Brain-penetrating peptide shuttles across the blood-brain barrier and extracellular-like space

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Abstract

Systemic delivery of nanomedicines into the brain is greatly impaired by multiple biological barriers—the blood-brain barrier (BBB) and the extracellular matrix of the extracellular space. To address this problem, we developed a combinatorial approach to identify peptides that are able to shuttle and transport through both barriers. A cysteine constrained heptapeptide M13 phage display library was iteratively panned against an established BBB model for three rounds to screen for brain-penetrating peptides. Using next-generation DNA sequencing and \textit{in silico} analysis, we identified peptides that were selectively enriched from successive rounds of panning for subsequent validation. Select peptide-presenting phage exhibited efficient intracellular uptake and shuttling across the \textit{in vitro} BBB model. Two clones, Pep-3 and Pep-9, exhibited specificity and higher efficiency of transcytosis and diffusive transport than other clones and controls and in particular, demonstrated better transcytosis and diffusion through extracellular matrix than gold
standard nona-arginine and clinically trialed Angiopep-2 peptides. From these in vitro studies, we demonstrated that systemically administered Pep-3 and Pep-9 peptide-presenting phage penetrate the BBB in vivo and distribute into the brain parenchyma. In summary, these in vitro and in vivo studies highlight that high-throughput screening can identify select peptides as promising carriers that are able to overcome the multiple biological barriers of the brain and shuttle different-sized molecules from small fluorophores to large macromolecules such as nanoparticles into the brain towards improved drug and gene delivery into brain.

Keywords

Blood-brain barrier, extracellular matrix, M13 phage, transcytosis, diffusion, peptide shuttle

Over 1 billion people globally suffer from neurological diseases, which account for 12% of total deaths annually\(^1\). Unfortunately, therapeutic delivery of drugs to the central nervous system (CNS) and into the brain parenchyma has remained a long-standing and significant challenge for CNS diseases\(^2\)–\(^4\). Upon their administration, drugs must penetrate multiple barriers such as the...
blood-brain barrier (BBB), the perivascular space, the cerebrospinal fluid, and the extracellular space surrounding the cells of the brain in order to reach target sites. In systemic delivery, the BBB is the primary barrier to the brain. The BBB consists of brain capillary endothelial cells and is regulated by supporting cells, such as pericytes, astrocytes and other glial cells, to form a tight and continuous barrier. During homeostasis, the polarized brain capillary endothelium simultaneously protects the brain from exposure to exogenous or toxic solutes and mediates selective exchange of essential nutrients, ions, and metabolites between blood and the brain interstitium by diffusion, transporters, and adsorptive- and receptor-mediated transport.

Typically, drugs exploit these pathways to permeate the BBB via the following: (1) hydrophobic small molecule drugs (< 400 Da) diffuse and penetrate through the endothelium; (2) other small molecules shuttle across the BBB by paracellular flux (i.e. openings between the endothelium); and (3) macromolecules including peptides and proteins use endogenous transport mechanisms (e.g. transferrin and insulin receptors, electrostatic adsorption) to actively transcytose, or go across, the BBB and enter the brain parenchyma. In spite of extensive efforts, drug delivery into the brain has not been successful, with ~98% of all small molecule drugs and nearly 100% of all biologics unable to shuttle across the BBB and reach the brain parenchyma.

After traversing the BBB, drugs and drug carriers must also navigate through the ubiquitous but underexplored extracellular space (ECS) prior to reaching the target cells. The ECS is a fluid-filled space (“water phase of a foam”) that surrounds all cells of the CNS and occupies 20% of the total brain volume. The ECS maintains dynamic flow of interstitial fluids and the ionic balance across the cell membranes. The ECS has an irregular structure around the cells with microdomains of void spaces. It consists of negatively charged, highly condensed extracellular matrix including high amounts of glycosaminoglycans (e.g. hyaluronan and heparin...
sulfate), proteoglycans\textsuperscript{16} and fibrous proteins (e.g. collagen and fibronectin). The geometry and composition of the ECS combine to hinder diffusive transport of molecules and drug delivery to the brain parenchyma\textsuperscript{17,21}. In the ECS, antibodies have been shown to bind to receptors, and lactoferrin\textsuperscript{18} binds to negatively charged heparin sulfate of the extracellular matrix\textsuperscript{22}; these molecules demonstrate significantly decreased transport in the ECS than in free medium. Consequently, solutes such as drugs need to circumvent size filtration and intermolecular interactions with the mesh-like network of brain extracellular matrix to diffuse through the ECS and reach target cells\textsuperscript{23,24}.

