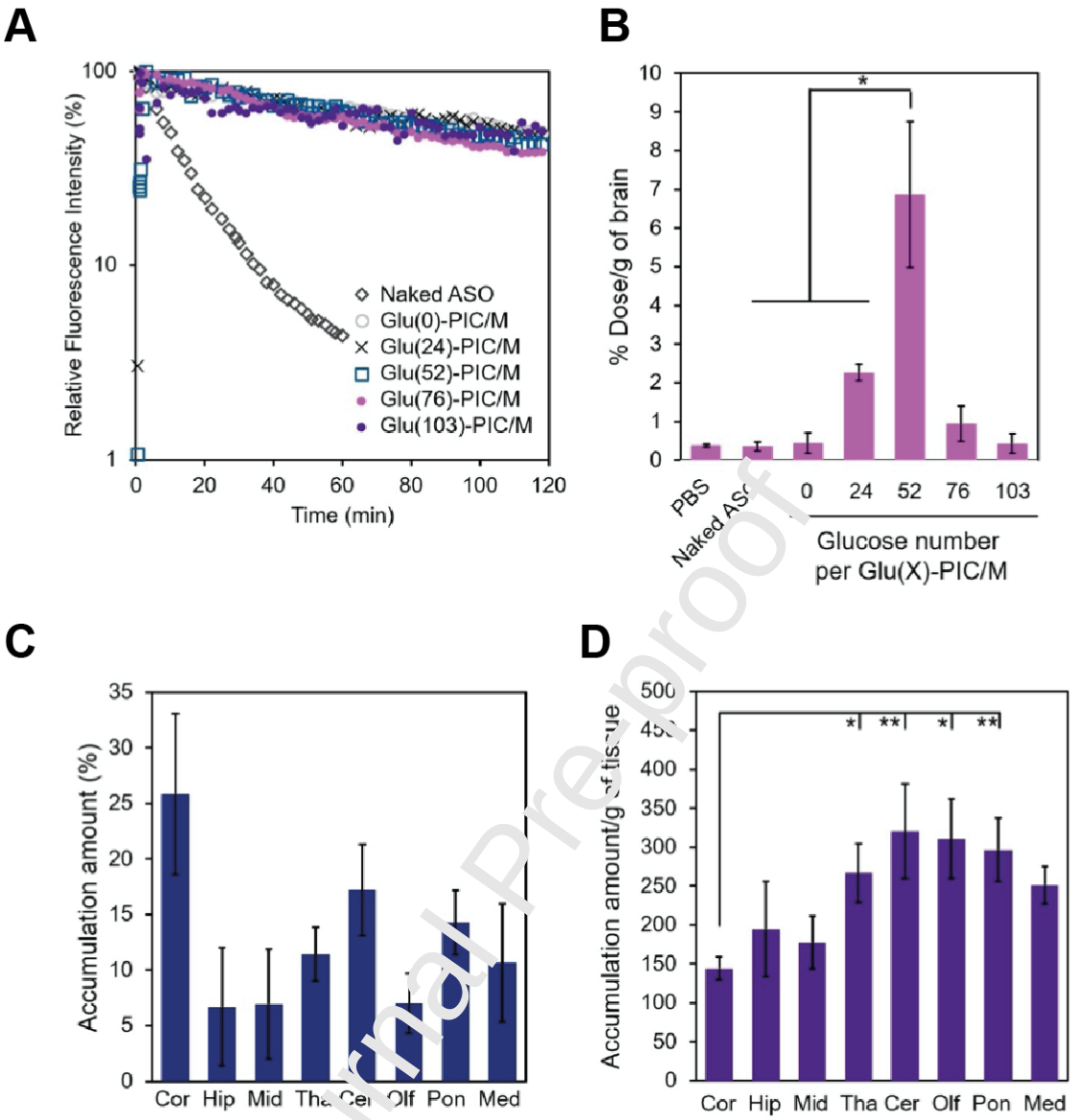

Figure 7. *In vivo* performance of Glu(X)-PIC/micelles (Ms) after intravenous administration. A) Blood circulation time of Glu(X)-PIC/Ms after intravenous injection. B) Whole brain accumulation of Glu(X)-PIC/Ms at 1 h post-administration. Glu(X)-PIC/Ms were intravenously injected 30 min after an intraperitoneal injection of glucose into fasting mice. C and D) Accumulation amount (%) in the different brain regions treated with Glu(52)-PIC/M at 1 h post-injection as obtained by normalising fluorescence intensity with the one obtained for the whole brain (C) and further weight-normalized (D). Cor: cerebral cortex, Hip: hippocampus, Mid: midbrain, Tha: thalamus/hypothalamus, Cer: cerebellum, Olf: olfactory bulb, Pon: pons, Med: medulla. Reproduced with permission [12]. Copyright 2020 Wiley-VCH.

In a different example, choline-derivatives based on bis-quaternary ammonium compounds with high BBB-choline transporter affinity have been conjugated to pegylated dendrigraft poly(Lys) to allow the transport of DGL-PEG/plasmid DNA (pDNA) NPs [165]. DGL-PEG NPs (≈ 90 nm), formed by electrostatic interactions, demonstrated an enhanced BBB permeability compared to pristine NPs *in vitro* (4-fold increase in permeability at 15 min) and significantly higher brain accumulation with almost 1.5-fold increase gene expression *in vivo* compared to pristine NPs. This system was

employed to co-deliver an encoding plasmid open reading frame of human tumour necrosis factor-related apoptosis-inducing ligand (pORF-hTRAIL pDNA) and DOX for the treatment of glioma [166]. NPs showed increased brain tumour accumulation, enhanced apoptosis *in vivo,* and superior antitumor efficacy when tested in glioma-bearing mice with prolonged median survival time (almost double compared to saline group and at least 1.2 times better than single therapy groups).

Another ligand to trigger CMT is the rabies virus glycoprotein peptide (RVG29), a 29 amino-acid peptide originated from the rabies virus glycoprotein that specifically binds to the nAchR. By using DGL-PEG-RVG29 NPs carrying a caspase-3 short hairpin RNA (shRNA) encoding plasmid DNA, the level of activated caspase-3 was reduced in a Parkinson's disease (PD) animal model with an increase in the rescue of dopaminergic neurons [167]. Consequently, PD rats treated with these nAchR-targeting NPs showed an enhancement of locomotor activity compared to rats treated with pristine NPs. Similar DGL-PEG-RVG29 NPs were employed to deliver a combination therapy consisting of a therapeutic gene and peptide for AD [168]. Using a gene therapy towards ß-secretase 1 (BACE1) and a specific D-amino acid based-peptide to target Aß accumulation and phosphorylated Tau, respectively, DGL-PEG-RVG29 NPs successfully reduced Aß plaques as well as neurofibrillary tangles in the cortex and hippocampus of an AD mouse model, which resulted in an improvement of memory.

