Introduction

A major hurdle hindering the diagnosis and treatment of neurological disorders is the difficulty for biotherapeutics to enter the central nervous system (CNS). This is due to the blood–brain barrier (BBB), which is comprised of tightly connected polarized endothelial cells that limit the passage of hydrophilic components and prevents the accumulation of material for transport at the BBB. Despite these limitations, specialized endogenous transport mechanisms exist to allow the transcytosis of nutrients and ions, thus enabling CNS homeostasis. Of these, absorptive mediated (AMT) and receptor mediated-transcytosis (RMT) pathways are key vesicular based transport systems which have become long-standing mechanisms surrounding trafficking at the BBB is more complex than initially thought, restricted by the barrier to those which have this capacity, and those routes have become widely exploited by conjugating molecules to the BBB model to reliably rank transcytosis of the vehicle library. Our results demonstrate heightened uptake and trafficking for the shuttles, with a structure–activity relationship for transcytosis emerging. Notably, due to their small size, the majority of shuttles demonstrated increased permeation compared to transferrin, with the highest performing shuttle affording a 4.9-fold increase. Consequently, we have identified novel peptide conjugates that have the capacity to act as promising brain shuttles.

Absorptive- and receptor-mediated transcytosis (AMT/RMT) are widely studied strategies to deliver therapeutics across the blood–brain barrier (BBB). However, an improved understanding of the molecular basis of uptake and trafficking is required that could promote delivery. Accordingly, we designed a flexible platform that merged AMT and RMT motifs on a single scaffold to probe various parameters (ligand, affinity, valency, position) in a screening campaign. During this process we adapted an in vitro BBB model to reliably rank transcytosis of the vehicle library. Our results demonstrate heightened uptake and trafficking for the shuttles, with a structure–activity relationship for transcytosis emerging. Notably, due to their small size, the majority of shuttles demonstrated increased permeation compared to transferrin, with the highest performing shuttle affording a 4.9-fold increase. Consequently, we have identified novel peptide conjugates that have the capacity to act as promising brain shuttles.

Whilst there is limited experimental data surrounding the molecular basis of uptake and trafficking for CNS delivery, it has been demonstrated that dissociation from receptors on the brain side is essential for trafficking mediated by the transferrin receptor (TfR), the quintessential receptor for RMT. Therefore transcytosis is more likely when the overall affinity towards the receptor is moderate to low, or when bivalent engagement of the receptor is discouraged to limit avidity.

Here we present the design, synthesis and validation of a diverse shuttle library to identify key physicochemical properties for transcytosis. We based our approach on two essential modular components to develop the chemical tool kit: (i) sweet-arrow-peptide (SAP), an isolated sequence derived from the N-terminal proline rich domain of γ-zein, and an innate CPP with a defined PPII secondary structure to act as a scaffold, and; (ii) a variety of validated RMT ligands to enable targeted delivery, Fig. 1 and Table 1.

**Fig. 1** Structure and modification of SAP into targeted brain delivery vehicles. (A) SAP as a PPII helix. Hydrophilic residues (R) are shown in blue while hydrophobic residues (V and L) are shown in yellow. (B) Simplification of the defined faces presented by SAP demonstrating the defined structure of the PPII helix. (C) Mutation of similar (V and V) or alternative residues (V and L; as shown in figure) on the SAP scaffold enables RMT ligands to be appended on the same or opposite face of the PPII helix in a bivalent format.
It was anticipated that uptake at the BBB could be probed through exploring ligand type (i.e. targeted receptor, affinity) and arrangement (valency, position) of the ligands fused to the scaffold, since previous studies have indicated that brain exposure is directly affected by these parameters.\textsuperscript{2,3,6,9,11} We provide compelling evidence that uptake and brain trafficking can be improved by combining AMT and RMT motifs on a single shuttle and that better understanding of receptor mediated trafficking within the brain endothelium is required at an individual and ligand-by-ligand basis, with ligand type, number and position effecting permeability in endothelial cells.