The complex molecular composition and the size capacity of the BBB and the ECS limit drugs and nanoscale delivery systems that possess the desired physicochemical properties to traverse these barriers. For example, nanocarriers greater than 150 nm in diameter are unable to penetrate the intact BBB\textsuperscript{25} and diffuse through the ECS\textsuperscript{19,26} due to their size. Also, while positive charged solutes can bind and enter the brain capillary endothelium, they are unable to efficiently dissociate from the plasma membrane and diffuse unhindered across the negatively charged ECS\textsuperscript{18,19,21}. Strategies such as intracerebral injection, hyperosmotic disruption, convection enhanced drug delivery, ultrasound-induced microbubble-mediated delivery, intranasal delivery, and functionalized nanoparticles\textsuperscript{27–32} have been used to transiently open, shuttle or bypass the BBB, but they have not yet achieved drug delivery in the CNS at therapeutic concentrations and/or require local delivery or a permeable BBB, which raises potential concerns over their safety.

One promising strategy to identify BBB-penetrating drug carriers is phage display technology. Phage, which are bacterial viruses, can be genetically engineered to display peptides or proteins on their surface. In particular, filamentous M13 phage are virus nanoparticles (~900 nm in length and 6–7 nm in diameter for M13) that present a collection, or library, of random
peptides. Subsequently, these peptide-presenting phage libraries can be combinatorially screened under selective pressure against a target, or panned, to select for peptides that possess the desired functionalities\textsuperscript{34}. These phage libraries are an attractive technology to identify brain penetrating peptides since they possess a larger chemical or design space than rationally designed peptides, and the selection requires no \textit{a priori} knowledge of the BBB targets. While phage display has been used to identify potential shuttle peptides, there are several limitations to current strategies. Traditional panning \textit{in vitro} using phage display identifies peptides that bind but may not transcytose the BBB and diffuse through the ECS. \textit{In vivo} panning may identify suboptimal peptides; since phages have short half-life in systemic circulation\textsuperscript{35,36}, it is possible that candidate peptides do not have sufficient time to bind and traverse the BBB. As a result, the first round of \textit{in vivo} selection becomes paramount to identify successful BBB penetrating peptides\textsuperscript{37}. Also, many peptides were identified using Sanger sequencing, which covers a limited sample space (5 – 1000 clones)\textsuperscript{38}.

To address the aforementioned challenges of delivery across the dynamic BBB and the ECS, we report the discovery of peptides that achieve both transport across the BBB and improved diffusion through the extracellular matrix (ECM). Here, cysteine constrained peptide-presenting M13 phage libraries were panned against an established \textit{in vitro} model of the BBB to identify phage that transported across the BBB. Through next-generation DNA sequencing and bioinformatics, select peptide sequences were identified and subsequently validated. Select peptide-presenting phage shuttle across the BBB and ECM using transport assays. Importantly, selected peptides demonstrate improved transcytosis and diffusive transport than gold standard nona-arginine\textsuperscript{39} and clinically trialed Angiopep-2\textsuperscript{40-42} peptides. Finally, from \textit{in vivo} studies, select peptide-presenting phage shuttle across the BBB and penetrate into the brain parenchyma. The
ability of these peptides to ferry small molecules and large macromolecules such as phage highlights their potential to effectively shuttle different nanomedicines into the brain to treat CNS diseases.