4.2 Receptor-mediated Transport

Large peptides and proteins, such as transferrin (Tf), low density lipoproteins, and insulin, rely on receptor-mediated transport (RMT) through specific receptors at the surface of BECs for transcytosis across the brain endothelium [169,170] (**Table 2**). Transcytosis via RMT includes four steps: 1) a circulating ligand binds to a cognate receptor expressed on the apical membrane of BECs (e.g., Tf binds to transferrin receptor, TfR); 2) endocytosis takes place through membrane invaginations and eventually the formation of an intracellular vesicle containing receptor-ligand complexes; 3) intracellular trafficking occurs through vesicular and/or vesicular-tubular structures; and then 4) exocytosis occurs with the release of the vesicular content into the brain parenchyma [171]. In BECs, the initial step of transcytosis - endocytosis - is mainly facilitated by clathrin-mediated endocytosis (CME). Conceptually, CME is a fairly simple process that consists of a few sequential and partially overlapping steps [172]. CME is initiated by the clustering of endocytic coat proteins on the inner leaflet of the apical membrane (initiation), which is further continued by the recruitment of other coat proteins (recruitment). Consequently, the assembly of these coat proteins prompts membrane bending, which transforms a flat membrane into a "clathrin-coated pit" (membrane bending). Scission constricts and cuts the neck of the invagination to separate the clathrin-coated

vesicle from the apical membrane. In mammals, this scission step is mediated by dynamin and Bin/Amphiphysin/Rvs (BAR)-containing proteins, including amphiphysin and endophilin. Finally, uncoating disassembles the endocytic protein machinery, releasing the receptor-ligand complex-filled vesicle (≈ 100 nm in diameter) for further intracellular trafficking [172]. So far, multiple receptors have been shown to undergo CME at the BBB, such as TfR and insulin receptors (IR), and also low density lipoprotein receptor-related protein 1 (LRP1) [173,174].

Notwithstanding the prevalence of CME, other clathrin-independent endocytic mechanisms regulate transcytosis at the BECs, such as caveolin-mediated endocytosis. Caveolae are small flask-shape organelles (60 to 80 nm) containing oligomeric caveolin-1 [175]. In BECs, caveolae assembly at the membrane is negatively regulated by the major facilitator superfamily domain-containing 2a (Mfsd2a), which maintains the integrity of the BBB [150,151]. In dysfunctions of the CNS, transcytosis rate is increased in BECs, and caveolae vesicles are implicated as the main contributors to leakage of the BBB [176–178]. Whether caveolin-1 is recruited for caveolin-mediated endocytosis at BECs under physiological conditions has yet to be elucidated. Further clathrin-independent pathways, including fast-acting tubulovesicular endophilin-mediated endocytosis [179], remain unexplored in BECs. Hence, while the mechanisms of endocytosis are investigated for epithelial or peripheral endothelial cells, comprehensive studies focusing on endocytic pathways at BECs are crucial to fully decipher transcytosis across the BBB.

Once the endocytic vesicles are internalised via an endocytic pathway, intracellular trafficking occurs through a vesicular endosomal sorting network. Endosomal sorting determines the fate of these vesicles filled with the receptor-ligand complexes – either degradation by trafficking into lysosomes or transcytosis by fusion with the basal membrane of the BECs. The itinerary of intracellular trafficking is a highly orchestrated process involving sorting of the endosomal content by numerous regulatory proteins [180]. Given that endosomal sorting occurs across other tissues, it is conceivable that BECs employ similar mechanisms. However, intracellular sorting mechanisms that control successful transcytosis in BECs are not yet fully identified. Interestingly, a few studies established that BECs regulate transcytosis mediated by transferrin receptor [181] and LRP1 [164] through sorting tubules that, consequently, facilitate a faster shuttling across the BECs. Yet, the specific intracellular itineraries associated with the regulatory proteins involved in transcytosis through the different endocytic pathways are still to be deciphered. What determines whether a cargo undergoes transcytosis or degradation? Hence, future studies addressing the mechanisms regulating transcytosis in the brain will reveal opportunities for hijacking RMT for brain drug delivery strategies. Despite all these open questions, RMT is currently exploited for shuttling peptides and

ligand-bearing NPs across the BECs, specifically, by transferrin, insulin, leptin and low-density lipoprotein receptors, as well as receptor for advanced glycation end product (RAGE).

Table 2. Receptors at the brain endothelium initiating receptor-mediated transport. Adapted from Abbott *et al.* [145]

Receptor	Ligands	Direction
TfR	Transferrin, monoclonal antibodies: 8D3, R17-217, and OX26, and B6, 9-mer CRT, 7-mer HAI and 12-mer THR peptides	Blood to Brain
IR	Insulin, monoclonal antibody HIRMAb	Blood to Brain
LepR	Leptin and leptin-derived peptides, Lep70-89 and leptin-30	Blood to Brain
LRP1	Lipoproteins, apolipoprotein E (ApoE), α_2-macroglobulin, aprotinin, amyloid-beta, angiopep-2, L57 and RAP12 peptides	Blood to Brain and Brain to Blood
RAGE	Glycosylated end products S100/calgranulins, HMGB, amyloid-beta, 16-23 and RP1 peptides	Blood to Brain

4.2.1 Transferrin Receptor

TfR is abundantly expressed by BECs to mediate transport of iron into the brain [182]. A cognate ligand for the TfR is the iron binding protein Tf, which is a transmembrane glycoprotein consisting of two 90 kDa subunits, linked by intermolecular disulfide bonds and with each subunit binding to one molecule of Tf [183]. Although Tf is a specific ligand to TfR, Tf is a questionable targeting moiety for drug delivery, mainly due to the fact that only a small amount of Tf is transcytosed across the BECs [184,185]. Thus, monoclonal antibodies against the TfR, which bind to epitopes on the extracellular domain of TfR distal to the Tf binding side circumventing competition with endogenous Tf, have been widely developed for RMT-based delivery [186,187]. OX26 (murine monoclonal antibody against rat TfR), 8D3 and R17-217 (murine monoclonal antibodies against mouse TfR) exhibited brain uptake in mice [188]. Both 8D3 and R17-27 antibodies exhibited high transport, with brain uptake of 3.1 and 1.6% of the ID/g, respectively, while OX26 uptake was 25 to 50 times lower, with only 0.06% ID/g [188]. Based on these findings, TfR antibodies have been explored for brain delivery by conjugation to a therapeutic cargo, such as brain-derived neurotrophic factor (BDNF) [189], basic fibroblast growth factor (bFGF) [190], and nerve growth factor (NGF) [191], or by genetically engineering chimeric proteins for tumour necrosis factor receptor (cTfRMAb-TNFR), anti-Aß antibodies (cTfRMAb-ScFv), glial cell line-derived neurotrophic factor (cTfRMAb-GDNF), and

erythropoietin (HIRMab-EPO) [192]. However, few studies investigating the transport of anti-TfR monoclonal antibodies have demonstrated that, despite the substantial binding to BECs, the transcytosis into the brain parenchyma is limited with TfR antibodies accumulating in the BECs upon endocytosis [193–195]. This accumulation occurs possibly either due to a lack of antibody dissociation from the TfR upon endocytosis or because the intracellular trafficking of the TfR is affected by the binding interaction with the antibody.