### Results and discussion

#### Design and synthesis of brain delivery shuttles

SAP, the core of our delivery vehicles, is formed from a short repeating sequence of \([VRLPPP]_3\) that is readily accessible in high yield through general automated solid-phase peptide synthesis (SPPS). It is noteworthy to mention that SAP retains a PPII conformation if 50\% of the sequence remains as proline, forming a left-handed helix of 3.0 residues per turn, which affords three distinct ‘faces’ in aqueous solution, Fig. 1B.\textsuperscript{7,12,13} Consequently, spatial parameters of the BBB shuttle could be readily studied through simple mutation of valine, arginine, or leucine amino acids to orthogonal residues at specific sites. For this, we included lysine (K) or propargylglycine (X) on the PPII scaffold to conjugate relevant RMT motifs through amide bond forming and copper alkyne–azide chemistry (CuAAC) conjugations respectively. The full scaffold library we generated is shown in Table 2. Mutations to the SAP primary sequence follow standard convention and, when conjugated to the specified ligand, the attachment point on the scaffold is given in brackets and in order of conjugation. All peptide scaffolds were made with the chemically inert C-terminal amide in place of a carboxylic acid and FAM was conjugated to the N-terminus as cargo and to allow \textit{in vitro} study.

To enable targeted brain delivery, we chose to functionalise our scaffold with established ligands that engage the transferrin receptor (TfR) and low-density lipoprotein-receptor related protein (LRP1). The relative abundance of these receptors on brain endothelial, alongside their high transport capacity, made them ideal targets for RMT mediated delivery and both have been actively explored in research.\textsuperscript{9,14–16} We focused on three main peptide ligands for these receptors, as shown in Table 1. Both TfRL1 and TfRL2 have been shown to interact with TfR at alternative sites compared to transferrin (Tf), and have either nanomolar (TfRL1; 15.0 nM) or micromolar affinity (TfRL2; 440 \(\mu M\) towards the receptor.\textsuperscript{9} Alternatively, Angiopep-2 (Apep2) displays a high transcytosis potential mediated by LRP1.\textsuperscript{8} While less is known about trafficking of LRP1, Apep2-conjugates have shown demonstrable success in clinical trials of neurological disease models.\textsuperscript{9} Peptide ligands were synthesised by general SPPS on rink-amide resin (see Table S1\textsuperscript{†} for characterisation data and yields). It is important to note that the stability of the peptide vehicles was not considered in this proof-of-concept screen. Recent investigations with \textit{retro-enantio} sequences of both TfRL1 and Apep2 have shown that metabolic limitations can be overcome without the loss of function, offering a plausible solution to degradation.\textsuperscript{14,17} This is also apparent for the \textit{d}-amino acid analog of SAP.\textsuperscript{18}

#### Chemoselective synthesis of brain delivery shuttles

Four conjugation approaches were used to decorate the scaffold in various formats, Scheme 1A–D. Solution phase CuAAC chemistry with microwave heating was most effective for affording PPII-ligand conjugates in either monovalent or bivalent formats that included identical ligands (Scheme 1A). Alternatively, for the attachment of orthogonal ligands to the scaffold, TfRL2 was first incorporated via continuation of Fmoc/\(\text{Bu}\) SPPS on the lysine side chain of PPII scaffolds, as generalised in Scheme 1B. Following FAM conjugation, unreactive N-terminal PPII was capped to ensure no unwanted reactions, before the lysine protecting group (ivDde) was selectively removed on resin. Resin was re-submitted to automated SPPS to afford the product following deprotection and cleavage. If propargylglycine (X) was also incorporated on the scaffold, bivalent conjugates with alternative ligands were afforded through subsequent reaction by CuAAC (Scheme 1C). While this approach afforded conjugates in high yield, it was somewhat limited, since conjugates of both TfRL1 and Apep2 were unattainable through continuation of SPPS on resin. To overcome this limitation, CuAAC could be used to attach one ligand to the