**Results and Discussion**

**Identification of BBB penetrating peptides by next-generation sequencing and analysis of their physicochemical properties**

Adapting from the pulse-chase assays used to study transcytosis of transferrin across the BBB^{43,44}, we developed a modified “pulse”-only assay to pan peptide-presenting M13 phage libraries *in vitro* against a human-derived BBB cell line hCMEC/D3, and through iterative screening, select for phage clones that transport across the BBB model and the underlying collagen matrix in a transwell system (**Figure 1**). Here, a cysteine constrained random heptapeptide (CX7C) M13 library was incubated in replicate against hCMEC/D3 cells plated on a collagen-coated transwell insert for 1 h at 37°C to allow phage internalization and/or recycling; inserts were subsequently washed and incubated for another 1 h to allow for phage transcytosis^{43}. hCMEC/D3 is a well-established *in vitro* BBB model used in drug transport studies that recapitulates the phenotype of the human BBB and avoids potential species differences with *in vitro* and *in vivo* rodent models^{45}. Cyclic peptide libraries were used because they have more conformational rigidity to bind to targets with high affinity, are stable, and less susceptible to protease degradation^{46}. The eluates of phage clones from each round were collected and amplified, and then they were either added to hCMEC/D3 for the subsequent round of panning, or their DNA was prepared for next-generation DNA sequencing (NGS). Since the M13 genome encodes for its phenotype, the phage displayed peptide sequences can be identified by DNA sequencing. Isolated phage DNA was barcoded and run on Illumina MiSeq to obtain a larger number of DNA sequences
Traditionally, the number of phage clones identified by Sanger sequencing is limited to 5 – 1000; using NGS, it is feasible to obtain up to 2.5 x 10^7 reads by the Illumina MiSeq platform, which allows for sufficient sampling of the CX7C library from biopanning and control experiments. Thereafter, NGS data was analyzed to identify peptide sequences and their frequency from each round of panning.

Figure 1 Scheme of CX7C peptide-presenting M13 phage library biopanning against in vitro BBB model and identification of peptide sequences. For the transcytosis assay, 10^11 plaque-forming units of CX7C peptide-presenting M13 phage library was added to the confluent hCMEC/D3 cells cultured on the transwell system in replicates. M13 phage clones in the eluate were grown in E. coli bacteria to amplify and make more copies for additional rounds of panning. Also, from each round, the DNA from the amplified eluate was isolated, purified, and prepared for next generation sequencing (NGS). NGS was used to obtain DNA sequences and then identify peptide sequences.

From NGS, DNA sequences were identified and translated into peptides from each round of biopanning in each replicate. After excluding insertless M13 phage from NGS dataset, the 20 most abundant peptides from the third round of biopanning were identified, and their frequency in the first two rounds was determined from the NGS dataset (Figure 2 and S1). From the third round of biopanning, both replicates shared 18 out of 20 of the most frequent sequences. The dominant peptide from the third round was Pep-1 with 7961 counts, which was approximately five-fold
higher than the second most frequent peptide Pep-2. The frequency of remaining sequences ranged from 121 – 618 counts in the third round of biopanning (Table 1). In both replicates, the eleven most abundant sequences (Pep-1 to Pep-11) exhibited apparent enrichment between successive rounds of biopanning (Figure 2 and Figure S1). With each successive round of panning, the increased frequency of the peptide is indicative of their affinity for the target\textsuperscript{48}. To ensure that the abundant sequences were due to selection enrichment and not because of their growth bias in bacteria\textsuperscript{47,49}, the naïve, original library was amplified in E. coli for three successive rounds without selection pressure, and the library DNA was sequenced by NGS. The twenty most frequent sequences from selection were not abundant in the third round of amplified naïve library; the counts ranged from 3 – 70. For example, there were only three reads of Pep-9 in the third round of amplified naïve library (Figure S2). This additional filter is needed to exclude potential false positives due to fast-growing phage clones\textsuperscript{50}. During biopanning, phage clones are selected against the target and subsequently these clones are amplified in host bacteria to make more copies; however, this results in phage clones that have affinity for the target and/or phage clones that easily amplify in bacteria. It has been demonstrated that amplification-based selection (i.e. clones that grow faster than other clones) is independent of target-based selection\textsuperscript{37,49–51}. Even without target-based selection, the diversity of phage libraries can collapse due to amplification-based selection. Our findings indicate that the twenty most frequent peptide-presenting phage clones from selection are not parasitic clones, and instead, their increased frequency with successive rounds of biopanning suggests that they bind to hCMEC/D3 cells.
Figure 2  Enrichment analysis of the 20 most frequent peptides from three rounds of biopanning against hCMEC/D3 cells. Peptides denoted from Pep-1 to Pep-20 refer to the 20 most abundant CX7C peptides present in the third round of biopanning. The sequence counts for each CX7C peptide from each round of screening were calculated as described in the Materials and Methods.