A recent series of studies suggests that engineering Tf antibody binding properties (affinity and avidity) affects intracellular trafficking and, consequently, transcytosis [181,196–198]. By engineering two constructs with a single chain (sc) Fab fragment of an anti-TfR monoclonal antibody fused either to one (sFab) or both (dFab) C-terminal ends of the heavy chain of an anti-Aβ monoclonal antibody, it was shown that the monovalent binding (sFab) increases Aß target by 55-fold compared to the bivalent (dFab) antibody [197]. *In vitro* and *in vivo* evidence demonstrated that monovalent binding mode (sFab) facilitates transcellular transport, whereas bivalent mode (dFab) leads to lysosomal degradation. In a later study, it was further shown that a monovalent TfR-based construct (sFab) is sorted for transcytosis through intracellular tubules while the bivalent construct (dFab) transport is impaired by cellular sorting into lysosomes [181]. Apart from these monoclonal antibodies, peptides interacting with TfR were found by phage display biopanning, including B6 peptide [199], 9-mer CRT peptide (CRTIGPSVC) [200], and 7-mer HAI or T7 (HAIYPRH) and 12-mer THR (THRPPMWSPV) peptide [201,202]. Due to practical reasons, the use of small peptides as targeting moieties in NPs to promote BBB entry, is preferred over conjugation of large antibodies. T7, was for instance, employed by Liu *et al.* to co-deliver to the brain pORF-hTRAIL pDNA and DOX using DGL-PEG [203]. DOX was conjugated through a pH-labile hydrazone linker for pH-dependent release. Combination-bearing T7 NPs of 170 nm diameter in size and 3 mV ζ-potential showed synergistic effects (*in vitro* and *in vivo*) and increased in almost twice the survival time of brain-tumour bearing mice when compared with any of the tested controls. DGL-PEG-T7 NPs efficiently delivered RNA interference (RNAi) to several brain areas (cortical layer, caudate putamen, hippocampus, and substantia nigra) for glioma treatment [204]. Gene silencing experiments revealed a more than 2-fold increase in *in vivo* gene silencing of T7-NPs compared to pristine NPs when tested in an orthotopic human glioma mice model using U-87 MG-luciferase cells. Apart from these studies using polypeptide nanocarriers, in a recent study, Wu *et al.* engineered an amphiphilic derivative from the GYR peptide (GYRPVHNIRGHWAPG), identified by phage display biopanning, that shows specific binding to BECs [205]. By adding a cysteine to induce cross-linking (*via* disulfide bridge formation) between two adjacent peptides and a fluorophore to the amino group of the cysteine to initiate π-π stacking among conjugates to the GYR peptide, the resulting amphiphile (CGY) self-

assembled into fiber-like structures [206]. *In vitro* studies showed that CGY PA interacts with BECs through TfR and RAGE, while intravenous injection of Cy5.5-CGY in mice resulted in targeting of the brain with a peak of the levels at 4 hours (5.7% of ID) [206]. Furthermore, CGY-BACE1-siRNA complexes showed an effective BACE1 down-regulation in the brain without toxicity and inflammation.

4.2.2 Leptin Receptor

Leptin receptor (LepR), or OB-R, is a single transmembrane glycoprotein belonging to the family of cytokine receptors. There are two isoforms of LepR: the short (OB-Ra), which is present in the brain endothelium, and the long one (OB-Rb) [207]. A cognate ligand for LepR is leptin, a protein (16 kDa) that regulates adipose tissue activity and appetite by acting in the CNS (i.e., hypothalamus), after secretion into the blood circulation by adipose tissues. At the BECs, leptin is transcytosed via the LepR (OB-Ra) [208,209], and consequently LepR targeting has been explored through the use of leptin-derived peptides, such as Lep70-89 [210] and 30-mer peptide (leptin-30) [211]. DGL-PEG-Leptin-30 was used to complex pDNA by electrostatic interactions forming NPs of around 140 nm in diameter [211]. *In vitro,* DGL-PEG-leptin-30 NPs demonstrated a transfection efficiency comparable to the commercial transfection agent Lipofectamine2000 and 4-fold increase in transfection when compared to pristine NPs. Additionally, DGL-PEG-leptin-30 NPs showed *in vitro* and *in vivo* permeability across the BBB with reduced cytotoxicity, and *in vivo* the DGL-PEG-leptin-30/luciferase pDNA NPs yielded a 2-fold increase in luciferase expression compared to that of DGL or DGL-PEG/pDNA NP controls.

4.2.3 Low-density Lipoprotein Receptors

The low-density lipoprotein receptor (LDLR), a single transmembrane glycoprotein that recognises low-density lipoprotein (LDL) particles, and LDLR-related proteins (LRP1 and LRP2) are abundantly expressed at BECs to mediate the transcytosis of lipoproteins and other ligands [212]. Specifically, LRP1 is a multifunctional receptor that interacts with a range of ligands, such as apolipoprotein E (ApoE), α_2-macroglobulin, aprotinin, amyloid precursor protein, and Aß [213]. One of the first examples of targeted gene delivery to the brain by means of P(Lys) nanocarriers was reported by Mousazadeh and co-workers using this receptor to shuttle them across the BBB [214]. An LDL receptor-binding peptide dimer (LRKLRKRLLR-LRKLRKRLLR) derived from its endogenous ligand ApoE was conjugated to $P(Lys)_{16}$. This $P(Lys)_{16}$ NP system was used to complex a reported DNA plasmid encoding for β-galactosidase (β-Gal). In this study, although a proof of concept was obtained with an

increased expression of β-Gal in the brain (increased by 600 times by using 30 ng/mg of NPs), brain targeting was lost due to a high level of expression of β-Gal in other organs. A variation of $P(Lys)_{16}$ non-viral vector functionalised with a different ApoE-derived sequence (LRVRLASHLRKLRKRLLRDA) has been successfully used for the delivery of an array of both small drugs and proteins to the brain via intravenous (IV) injection [215–218]. However, the development of these $P(Lys)_{16}$ NPs has been hampered by a peptide dose-dependent toxicity associated to an unexpected inhibition of acetylcholinesterase, which still remains to be fully understood [219].

In 2008, Demeule *et al*. identified angiopep-2, a 19-amino acid peptide (TFFYGGSRGKRNNFKTEEY-OH), by aligning the amino acid sequence of aprotinin and other LRP1-binding proteins containing a Kunitz domain [220,221]. Angiopep-2 peptide showed improved transcytosis across BECs (3-7-fold increase) and parenchymal accumulation in comparison to aprotinin [221]. Based on these findings, Angiochem (Montreal, Canada) developed a range of angiopep-2-drug conjugates such as, paclitaxel (ANG1005), anti-HER2 monoclonal antibody (ANG4043), DOX (ANG1007), and neurotensin (ANG2002), for the treatment of brain and brain-related disorders. In particular, the ANG1005 is currently in Phase II clinical trial for the treatment of recurrent brain metastases [222,223]. Therefore, angiopep-2 is, possibly, the most exploited LRP1-ligand for the design of nanocarriers targeting the brain. In one example concerning peptidic nanocarriers, angiopep-2 was attached to DGL-PEG [224]. Spherical DGL-PEG NPs (≈ 120 nm in diameter) were formed from condensing the neuroprotective gene encoding human *GDNF*. However, angiopep-bearing NPs showed only moderate improvements in locomotor activity and dopaminergic neuron recovery, after multiple IV administrations in a rotenone-induced chronic PD rat model. Gao *et al.* attached angiopep-2 to PEI-P(Lys)-PEG polymers, and complexed PEI-P(Lys)-PEG with Herpes Simplex Virus Type 1 Thymidine Kinase (HSV-TK) pDNA for the treatment of GBM [48]. Imaging studies demonstrated NP accumulation in the brain cortex and striatum, while IV injection of HSV-TK-carrying NPs together with intraperitoneal (IP) injection of the antiviral ganciclovir, exhibited significant anti-tumour effects in an orthotopic GBM mouse model increasing the survival by 10 days. Although promising results were observed *in vivo*, there is a lack of ligand density optimisation in these examples, and thus only moderate therapeutic outcomes were reported so far. Interestingly, our recent study demonstrated that avidity of angiopep-2-functionalised nanocarriers affects intracellular trafficking and ultimately transcytosis across BECs [164]. A high number of angiopep-2 peptides at the surface of the nanocarriers (polymersomes) directs LRP1 towards an endosomal sorting and lysosomal degradation, while a mid-avidity favours the transport across BECs through syndapin-2-stabilised tubular structures.