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**Table 1** Sequences and target of peptide ligands used for delivery vehicle construction. Peptides were synthesised with either FAM conjugated to the N-terminus or azidolysine (K(N3)) for use as a control or for conjugation to SAP respectively.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>RMT target</th>
</tr>
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<tbody>
<tr>
<td>SAP</td>
<td>VRLPPPVRLLPPVRLPPP</td>
<td>NA</td>
</tr>
<tr>
<td>TfRL1</td>
<td>TRHPPMWSPVWP</td>
<td>TR</td>
</tr>
<tr>
<td>TfRL2</td>
<td>HAIYPRH</td>
<td>TR</td>
</tr>
<tr>
<td>Apep2</td>
<td>TFFYGGSRGRKNNFKTE</td>
<td>LRP1</td>
</tr>
</tbody>
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**Table 2** Peptide scaffolds synthesised and used for delivery vehicle construction. Peptides were synthesised with FAM conjugated to the N-terminus. Monovalent PPII(R8K) incorporated K(ivDde) for continuation of peptide synthesis while PPII(L5X)L15K and PPII(L3X)V13K included both K(ivDde) and K(Boc). Mutated residues in the sequence are shown in bold.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Conjugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPII(R8X)</td>
<td>VRLPPPVPXLPPVRLPPP</td>
<td>CuAAC</td>
</tr>
<tr>
<td>PPII(R8K)</td>
<td>VRLPPPVPXLPPVRLPPP</td>
<td>Amide</td>
</tr>
<tr>
<td>PPII(L3X)</td>
<td>VRKPPPVRLLPPVRLPPP</td>
<td>CuAAC</td>
</tr>
<tr>
<td>PPII(L13X)</td>
<td>VRLLPPPVRLLPPVRLPPP</td>
<td>CuAAC</td>
</tr>
<tr>
<td>PPII(L3X)L15X</td>
<td>VRKPPPVRLLPPVRLPPP</td>
<td>CuAAC</td>
</tr>
<tr>
<td>PPII(L3X)V13X</td>
<td>VRKPPPVRLLPPVRLPPP</td>
<td>CuAAC</td>
</tr>
<tr>
<td>PPII(L3X)L15K</td>
<td>VRKPPPVRLLPPVRLPPP</td>
<td>Amide, CuAAC</td>
</tr>
<tr>
<td>PPII(L3X)V13K</td>
<td>VRKPPPVRLLPPVRLPPP</td>
<td>Amide, CuAAC</td>
</tr>
</tbody>
</table>
To evaluate functionality of the conjugated peptides, in vitro binding capacity of shuttles towards TfR was assessed. As predicted, TfRL1-vehicles contained FAM-labelled molecules detected by an anti-FAM HRP-diluted and added to plates that were coated with TfR protein. Analyses were performed. TfR binding was assessed through an incorporated TfRL2 (PPII(L3X;L15X)TfRL1: 0.26\(\times\)C6; PPII(L3X;V13X)TfRL2: 0.31\(\times\)C6) (Scheme 1D). As demonstrated here and elsewhere, CuAAC mediated convergence provided rapid access to versatile bioconjugates that was reproducible.

**Secondary structure determination of brain shuttles**

We determined structural integrity of the PPII helix by circular dichroism (CD) spectroscopy, where spectra of unmodified SAP and the N-terminal FITC-conjugated analogue were collected as controls, Fig. 2A. Importantly, the results suggest preservation of the secondary PPII structure following modification to the backbone since all peptides demonstrated strong absorption at 203 nm, Fig. 2B–D. Specifically, PPII helicity was insensitive to the conjugate linkage chemistry, ligand type and number of additions to the PPII scaffold.