The physiochemical properties of the twenty most abundant peptides (Pep-1 to Pep-20) were determined in silico (Table 1). Here, 12 were basic, 1 was acidic, and 7 possessed a net neutral charge. The grand average of hydropathy (GRAVY) score was calculated as a value of the hydrophobicity (or hydrophilicity) of the peptides; the more positive GRAVY score correlates with greater hydrophobicity of the peptide sequence. From our listed sequences, 11 of them were hydrophobic (Table 1).

Table 1 Physiochemical properties of 20 most frequent peptides in the BBB biopanning
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<th>Peptide name</th>
<th>Charge Attribute</th>
<th>Net charge</th>
<th>PI</th>
<th>m.w. (g/mol)</th>
<th>Hydropathy (Gravy score)</th>
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**Transcytosis of select CX7C peptide-presenting phage in vitro**

After identifying peptide sequences, we confirmed the ability of individual phage-presenting phage to shuttle across the BBB in vitro. The eleven most abundant and enriched sequences (denoted as Pep-1- Pep-11) and the consensus motif were individually cloned into the M13KE vector for peptide display. In addition, negative control-NC, which demonstrated decreased frequency with successive rounds of panning (18 counts in round 1, not present in
subsequent rounds), and two BBB peptide shuttles from other groups\textsuperscript{56,57}, were also cloned into M13KE phage vector DNA. Transport of these individual peptide-presenting phage across hCMEC/D3 cells was quantified following the same transcytosis assay used for biopanning. The number of phage that shuttled across hCMEC/D3 over the total number of input phage for each M13 clone was calculated to compare their transcytosis or transport efficiency across the BBB (\textbf{Figure 3}). In particular, Pep-8 and Pep-9 presenting phages demonstrated highest shuttling efficiency, with output to input ratios of $1.48 \times 10^{-3}$ and $1.78 \times 10^{-3}$, respectively. The five most frequent peptide-presenting clones (Pep-1 - Pep-5) had transcytosis efficiencies of $2.34 \times 10^{-4}$, $1.09 \times 10^{-4}$, $2.02 \times 10^{-4}$, $4.51 \times 10^{-4}$, and $1.01 \times 10^{-4}$ respectively; the other clones amongst the top 11 had efficiencies $\sim 10^{-5}$. Here, the motif-presenting M13 clone had $3.76 \times 10^{-4}$ transcytosis ratio and the positive controls PC-1 and PC-2 had $5.34 \times 10^{-4}$ and $2.42 \times 10^{-4}$ respectively. As expected, the negative control NC demonstrated the lowest BBB shuttling efficiency, with a ratio $\sim 2.50 \times 10^{-5}$ (\textbf{Figure 3}). It has been demonstrated that M13 phage display libraries have limited diversity and have bias for individual amino acids at specific positions. Similar to these findings, other libraries demonstrate less diversity at the first and last position\textsuperscript{52} and compositional bias for specific amino acids\textsuperscript{58,59}. As observed by others\textsuperscript{60}, the motif sequence-presenting M13 clone may not bind and shuttle across hCMEC/D3 better than the selected clones, which may have a specific target or transport mechanism.
hCMEC/D3 cells were cultured to form tight and continuous BBB cell monolayer following the same procedures as prior experiments. The equivalent amount of each peptide-presenting M13 phages (Pep-1 to Pep-11, negative control NC, and positive controls PC-1 and PC-2) were added to the BBB model in the transwell system. The ratio of output phage that went across hCMEC/D3 to initial input phage was calculated to compare the transcytosis efficiency of each M13 clone. The transcytosis efficiency for all the validated clones varied within the range of $1.92 \times 10^{-5}$ to $3.5 \times 10^{-3}$.