Apart from angiopep-2, L57 peptide (TWPKHFDKHTFYSILKLGKH-OH) was identified by phage display biopanning for the targeting of LRP1, showing significant brain uptake in mice [225]. The receptor-associated protein (RAP) is a chaperone protein that, tightly binds to LRP1, and participates in folding and trafficking of LRP1. Pan *et al.* demonstrated that RAP is relatively stable in blood for ≈ 30 minutes, and permeates intact across the BECs (0.5-1% ID/g in the mouse brain, 30 minutes after IV injection) [226]. Recently, using computer-aided design, a short RAP12 (EAKIEKHNHYQK, amino acids 251-262 of RAP) peptide was identified for LRP1-mediated transport across the BBB [227].

4.2.4 RAGE

RAGE, a transmembrane protein of the immunoglobulin superfamily, is a multiligand receptor, which binds to advanced glycation end products (AGEs) (i.e., adducts from non-enzymatic glycation of proteins and lipids), S100/calgranulins, HMGB and Aß protein [228]. At the BBB, RAGE mediates the transport of Aß from blood-to-brain contributing to the formation of Aß plaques [229]. In the Aß peptide, the major binding site to RAGE is localised to an eight amino acid stretch of residues at position 16-23 (KLVFFAED) – a series of hydrophobic residues flanked by two negatively charged amino acid residues at the C-terminal [230]. In addition to the 16-23 peptide, the RP1 peptide (APDTKTQ), identified by phage display biopanning with homology to 16-23 regions of Aβ shows a high-affinity to RAGE [231]. Due to its function in Aß transport, this receptor has been widely explored to shuttle NPs towards AD treatments. In one example, KLVFFAED-tagged PEG-P(Lys)-based micelles with reactive oxygen species (ROS)-responsiveness and scavenging ability provided by the phenylboronic groups attached to the polymer backbone, were used to encapsulate the AD multitarget drug curcumin [232]. Curcumin targets several pathological hallmarks of AD by inhibiting/disrupting Aβ plaques formation [233,234], clearing phosphorylated Tau [234], inhibiting acetylcholinesterase (AChE) [235], acting as antioxidant activity for oxidative stress [236] and modulating several steps of the inflammatory cascade [237]. The peptide KLVFFAED [238] was attached to PEG prior micelle formation and drug encapsulation leading to Aβ-PEG-P(Lys)B/CUR spheres of ≈ 65 nm in diameter. In an AD mouse model, Aβ-PEG-P(Lys)B/CUR elicited *in vitro* neuroprotection towards oxidative stress and Aβ toxicity, as well as, an improvement in memory and Aβ burden *in vivo* (**Figure 8**). The neuroprotection effects of these NPs were attributed to a synergy caused by the boronic units of the polymer and the payload (curcumin).

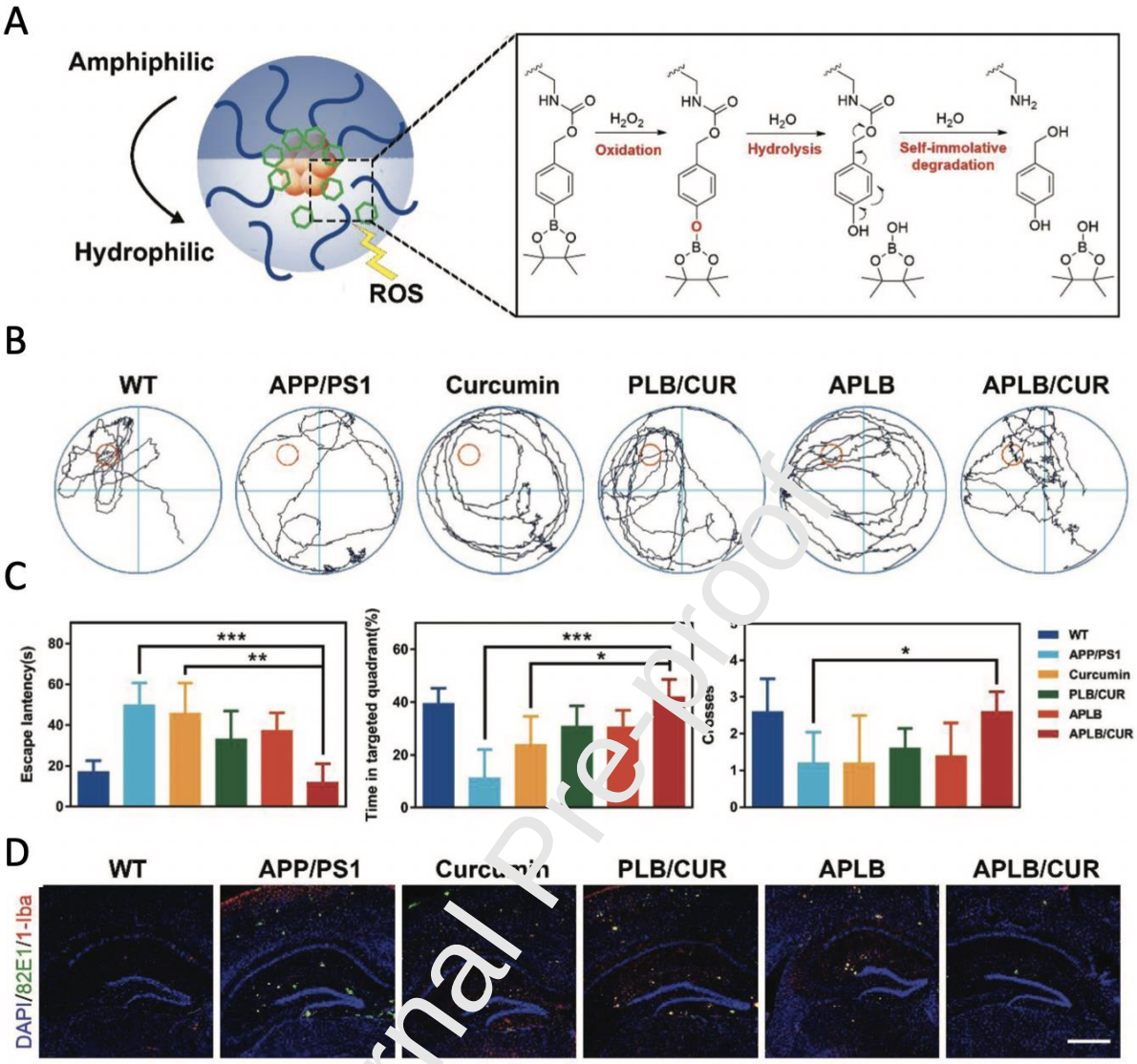

Figure 8. KLVFFAED-tagged PEG-P(Lys)-based micelles with ROS-responsiveness and scavenging ability and their in vivo performance in AD mice model. A) Schematic representation of the mechanism of ROS induced self-immolative degradation. B-C) Morris water maze test for memory studies showing B) Representative swimming paths of WT mice and APP/PS1 transgenic mice under different treatments: control, curcumin, PLB/CUR: unlabelled NPs bearing curcumin, APLB: empty labelled NPs, and APLB/CUR: labelled and curcumin loaded NPs; and C) Quantification of the escape latency, time in the targeted quadrant and crosses over the platform site. D) Reduction of Aβ burden (green) and microglia activation (red) in mice hippocampus after three months of treatment with APLB/CUR showed by immunostaining. Scale bar: 250 µm. Reproduced under the terms of the CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/) [232]. Copyright 2018 the Authors.

4.2.5 Targeting Alternative Receptors at the BBB

Although not-BBB specific, other vascular receptors have been used to deliver peptide NPs to the brain. The widely explored RGD peptides, which target αVβ3 and αvβ5 integrins, have been used in conjunction with polypeptide nanocarriers for brain delivery. RGD peptides have a selective affinity for the integrins overexpressed in the endothelial cells of tumour angiogenic vessels [14].