**In vitro binding capacity of shuttles towards TfR**

To evaluate functionality of the conjugated peptides, in vitro analyses were performed. TfR binding was assessed through an ELISA format, whereby FAM-conjugated shuttles were serially diluted and added to plates that were coated with TfR protein. FAM-labelled vehicles were detected by an anti-FAM HRP-conjugated antibody. As predicted, TfRL1-vehicles contained a higher affinity towards the TfR than the equivalent vehicle incorporating TfRL2 (PPII[L3X;L15X]TfRL1: 0.26 \(\pm\) 0.17 nM; PPII[L3X;V13X]TfRL1: 0.31 \(\pm\) 0.26; cf. PPII[L3X;L15X]TfRL2: 11.8 \(\pm\) 1.11 nM; PPII[L3X;V13X]TfRL2: 12.7 \(\pm\) 1.37), Fig. 2E. Notably, TfRL2 bivalent shuttles contained a similar affinity towards TfR compared to Tf-biotin when assayed in a similar format (EC\(_{50}\): 20.9 \(\pm\) 2.1 nM). It is assumed that an avidity effect is demonstrated by the bivalent vehicles since monovalent TfR-targeting shuttles contained too low-affinity towards TfR to be detected. Further, positioning of the ligand did not affect TfR binding affinity since scaffolds that incorporated ligands on the same face afforded affinity values similar to those with ligands fused on different sides.

**Evaluation of the BBB-shuttle properties of the conjugates in brain endothelial cells**

Having demonstrated preservation of receptor binding, we next used bEnd.3 cells to screen the uptake capacity of the shuttle library by flow cytometry. We determined peptide uptake and internalisation in a confluent monolayer of bEnd.3 cells which is an immortalised mouse brain endothelial cell line that has similar characteristics to the BBB. TfR is an established receptor for clathrin dependent uptake of Tf, favoured in an iron bound form when at physiological pH (i.e. holo-Tf). Flow cytometry (FC) and microscopy data obtained with human derived Alexa-Fluor 647-conjugated Tf (Tf-A647), confirmed functional, membrane resident TfR (see Fig. S1 and S2†).

**Evaluation of the internalisation capacity in brain endothelial cells**

For library screening, cells were exposed to equal concentrations (500 nM) of FAM labelled molecules in media for 3 h to allow binding, internalisation and sorting, and external FAM fluorescence was quenched by addition of trypan blue. Low temperature was shown to inhibit cellular uptake of the vehicles (Fig. S3†), demonstrating an energy-driven internalisation process. Furthermore, using DAPI as a viability indicator, no...
cytotoxicity of the vehicles was demonstrated during these experiments and over a concentration range of up to 100 μM, where viability remained over 98% (Fig. S4†).

It can be seen from Fig. 2F that, in general, brain shuttles contained heightened, non-additive, uptake compared to their unconjugated counterparts, implying a synergistic effect for uptake. TfRL1 demonstrated the highest capacity for endocytosis when incubated alone, however appreciable difference was shown for the monovalent vehicle in this format (TfRL1 cf. PPII(R8X)TfR2 \( P = 0.798 \)); TfRL2 cf. PPII(R8X)TfR2 \( P = 0.018 \); Apep2 cf. PPII(R8X)Apep2 \( P = 0.002 \). Notably, when arranged in a bivalent format, TfRL1 modified vehicles showed a dramatic increase in cellular internalisation that exceeded additive contributions. Interestingly, endocytosis of TFR-targeting bivalent vehicles was not significantly influenced by its positional arrangement on the scaffold (PPII(L3X;L15X)TfR1 cf. PPII(L3X;V13X)TfR1 \( P = 0.408 \); PPII(L3X;L15X)TfR2 cf. PPII(L3X;V13X)TfR2 \( P = 0.050 \)), compared to those targeting LRP-2 which favoured ligands attached to opposite faces of the scaffold (PPII(L3X;L15X)Apep2 cf. PPII(L3X;V13X)Apep2 \( P = 0.0059 \)). This phenomenon could be attributed to increased steric strain of the larger Apep2 peptide when incorporated on the same face, or possibly linked to the ligands ability to influence receptor clustering on the extracellular membrane. Again, higher uptake was demonstrated for scaffolds that combined alternative transcytosis ligands on the same molecule compared to individual ligands. However, uptake was not improved compared to bivalent scaffolds containing identical ligands.