To study cellular uptake of transcytosed phage in hCMEC/D3 cells, we tested phage that had transcytosis efficiencies above $10^{-4}$ (Pep-1 – Pep-5, Pep-8, and Pep-9) and compared to control phage (NC and PC-1). Phage were incubated with confluent hCMEC/D3 cells, and internalized phage were collected and quantified relative to the amount of their input (Figure 4). The selected clones demonstrated uptake ratios (i.e. number of internalized phage/input phage) ranging from $5.66 \times 10^{-4}$ to $2.07 \times 10^{-3}$ (Figure 4, in black filled bars). Interestingly, Pep-9 phage exhibited highest cellular uptake with a ratio of $2.07 \times 10^{-3}$, and the negative control NC phage exhibited the lowest uptake ratio of $4.17 \times 10^{-4}$. Cellular uptake of the phage clones correlated with the efficiency of transcytosis (Figure 3), which is expected since cell uptake is part of transcytosis. In
addition, the intracellular motion of phage clones was imaged and quantified using 2D particle tracking, which is able to track passive and active transport. Selected M13 clones were fluorescently labeled and incubated with confluent hCMEC/D3 cell monolayer for 1h. Intracellular transport of these clones was recorded as 30 s movies, and the trajectories of intracellular motion for each clone was analyzed using a 2D particle tracking method\(^{61,62}\). Here, the intracellular diffusion coefficient ranged from \(5.17 \times 10^{-2} - 7.98 \times 10^{-2} \, \mu m^2/s\) for the selected M13 clones (Figure 4). The active transport behavior of the M13 clones can be extracted and calculated from particle tracking trajectories; selected phage clones had velocities ranging from 1.08–1.41 \(\mu m/s\) (Figure S3). The velocities for active transport are within the observed values of active intracellular transport via motor proteins (0.5 – 2 \(\mu m/s\)) in EGFR trafficking\(^{62}\) and in other studies\(^{63–65}\). The velocities for passive diffusion were within the range measured for confined diffusion, which is observed during events associated with ligand-receptor binding\(^{62,66}\). The intracellular trajectories of a representative M13 clone and segmentation of their motion into active transport and passive diffusion are shown in Figure S4. The intracellular trajectories of the phage clones and their calculated velocities suggested that identified M13 phage clones may use active transport to shuttle across the BBB model.
Figure 4 Cellular uptake and intracellular diffusion of the M13 clones in hCMEC/D3 cells. hCMEC/D3 cells were cultured to confluency in 12-well plates. Equivalent amount of M13 clones (input) were added to cells for 1 h at 37°C. The amount the clones accumulated intracellularly were quantified (output). The ratio of output to input represents the efficiency of cellular uptake for each clone, which is shown on the left y-axis (black-filled plots). The uptake efficiency ranged from $5.66 \times 10^{-4}$ to $2.07 \times 10^{-3}$ for all the clones. 2D particle tracking method was used to monitor and calculate the trajectories of each clone trafficking inside of hCMEC/D3 cells. Multiple 30 s movies were recorded (20 frames/s) to track the motion of Alexa Fluor® 488 conjugated M13 clones inside of hCMEC/D3 cells. Then, three steps of processing methods were performed on the movies: (i) Identifying contiguous regions of pixels; (ii) Gaussian fitting; and (iii) building trajectories from coordinates. About 193 - 1299 qualified trajectories were chosen to calculate the mean-squared displacement (MSD) for each clone. The diffusion coefficient (D) ranged from $5.17 \times 10^{-2}$ - $7.98 \times 10^{-2}$ µm²/s for all the tested M13 clones. The data is shown with the pink-filled bars, with scale on the right y-axis.

To confirm that transport across the BBB is sequence-specific, the transcytosis of phage clones (with transcytosis efficiencies above $10^{-4}$) was compared to their scrambled controls, i.e.
phage displayed peptide with same amino acid composition but in random order of the 7-mer in the CX7C region (Figure 5). Here, Pep-3, Pep-4, Pep-5, and Pep-9 clones exhibited statistically greater transcytosis efficiency \((3.99 \pm 0.44) \times 10^{-4}, (5.87 \pm 0.66) \times 10^{-5}, (5.00 \pm 0.12) \times 10^{-5}, (7.71 \pm 0.23) \times 10^{-5}\), respectively) than their respective scrambled controls \((1.13 \pm 0.26) \times 10^{-4}, (3.03 \pm 0.64) \times 10^{-5}, (3.00 \pm 0.91) \times 10^{-5}, (3.61 \pm 1.58) \times 10^{-5}; p \leq 0.05\). If phage clones did not demonstrate specific transport in hCMEC/D3 cells, altering the sequence order would not change their transport\(^{67-69}\), as seen with other phage displayed peptide ligand-target binding studies\(^{70,71}\). This result suggests that these highlighted clones have specific interactions with targets present on the hCMEC/D3 cells.