Thus, conjugation of RGD to nanocarriers appears as a way to cross the brain-blood tumour barrier for both imaging and treatment of GBM. The Kataoka group have employed cRGD-installed PEG-P(Glu) micelles to entrap a platinum (II) analogue, DACHPt, via complexation with carboxyl groups for treatment of GBM [14]. Different ligand densities were examined with the 20% cRGD containing micelles showing a higher level of brain accumulation (≈ 6 % ID/ g in the tumour after 10 hours of IV administration) and a significant decrease in tumour volume compared to saline (10-fold decrease in tumour volume). Recently, these cRGD-PEG-P(Glu) were also used for delivery of epirubicin for treatment of GBM [47]. Epirubicin was attached by post-polymerisation of modified PEG-P(Asp) polymer backbone through pH-sensitive hydrazine-linker. cRGD-epirubicin micelles showed a 12-fold increase in anti-tumour activity in an orthotopic GBM model compared to pristine micelles. Conjugation of cRGD was further employed to image neovasculature in an orthotopic model of GBM [239]. PIC vesicles (PICsomes) formed by PEG-P(Asp) and the homocatiomer n-butyl-poly([5-aminopentyl]-α,β-aspartamide) (Bu-P(Asp)-AP) were synthesised with a range of cRGD densities and tested *in vitro* and *in vivo*. cRGD-PICsomes bearing 4% of cRGD showed greater accumulation within the neovasculature remaining for longer times (> 24 h). Furthermore, the cRGD PICsomes were loaded with the supermagnetic iron oxide to use as a magnetic resonance imaging (MRI) contrast imaging of tumour neovasculature *in vivo*. Kulhari *et al*. conjugated a different RGD sequence (cRGDfK peptide) to P(Glu)-P(Phe) nanocarriers for the encapsulation and delivery of camptothecin (CPT) against GBM [52]. CPT containing cRGDfK-grafted NPs of ≈ 100 nm in size elicited the internalisation in glioma U-87 MG cells, which resulted in a more efficient ROS generation, induction of apoptosis and improved control over cell migration. Nevertheless, *in vivo* studies are yet lacking.

The BBB plays a critical role in maintaining suitable brain folic acid (FA) concentrations (1.5 to 3-fold increase compared to the serum) through its active transport via folate receptor (FR). Although widely expressed, FR is upregulated in numerous solid tumours, including GBM, and neovasculature. Based on this, folate has been used as a ligand to functionalise nanocarriers for the transport of cargo to the brain. Du Chen and co-workers used FA to co-deliver DOX and BCL-2 (an anti-apoptotic protein) siRNA as a way to overcome drug-resistance in the treatment of glioma [240]. FA-PEG-P(Glu) was used to coat self-assembled NPs from linear (PEI)-poly(e-caprolactone) (PCL) PEI-PCL pre-loaded with siRNA and DOX [241]. FA-functionalised NPs effectively delivered BCL-2 siRNA and sensitized C6 rat glioma cells to chemotherapy both *in vitro* and *in vivo*, showing greater efficacy almost suppressing tumour growth and showing extended median survival times [241]. A dually targeting polymersome formulation of DOX was designed by Chen and co-workers [242]. Poly-gamma(Glu)-based NPs with backbones decorated with FA and des-octanoyl ghrelin, a 28 amino acid

endogenous ligand known to transport from blood-to-brain direction via des-octanoyl ghrelin binding sites RMT [243,244], demonstrated an enhanced BBB crossing *in vitro* and enhanced anti-tumour effects compared to controls.

Several infectious pathogens such as prions, bacteria or some neurotropic viruses are able to reach the brain *via* laminin receptor binding [245–247]. Laminin is the major structural component of the basal membrane at the extracellular matrix, and its receptor abundantly expressed in adult neurons and glial cells has demonstrated essential roles in tumour invasion and metastasis [247,248]. To target laminin receptor, DGL-PEG nanocarriers were decorated with a *Streptococcus pneumoniae*-derived peptide EPRNEEK [119]. EPRNEEK-DGL-PEG NPs containing a plasmid DNA encoding for luciferase exhibited increased BBB translocation and accumulation within a glioma tumour compared to pristine NPs.

Yi *et al.* designed an amphiphilic R_3V_6 peptide, which self-assembled into small NPs, for the delivery of vascular endothelial growth factor (VEGF)-siRNA and carmustine to C6 glioma cells [249]. It was shown that carmustine is efficiently entrapped into the hydrophobic core of the micelles, and that the positively charged R_3V_6 peptide forms a stable complex with the VEGF siRNA. However, this study offered no details regarding *in vivo* efficacy of the VEGF-siRNA/carmustine-PA complexes and targeting to the BECs or tumour. To target the tumour vasculature, CREKA peptide was employed for an active binding to fibrin in tumour vasculature. Fluorescently labelled Cy7-PEG-CREKA micelles, when administered intravenously in an intracranial GL261 glioma mouse model, exhibited accumulation at the tumour site within 1 hour [250].

4.3 Adsorptive-mediated Transport of peptides

Adsorptive-mediated transport (AMT) is a mechanism of transport triggered by the interaction of polycationic peptides to anionic components, including carboxylic acid groups of sialoglycoproteins and sialoglycolipids and sulphate groups of heparan sulphate proteoglycans, at the surface of BECs [251]. Although AMT is described in drug delivery for the brain, the mechanism of intracellular trafficking and, consequently, transcytosis is not well-understood. In particular, it is established that polycationic species interact strongly with plasma proteins such as albumin, fibrogen, and immune-globulins, most of which are negatively charged, forming the so-called protein corona [252,253]. As such, rather than being due to electrostatic interactions, many of the transport processes attributed in the literature to this adsorptive mechanism may rather be driven by the protein corona *via* an undisclosed RMT pathway.

Regardless of the precise details of the underlying transport mechanism, cationic albumin, basic polypeptides (including, ebiratide, 001-C8, E-2078, histone, avidin, wheat germ agglutinin and

protamine), and cell penetrating peptides (CPPs) have been reported in the literature as undergoing AMT-based delivery into the brain [251]. In particular, CPPs are short peptides (< 30 amino acids in length), which are classified according to their physicochemical properties; cationic, amphipathic or hydrophobic [254]. The cationic class consists of peptides abundant in positively charged amino acids (Arg and Lys), such as peptides derived from human immunodeficiency virus (HIV) transactivator of transcription (TAT) protein (TAT_{48-60}, TAT_{49-57}), penetratin, polyarginines and DPV1047. The majority of reported studies exploiting CPPs as delivery vectors for transport across the BBB include TAT. However, additional CPPs have shown potential for brain delivery, such as dNP2, that was used in the delivery of the cytotoxic T-lymphocyte antigen for autoimmune encephalomyelitis [255].