**Intracellular location of BBB shuttles**

To obtain information on the intracellular fate of the shuttles, bEnd.3 cells were pulsed with bivalent vehicles, Tf-A647 and unconjugated TfRL1 and Apep2 ligands for 3 h, unbound compounds were removed and cells fixed and stained for immunofluorescence microscopy. Images were processed as described in Fig. S5†. Fig. 3A–C shows colocalisation of PPII(L3X;V13X)TfR1 with DAPI, Tf-A647 or LAMP1 respectively and represents a typical image generated from the screen following deconvolution and segmentation. It can be seen from Fig. 3D that all vehicles gave highest colocalisation with Tf-A647 as opposed to LAMP1, indicating uptake into endosomal vesicles with minimal degradation through lysosomal sorting. In addition, Tf-A647 colocalisation values were higher for vehicles compared to the individual RMT ligand components. Notably, the degree of Tf-A647 and LAMP1 colocalisation was comparable for TfRL1 vehicles incorporating this ligand on the same or opposite face. In addition, Tf-A647 colocalisation for bivalent TfRL1 vehicles and PPII(L3X;L15X)TfR2 was also comparable. However, increased levels of LAMP1 colocalisation was observed for TfR2 bivalent vehicle, indicating that the higher affinity TfR ligand is more efficient for non-degradative cellular uptake in this system. Colocalisation of Apep2 bivalent vehicles with Tf-A647 were affected by the position of the ligand, demonstrating a similar trend to that shown with endocytotic capacity, Fig. 2F. Trivalent vehicles decorated with Tfr ligands demonstrated similar colocalisation with Tf-A647 to those in a bivalent format. Whilst vehicle PPII(L3X;L15X)TfR2(K) TfRL1(X) demonstrated highest Tf-A647 colocalisation, it also had heightened association in the lysosome. Notably,
PPII(L3X;L15K)TfRL2(K)ApPe2(X) had low TF-A647 colocalisation and the highest LAMP1 association out of the vehicles screened. This could imply that the ligands are incompatible when combined on the same vehicle, or that endocytosis is supported through a different mechanism of uptake. Alternatively, trivalent vehicle PPII(L3X;L15K)TfRL1(X)ApPe2(Kx) gave high TF-A647 colocalisation and moderate LAMP1 association.

Permeability experiments

To study transcytosis potential of the vehicles in vitro we optimised a BBB model using a 3D-transwell format. We found that a bEnd.3/mesenchymal stem cell (MSC) co-culture reproducibly afforded the highest resistance to paracellular diffusion through assessment with transepithelial electrical resistance measurements (TEER) and small molecule permeability studies (Fig. S6†). This is similar to results shown by others.† Immunostaining for TJ protein ZO-1 in the bEnd.3 cell line confirmed its presence and trafficking towards the cell junctions (Fig. S7†). While this indicates that an adequate and reproducible barrier was formed from the bEnd.3/MSC co-culture model, we found that it was of paramount importance to distinguish transcytosis from the expected background of paracellular flow since others have reported issues distinguishing these values.22

Optimisation and adaptation of BBB in vitro model

Often in vitro assays of the BBB overlook contributions of paracellular flux that can lead to overestimations of brain exposure, and offer no valid comparison between the individual molecules being screened. We believed that without a quantifiable probe for paracellular diffusion, it would be difficult to accurately screen the transcytosis capacity of our vehicles.