Figure 5 Transcytosis assay of selected peptide-presenting M13 phages compared to their respective scrambled controls in hCMEC/D3 cells. The efficiency of transcytosis (i.e. output to input) of Pep-1,
Pep-2, Pep-3, Pep-4, Pep-5, Pep-8, Pep-9 and motif-presenting M13 clones was calculated from their transcytosis in hCMEC/D3 cells in the transwell system. The transcytosis efficiency of each clone was normalized to the efficiency of their respective scrambled control M13 clone.

To determine if selected phages transport across hCMEC/D3 cells through a temperature-dependent mechanism, the transcytosis assay was performed at 37°C and 4°C. Transcytosis efficiency was 25 to 402-fold higher for the phage clones at 37°C than 4°C (Figure 6). Of note, Pep-8 and Pep-9 phage clones demonstrated the greatest difference in their transport, with a 402- and 169-fold decrease at 4°C, respectively. Our findings are in agreement with other peptide-mediated transport studies with D1 peptide72 and tympanic membrane transport peptide TMT-373, which showed energy-dependent active transport. Combined with the results in Figure 4 and S4, our data suggests that phage clones are actively transported across hCMEC/D3 cells.

![Figure 6 Temperature dependent transcytosis assay of peptide-presenting M13 phages in hCMEC/D3 cells](image)

**Figure 6 Temperature dependent transcytosis assay of peptide-presenting M13 phages in hCMEC/D3 cells.** Transcytosis assays were performed at 37°C and 4°C for selected M13 clones against confluent hCMEC/D3 cells. The transcytosis efficiency was calculated as the ratio of output to input phage for each clone. The transcytosis efficiency for all the clones ranged from $3.28 \times 10^{-6}$ - $1.03 \times 10^{-5}$ at 4°C and $1.01 \times 10^{-4}$ - $1.78 \times 10^{-3}$ at 37°C.
Transcytosis of selected peptides against BBB in vitro

While our prior experiments focused on validating transport of peptide-presenting phage in vitro, we wanted to confirm the ability of the peptides to facilitate BBB transport without the structural context of the M13 phage. Here, fluorescently-labeled Pep-1, Pep-3, Pep-4, Pep-5, Pep-8, Pep-9, cell-penetrating peptide nona-arginine (R9)\textsuperscript{39}, and Angiopep-2\textsuperscript{74} peptides were synthesized and tested for their ability to traverse the hCMEC/D3. R9 is an arginine-rich peptide that efficiently binds to cells via electrostatic interactions and has been shown to penetrate the cellular membrane by macropinocytosis\textsuperscript{73} at low nanomolar to micromolar concentrations and by pore-forming translocation into the cytosol at higher concentrations\textsuperscript{75,76}. Angiopep-2 is a rationally designed peptide from aprotinin, a low-density lipoprotein receptor-related protein, that has been shown to cross the BBB\textsuperscript{74,78} and used in clinical trials to shuttle different drugs such as small molecule paclitaxel\textsuperscript{79}, neurotensin peptide\textsuperscript{80}, and Her2 antibody\textsuperscript{81}, across the BBB. Using the transcytosis assay described earlier, equivalent molar weight of each FAM-labeled peptide was incubated with confluent hCMEC/D3 cells in the donor compartment of the transwell system and their fluorescence was measured in the receiving compartment at a series of timepoints up to 120 min. The ratio of output to input fluorescence intensity was calculated to represent the ability of each peptide to shuttle across the in vitro BBB and account for any potential differences between fluorescent labeling of synthesized peptides. The output/input ratio for each peptide at different time points varied from the range of 0.00159 - 0.361. At 120 min, the output/input for all the tested peptides was within the range of 0.130 - 0.361. Interestingly, the selected peptides demonstrated transcytosis efficiency comparable to Angiopep-2 up to 60 min. Pep-3 and Pep-9 had equivalent transport compared to Angiopep-2 at 90 min, whereas only Pep-3 had similar transport at 120 min (Figure 7, p < 0.05, two-way ANOVA). All CX7C peptides demonstrated improved transport
compared to R9 throughout the duration of the assay (except at 5 minutes; Figure 7, p < 0.05, two-way ANOVA). However, while R9 has been used as a cell-penetrating peptide\textsuperscript{21,77}, there are no reports of the ability of R9 to transcytose and exit the BBB. These results suggest that our CX7C peptides are more efficient to shuttle across hCMEC/D3 cells than R9, and certain CX7C peptides exhibited similar transport to Angiopep-2.