The CPP platform has been combined with a singular class of polyamino acids used in the delivery of therapeutics, the ELPs discussed in Section 3.1 [256,257]. Their relevance to the field of drug delivery arises from their convenient LCST (40-41ºC), which allows for the application of mild hyperthermia, a mature clinical modality currently used in clinics [258]. In one example, Hearst *et al.* used ELPs fused to Synb1 CPP to demonstrate that external thermal cycling increased localisation of fluorescent-labelled Synb1-ELP into the brain, particularly, in the cerebellum after IV injection [259]. The group used the tandem Synb1-ELP to deliver a Notch inhibitory peptide, dominant negative MAML (dnMAML) [260] or DOX through a pH sensitive hydrazine linker [261] for the treatment of GBM. By combining the Synb1-ELP nanocarrier and hyperthermia, cellular uptake and pharmacological effects were enhanced *in vitro*. Bac CPP-fused-ELP nanocarriers were used to deliver a therapeutic peptide (TP) targeted to the proto-oncogenic protein c-Myc for the treatment of glioma [262]. Bac CPP-fused ELP elicited a 5-fold increase in the accumulation of the peptide within the intracerebral tumours in glioma bearing mice, while hyperthermia further enhanced brain accumulation (3-fold increase). Overall, Bac-ELP-TP exerted up to 80% of tumour volume inhibition, delayed onset of cognitive deficits, and doubled the survival rate in a glioma mouse model. In a different study, Yao *et al.* linked LIM kinase 2 Nuclear translocation signal peptide (LNP) CPP [263] to DGL-PEG polymers, and used them to complex a plasmid DNA encoding for inhibitor of growth 4 (ING4) as a therapy for glioma [15]. LNP conjugation to DGL-PEG nanocarriers resulted in a 3-fold increase in the *in vitro* BBB crossing (from ≈ 100 pmol of untagged NPs to ≈ 300 pmol in LNP-tagged NPs at 1 hour). Moreover, DGL-PEG-LNP/ING4 pDNA NPs increased median survival time (47 days *vs* 29 days of saline and 38 days of DGL-PEG/ING4 pDNA NPs) in mice bearing human U-87MG glioma.

Table 3. Representative examples of polypeptide NPs used for brain delivery following ligand-installed strategies

Polymer	Ligand	Receptor/transporter	Therapeutic Cargo	CNS Application	Ref
P(Lys) (K16)	ApoE derived peptide	LDLr	β-galactosidase pDNA	PK/Biodistribution study	[214]
P(Lys) (K16)	ApoE derived peptide	LDLr	Cisplatin, methotrexate, Insulin	PK/Biodistribution study	[215]
P(Lys) (K16)	ApoE derived peptide	LDLr	β-galactosidase and antibodies	PK/Biodistribution study	[216]
P(Lys) (K16)	ApoE derived peptide	LDLr	Tripeptidyl peptidase I enzyme (TPP1)	Lysosomal storage disease	[218]
DGL-PEG	Angiopep-2	LRP1	hGDNF pDNA	Parkinson's Disease	[224]
PEI-g-P(Lys)-PEG	Angiopep-2	LRP1	HSV-TK pDNA	Glioblastoma	[48]
DGL-PEG	Leptin30 peptide	LepR	Luciferase pDNA	PK/Biodistribution study	[211]
DGL-PEG	Choline derivatives	Choline	pORF-hTRAIL pDNA and DOX	Glioma	[166]
DGL-PEG	RVG29	nAchR	caspase-3 shRNA encoding plasmid	Parkinson's Disease	[167]
DGL-PEG	RVG29	nAchR	BACE1-AS shRNA encoding plasmid and d-peptide	Alzheimer's Disease	[168]
DGL-PEG	T7	TfR	pORF-hTRAIL pDNA and DOX	Glioma	[203]
DGL-PEG	T7	TfR	Luciferase siRNA	Glioma	[204]
DGL-PEG	EPRNEEK	Laminin receptor	Luciferase pDNA	Glioma	[264]
PEG-P(Lys)B	KLVFFAED	RAGE	Curcumin	Alzheimer's Disease	[232]
PEG-P(Glu)	cRGD	αVβ3 and αvβ5 integrins	DACHPt	Glioblastoma	[14]
PEG-P(Asp)	cRGD	αVβ3 and αvβ5 integrins	Epirubicin	Glioblastoma	[47]
PEG-P(Asp)+P(Asp-AP)	cRGD	αVβ3 and αvβ5 integrins	Superparamagnetic iron oxide	Glioblastoma/imaging	[239]
P(Glu)-P(Phe)	cRGDfk	αVβ3 and αvβ5 integrins	Camptothecin	Glioblastoma	[52]
PEG-P(Asp)+PEG-P(Asp-AP)	Glucose-6	GLUT1	-	PK/Biodistribution study	[57]
PEG-P(Lys) modified	Glucose-6	GLUT1	Antisense oligonucleotide (ASO)	PK/Biodistribution study	[12]
PEG-P(Lys) modified	Glucose-6	GLUT1	3D6-Fab	Alzheimer's Disease	[13]
PEI-PCL NPs coated with PEG-P(Glu)	Folic Acid	FR	DOX and BCL-2 siRNA	Glioma	[240]
Poly-gamma(Glu)-	Folic Acid +des-octanoyl ghrelin	FR and ghrelin binding sites	DOX	Glioma	[242]
ELP	Synb1 (CPP)	-	Dn(MAML) (Notch inhibitor)	Glioblastoma	[260]
ELP	Synb1 (CPP)	-	DOX	Glioblastoma	[261]
ELP	Bac (CPP)	-	c-Myc targeted TP	Glioma	[262]
ELP	LNP (CPP)	-	ING4 pDNA	Glioma	[15]
R3V6	-	-	VEGF siRNA and carmustine	Glioma	[249]
PEG-CREKA	CREKA	Fibrin	-	Glioma	[265]
5-FAM-CGYRPVHNIRGHWAPG		TfR and RAGE	BACE1 siRNA	Alzheimer's Disease	[206]

5 Alternative Administration Routes to the Brain

Rather than engineering peptide NPs to cross the BBB, it may be easier to reach the brain by means of a different administration route. The strategies summarised in this section may or may not rely on the use of ligands and include localised administration via intracerebroventricular (ICV), intrathecal (IT) or stereotactic injection directly into tumours; convection-enhanced delivery (CED) and intranasal administration (IN). These strategies have a number of benefits and disadvantages: IN administration potentially bypasses a number of challenges associated with brain delivery, while ICV administration and CED are extremely invasive procedures, raising questions about their clinical viability.

5.1. Localised Delivery

Although considered an invasive and aggressive method, FDA approval of Gliadel wafers in 1997 demonstrated the efficacy and safety of local delivery of therapeutics to the brain [266]. In one example, Abid Sheikh and co-workers [267] used PEI-P(Lys) to complex and deliver VEGF pDNA to PD holding rats through direct administration to the brain by stereotactic brain surgery. These polyplex NPs exerted great transfection efficiencies both *in vitro* and *in vivo* and the delivery of VEGF decreased neuronal loss, reduced apoptosis events and microglia activation, and therefore prevented motor deficits in 6-hydroxydopamine (6-OHDA) induced rat PD model. The Kataoka group has also explored the ICV route for the delivery of therapeutics to the brain using polyplex nanomicelles. In one study, the polycation PEG-P(AspDET), a PEG–P(Asp) polymer carrying diethylenetriamine (DET) at the side chains, was used to produced pH-sensitive micelles of 50 nm in diameter and core-shell structure [268]. DET moieties with a two-step protonation behaviour at a pK_as of 9.5 and 6.0 were introduced to provide pH-triggered destabilization of the cell membrane and endosomal escape [269]. Delivery of messenger RNA (mRNA) by ICV infusion into mice brains using these polyplex micelles resulted in sustained protein expression in cerebrospinal fluid (CSF) and overcome the two main limitations of mRNA, its stability and immunogenicity. mRNA encoding the mouse neprilysin protein (NEP), a protease that degrades Aβ [270] was efficiently introduced in mice brains by ICV using these polyplex micelles. Mice pre-treatment with NEP mRNA micelles increased NEP concentration and almost doubled its proteolytic activity in brain and diminished almost to half the amount of exogenous $A\beta_{1\text{-}40}$ injected thereafter as compared to other groups. The authors recently reported an upgrade of these polypeptide nanocarriers by fine-tuning the hydrophobicity (logP) of the P(Asp) residues in the side chains [271]. A direct correlation among logP and polyplex stability and cell internalisation efficiency was found.