It seemed plausible that a similar sized marker (such as TexasRed labelled dextran (dex-TexR; 3000 g mol\(^{-1}\))) that is incapable of AMT or RMT uptake could serve as an internal standard to quantify paracellular contribution of the vehicles. As exemplified in Fig. 4A, experiments are performed in parallel with probes (both dex-TexR and FAM-PPII vehicle or TF-A647) fed to insets containing either the cell monolayer (PS) or filter alone (PS)). The corresponding \(P_{app}\) for dex-TexR in both scenarios is first calculated and the extent in which movement is reduced by presence of the monolayer (defined here as the transport ratio (TR)) is used to determine the expected paracellular diffusion rate of the individual vehicles. We employed TF-A647 and a similarly sized TexasRed labelled dextran (dex-TexR; 70 000 g mol\(^{-1}\)) as a positive control for transcytosis. Typical clearance profiles when compounds are incubated with and without cells, are shown in Fig. 4B–C. As expected, due to their similar molecular weight, both dex-TexR and TF-A647 afforded comparable clearance when incubated without cells (PS)). When incubated in the presence of cells, dex-TexR was detected in the basolateral compartment indicating back- ground paracellular flux. However, TF-A647 was cleared at a faster rate in comparison, confirming transcytosis of the molecule.

Corrected values were subsequently calculated to internally rank the transcytosis capacity of BBB-shuttles. In addition, to verify validity of the model, a selection of high permeability vehicles were selected for further studies at low temperature, where uptake and transcytosis should be inhibited. In agreement, shuttles showed permeability similar to dex-TexR (3000 g mol\(^{-1}\)), demonstrating negligible true transcytosis in this condition (Fig. S8†).

Permeability screen of BBB shuttles

Our screen shows that the majority of functionalised vehicles had higher rates of permeability than TF-A647 with PPII(R8X) TfRL1, PPII(L3X;L15X)ApPe2 and both bivalent TfRL2 vehicles being the exception, Fig. 4D. TfRL1 modified vehicles preferred a bivalent format (PPII(L3X;L15X)TfRL1: 3.5-fold increase relative to TF-A647; PPII(L3X;L15X)TfRL1: 3.0-fold), whereas TfRL2 and ApPe2 monovalent vehicles experienced higher levels of basal transport (PPII(R8X)TfRL2: 1.4-fold; PPII(R8X)ApPe2: 4.9-
towards the TfR does not govern transport ability for our peptidic BBB shuttles since trivalent vehicles combining high and low affinity ligands showed lower permeability than the corresponding bivalent high affinity TfR1 vehicles.

Whilst vehicle PPII(L3X;L15K)TfRL2(K);Apep2(X) gave the highest level of transcytosis for a highly functionalised shuttle (3.8-fold increase relative to Tf-A647), the increased permeability may be attributed to the ligands interacting independently with their receptors, since both ligands enjoyed higher transport in a monovalent form. In line with this hypothesis, transcytosis for PPII(L3X;L15K)TfRL2(K);Apep2(X) was lowest for trivalent vehicles with ligands merged to the same face (1.8-fold), implying that the ligands are not working in synergy. This result is in contrast to when ligand TfR1 and Apep2 are combined, which showed improved permeability when ligands are arranged on the same face.

**Conclusions**

Here we report the first use of CPP SAP as a scaffold for developing targeted BBB penetrable shuttles, which constitutes one of the most restrictive barriers in the body. In this regard, we designed a versatile vehicle library by a convergent approach, strategically introducing mutations within the sequence to graft RMT ligands in a selective manner using either amide bond forming or CuAAC mediated reactions. Notably, CuAAC provided a superior and flexible reaction for these modifications. Notably, SAP retained a helical PPII structure influencing both uptake and transcytosis for individual ligands. The majority of vehicles screened demonstrated heightened transcytosis rates compared to Tf-A647. Here we believe the small size of the peptide conjugates, compared to Tf and other macromolecule shuttles, afford them an advantage for targeted transcytosis due to higher diffusion rates. Within our permeability screening campaign, dextran-TexR was successfully included as an internal standard for quantifying paracellular and non-specific movement. Consequently, the results presented demonstrate that PPII derived shuttles represent novel, exciting and promising classes of bioconjugates for enhancing uptake at the BBB. The flexibility of the screening approach could be readily adopted to investigate other ligands for AMT and RMT uptake at the BBB to validate and identify optimal ligands and shuttles for delivery.

**Conflicts of interest**

There are no conflicts of interest to declare.

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Notes and references