5.2. Convection-Enhanced Delivery

Convection-enhanced delivery (CED) is an alternative method that bypasses the BBB entirely, potentially greatly increasing delivery of therapeutics [272]. This technique is based on the direct pumping of therapeutics to the brain by means of a pressure gradient at the tip of an infusion catheter. Since small drugs usually fail to distribute within the tissue, the combination of CED and nanomaterial-based delivery systems has demonstrated great potential in dealing with brain tumours [273]. Inoue and co-workers applied CED to delivery PEG-P(Asp)-DOX conjugated micelles in rat brains with 9L (gliosarcoma cells) intracranial tumours [274]. DOX-bearing micelles infused by CED achieved significantly wider distribution both in brain tumour and surroundings and exhibited enhanced antitumor effects as reported by an almost twice increased median survival time compared with free DOX. The same authors used PEG-P(benzyl-Asp) to deliver Am80 [275], a synthetic agonist of the nuclear retinoid acid receptor (RAR) highly expressed in glioma cells, reported to inhibit the growth rate of several cancers [276]. Combination of CED of the micellar Am80 together with the systemic administration of temozolomide (TMZ) resulted in significantly enhanced survival of U-87 MG-bearing rats compared to single therapies and controls. CED was recently applied to enhance the brain delivery of NK012 [277], a PEG-P(Asp) polymeric micelle carrying the antitumor drug 7-ethyl-10-hydroxy-camptothecin (SN-38), developed by Nippon Kayaku Co. that received FDA Orphan Drug Designation for small cell lung cancer [278]. Unlike free SN-38, NK012 was able to significantly increase the median survival with minimal brain damage when tested in combination with CED against 9L (42 vs 28 days of SN-38) and U-87 MG (28 vs 21 days of SN-38) mice brain tumour.

The Hanes group studied the influence of NP size and PEG corona density in brain diffusion of NPs [279]. While NPs with low density PEG coating showed adhesive interactions with the brain extracellular matrix regardless of size, surface shielding with a dense PEG corona enhanced diffusion in the brain of 40 and 100 nm NPs, but not 200 nm NPs [280]. These results defy the convectional opinion that only NPs smaller than 64 nm access the brain parenchyma through the extracellular space [281]. Based on this knowledge, biodegradable PEGylated P(Lys) NPs were used by the group as non-viral vectors [282]. Ellipsoidal PEGylated NPs carrying enhanced green fluorescence protein eGFP-expressing DNA showed enhanced brain distribution *in vivo* following CED with efficient transfection. In a more recent example, the authors used the same concept to deliver anticancer agents. NPs based on P(Asp) to which PEG chains were conjugated to the polymer backbone were used to complex cisplatin yielding 70 nm NPs with a dense PEG corona [283]. CED of NPs in F98 rat glioma tumours resulted in a significantly enhanced antitumor efficacy with 80% of the rats exhibiting long-term survival. This technique has also been employed to deliver MRI macromolecular

probes such as gadolinium diethylenetriaminepentaacetic acid-installed PLys derivatives developed by Hardy et al [284].

5.3. Intranasal Administration

Nose-to-brain delivery or intranasal (IN) administration of therapeutics has recently arisen as a promising approach to directly reach the brain and bypass the BBB. The possibility for self-administration, as well as low systemic accumulation and the rapid and enhanced absorption of therapeutics following this route, provides fast action with reduced off-target effects making IN an interesting alternative for the treatment of CNS disorders [285]. Although IN administration allows for higher brain accumulation (up to 10-fold) when compared to IV, only a few therapeutics hold specific mucoadhesion/mucodiffusion balance to be applied within this route. This fact, together with the need for further navigation once at the olfactory bulb to access other brain areas, highlights the need for its combination with nanosized delivery systems [286]. To this end, Baba and co-workers used the previously mentioned polyplex nanomicelles based on PEG-P(AspDET) block copolymers to deliver mRNA via IN administration to treat sensory nerve diseases [287]. Mice with drug-induced olfactory dysfunction treated daily with brain-derived neurotrophic factor (BDNF)-expressing mRNA using these micelles through the IN route allowed for almost complete rescue of olfactory function and the regeneration of the olfactory epithelium structure as observed by behavioural test and histology respectively. Similarly, GDNF pDNA was intranasally delivered to the brain of PD rats complexed to a PEG-P Lys)$_{30}$ carrier [288]. The generated pGDNF DNA NPs were intranasally administered 1 week prior to a 6-OHDA lesion in rats used as a PD model. Administration of the NPs yielded longer-lasting GDNF brain expression compared to naked GDNF and consequently, exerted greater neuroprotection.

The abovementioned combination of ELP and CPP carriers have also been explored through the IN route. cAMP-dependent protein kinase A (PKA) inhibitor was delivered efficiently to the brain following IN route by the aid of the tandem Synb1-ELP in order to treat spinocerebellar ataxia-1 (SCA1) [289]. However, a follow-up study showed that the addition of CPPs (either Tat or Synb1) to ELP significantly affected the biodistribution of ELP after IN administration by increasing their retention at the nasal epithelium in detriment of the amounts reaching perivascular spaces at the brain [290].

Samaridou et al recently produced polymer-coated nanocomplexes based on octaarginine (R8)-lauric acid (C12) conjugate and a therapeutic miRNA mimic [291]. These complexes were coated with PEG-P(Glu) to provide stability and enhance their mucodiffusion leading to what the authors called enveloped nanocomplexes (ENCPs) of 100 nm in size and slightly positive z-potential. When IN

administered *in vivo* in APP^{NL-G-F} AD mice, ENCPs carrying the AD-relevant miR-132 reached the hippocampus, one of the first AD affected areas, and increased the endogenous levels of miR-132 levels with the consequent improvement of its function.

6. Targeting the BBB in pathological conditions

In most of the examples described above, the design of these peptide NPs is based on the physiology of a healthy BBB and thus overlooks the significant effects of brain disorders on integrity of the barrier and transport across the BECs [144,292]. Indeed, reductions in the cerebral blood flow (CBF), microvascular pathologies, aberrant angiogenesis, breakdown of the BBB due to disruption of the TJs, and altered transport systems in BECs, almost certainly affect the fate of peptide nanocarriers.

In terms of blood flow, reductions in resting CBF or altered responses to brain activation may occur in different regions of the brain in AD, PD, stroke, and other disorders [293,294]. In normal ageing, a modest reduction of 20% in CBF is observed while more severe regional reductions in CBF (> 50%) occur in chronic neurodegenerative disorders (AD and PD) and stroke [144,295]. Additionally, an increase in blood vessel tortuosity observed in neurodegeneration results in changes in blood rheology that impair the ability of nanocarriers to cross the BECs, thus magnifying the issue of CBF [296]. Similarly, in gliomas, owing to the confined space within the brain, tumour growth can impair CBF and alterations occur in blood vessel geometry, leading to increased tortuosity compared to healthy vasculature [297,298]. Significant microvascular pathologies, including reduced microvascular density, an increased number of fragmented vessels with few intact branches, atrophic string vessels, alterations in vessel diameter, and capillary basement thickening with an accumulation of collagen, have been described in AD and PD [299,300]. Furthermore, findings suggest that degeneration of BECs in AD may reflect an aberrant angiogenesis [301,302], which is also characteristic of brain tumours, such as gliomas [303]. Altogether, these alterations in the architecture of the vasculature along with the characteristic abnormal angiogenesis may again impact flow through the blood vessels as well as BEC-crossing mechanisms. Thus, access to the brain parenchyma may be restricted in brain disorders. On the other hand, breakdown of the BBB has been demonstrated in AD and PD [304–306], glioma [307,308], stroke [309] and traumatic brain injury [310], which may facilitate diffusion of nanocarriers from the blood to the brain parenchyma. Importantly, in addition to the structural changes of the brain blood vessels, molecular alterations in terms of receptors expressed at BECs have been identified, impacting the design of NPs for the crossing across the BBB. In ageing and AD, reduced levels of LRP1 [311] and GLUT1 [312] has been found in rodents and AD individuals, while RAGE levels are increased in AD [313]. Based on these, it is important to understand the physiology of the BECs to precisely target the BBB and achieve

efficient crossing. As example of that, RAGE has been considerably employed for brain delivery in AD, while the peptide RGD sequence targeting the integrins overexpressed in BECs of tumour angiogenic vessels is exploited for delivery in glioma (Table 3).

Although it is essential to understand the biology and pathology of the BBB to precisely design nanocarriers for brain delivery, challenges remain. At the BBB level, a number of carrier- and receptor-mediated transporters remain to be studied in pathological conditions to further understand their role and their potential as a targeting for brain delivery. Developing new genomic and proteomic discovery platforms will allow us to identify transporters that can be potentially used as portals of entry for the peptide nanocarriers.

7. Conclusions and Future Outlook

There is an unquestionable increase in the prevalence of brain disorders, including brain tumours, PD, and AD. This trend will almost certainly continue due to an ageing population, generating a major problem in healthcare systems across the world. While many efforts have been made in the field to improve current therapies and develop new disease-modifying therapies, there is a clear need for further investment. The treatment of brain disorders is particularly challenging due to (i) the presence of the most impenetrable metabolic and structural barrier, the BBB, and (ii) the lack of diffusivity of the majority of the CNS drugs and therapeutics once at the brain parenchyma. This second issue leaves compounds unable to reach all affected areas, leading to poor therapeutic outcomes. Nanotechnology and nanomedicine are promising tools to overcome these limitations. Despite being in their early development for brain delivery, biodegradable peptide-based NPs in particular show great potential as illustrated here by the many examples in preclinical stages. To reach the brain, choosing the right administration route (IV, IN or local administration) as well as a careful selection of the targeting ligand (according to the route, disease condition and stage) needs consideration. Additionally, as recently demonstrated, optimisation of ligand densities to reach the brain parenchyma within sufficient concentrations to provide enough therapeutic response is key in the design of nanocarriers [164].

However, the challenges in translating peptide NPs to market are manifold. Many of the limitations in this area are generic to the field of medicine development, such as the need for more realistic *in vitro* and *in vivo* models to establish structure-activity relationships and predict and test NP outcomes [314,315]. Normalisation of quantitative methods and parameters to report pharmacokinetics and pharmacodynamics, including percentage of brain accumulation, will help in the understanding of the use and concentration of certain targeting ligands and the true potential of nanocarriers. The frequent neglect at the preclinical stages of potential NP-induced neurotoxicity,

off-target and long-term effects, as well as their understudied brain and whole-body clearance and degradation routes, pose a barrier to their evaluation at later stages and translation from bench to bedside. The degradation pathways of peptides must be carefully considered, since several amino acids such as γ-aminobutyric acid (GABA), glycine, and glutamic or aspartic acid function as key primary excitatory transmitters in mammals. As such, the degradation of peptide nanocarriers may disrupt their concentrations at the synaptic feet of neurons and therefore interfere with amino-acid mediated synaptic transmission [316,317].

There remain significant shortcomings in our understanding of how NPs can be designed to cross the BBB. Although it is generally recognised that BECs facilitate the transport of essential molecules by using a CMT or RMT, these mechanisms of transport are yet to be fully understood, particularly in a scenario of pathology. Recent findings on intracellular transport across the BBB raise important questions. How many pathways can actually drive transcytosis? What determines which pathway will sort different receptors and whether a cargo undergoes transcytosis or lysosomal degradation? Intracellular sorting mechanisms (e.g., tubules or vesicular endosomes) that control successful transcytosis in BECs are still to be deciphered. By answering these fundamental questions, we then will be able to fully understand what exactly affects the intracellular transport within BECs to improve the delivery of therapies into the brain. Furthermore, developing genomic and proteomic discovery tools will enable us to identify new transporters in healthy or diseased BECs, which can then be hijacked for CMT- or RMT-based strategies for delivery of drugs into the brain.

There are also significant shortcomings in peptide NP synthesis and characterisation techniques, which have led to major reproducibility issues in the field [318–320]. Despite being the subject of study for decades, peptide and polypeptide self-assembly remains remarkably poorly understood. The overwhelming majority of the studies discussed in this work use polyplexes to achieve brain delivery. This is, at least in part, due to their simplicity and the relative ease with which they can be produced and functionalised. There is another reason for their popularity, however, which is that the methods by which more complex, self-assembled peptide structures can be produced at a scale and reproducibility suitable for drug delivery studies are simply not known. Traditional top-down approaches rooted in statistical mechanics have to-date generally only been successful in describing the formation and self-assembly of the more commonly seen motifs in biology, such as β-sheets, helices, and turns. At the same time, simulations-based approaches to study peptide self-assembly are limited by the colossal computational requirements needed to simulate large ensembles of polypeptides in water on microsecond timescales. Experimental studies that apply the evolutionary approaches discussed in Section 3.3 may prove much more productive, while having far lower technical barriers to entry.

Problems in peptide NP fabrication are compounded by challenges in their characterisation. Popular techniques such as DLS and TEM are rather limited and suffer from selective reporting. Newer methods, such as tunable resistive pulse sensing and nanoparticle tracking analysis are potentially useful, although older methods such as asymmetric flow field flow fractionation with in-line DLS and multi-angle light scattering would represent a significant improvement over the norm. Surface functional group density remains an under-scrutinised quantity; the development of techniques that can report more than just an average presents a particular challenge. Assays built on the recently developed principles of superselectivity [321–323], in which NP binding is a strong function of receptor density over a narrow range of values, may prove promising here. Finally, there is a striking shortage of studies on protein corona formation in peptide NPs [324–327]. Understanding how the protein corona reduces, or even enhances, the efficacy of engineered NPs is of paramount importance for their rational design. Indeed, the highly controlled interactions between proteins and biomolecules observed in nature suggest that peptide NPs are a particularly promising platform not for removing, but for *engineering* the protein corona for drug delivery applications [328–330].

Despite the challenges listed above, peptide nanocarriers are perfectly positioned to overcome many of the obstacles that prevent successful delivery of therapeutics to the brain. The material diversity of the building blocks of peptides allows for design and tuning of a multitude of supramolecular interactions. Polypeptides can be synthesised with a low polydispersity, complexity, and reproducibility almost entirely unique among polymers. The body of work surrounding protein folding provides an unparalleled toolkit for rational design and characterisation of peptide NPs. On top of this, peptide synthesis and self-assembly techniques make them highly amenable to automated, high-throughput study and discovery that is driven by machine learning methods and affordable lab-made robotics. Improved peptide NP synthesis methods will, in turn, provide researchers with new probes for identifying novel pathways and mechanisms for the delivery of therapeutics to the brain. We believe that this diversity of both material properties and experimental approaches make peptide NPs an extremely promising platform for brain delivery.

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