Figure 7 Transcytosis assay of 5’carboxyfluorescein (FAM)-conjugated CX7C, R9, and Angiopep-2 peptides against hCMEC/D3 cells. 10 nmol of each FAM-labeled CX7C peptides and controls were added to confluent hCMEC/D3 cells in the donor compartment of the 24-well transwell and incubated up to 120 min at 37°C. For each peptide at each time point, the fluorescence intensity of the receiving compartment was measured by a plate reader and the ratio was calculated relative to the fluorescence of the initial amount added to the donor compartment.
To confirm that the selected peptides transport across the BBB through energy-dependent pathways, selected FAM-CX7C peptides were incubated against hCMEC/D3 cells at 37°C and 4°C respectively, and transcytosis efficiency was quantified as before. All peptides demonstrated decreased transport at 4°C (Figure 8). R9 demonstrated the greatest decrease in transport at 4°C, with an almost ten-fold reduction in transcytosis efficiency; this is most likely due to its inability to be internalized by the cells. The other peptides exhibited ~1.5- to 2.4-fold reduction in transcytosis efficiency at 4°C. In particular, Pep-8 and Pep-9 demonstrated the greatest decrease in transcytosis efficiency amongst the selected CX7C peptides at 4°C compared to 37°C, with a 2.0- and 1.8-fold decrease, respectively. This trend is consistent with the results from energy-dependent transcytosis of peptide-presenting phage (Figure 6).
Figure 8 Temperature-dependent transcytosis of the FAM-labeled peptides against hCMEC/D3 cells. Transcytosis assay of 10 nmol of each fluorescently-labeled peptide was performed against hCMEC/D3 cells in the 24-well transwell plate at 37°C and 4°C up to 120 min. At each timepoint, the ratio of fluorescent intensity was calculated as in prior studies to represent the transcytosis efficiency. In each panel, the transcytosis efficiency of each peptide at 37°C (black line) and 4°C (red line) was plotted versus time.

**Diffusive transport of selected peptides through extracellular matrix**

After penetrating the BBB, molecules must also traverse the extracellular matrix (ECM) in the extracellular space of the brain to reach the parenchyma. Since transport through the brain ECM is mainly driven by diffusion\(^3\,\text{82}\), we did two experiments to study diffusion of the selected peptide shuttles through Matrigel, an ECM mimic that has been extensively used to study diffusive transport of solutes and nanoparticles\(^85\,\text{–}\,87\). In one experiment, equivalent molar weight of each FAM-labeled peptide was added to the donor compartment of a transwell insert coated with Matrigel and allowed to diffuse into the receiver compartment of the transwell. The fluorescent intensity of peptides was measured in the receiver compartment up to 6 h. The ratio of output to
input fluorescence represents the efficiency of each peptide to diffuse through Matrigel. For the duration of the study, R9 diffused slowest through Matrigel among all tested peptides (Figure 9, two-way ANOVA, p < 0.05). Importantly, Pep-1, Pep-3, Pep-4, Pep-5, Pep-9 peptides exhibited improved transport through Matrigel than Angiopep-2 (except at 300 and 360 min timepoints, where Pep-5 was comparable to Angiopep-2); Pep-8 demonstrated equivalent transport to Angiopep-2 during the entire time course (two-way ANOVA, p < 0.05).

Next, diffusive transport of the peptides was imaged and in a microfluidic chamber with embedded Matrigel to exclude possible interactions between the peptides and the polycarbonate membrane insert from the transwell. Here, equivalent molar weight of FAM-CX7C peptides and
controls were incubated with Matrigel embedded in the channels of the chamber slide under sink conditions, and the fluorescence was visualized by time-lapse imaging to observe migration of peptides through the gel-embedded channels. Whole field-of-view fluorescent images of each channel was taken every 30 min up to 12 h, and representative images at 0, 6, and 12 h are shown in Figure 10 for each peptide. All selected CX7C peptides migrated faster than R9 and Angiopep-2 during the diffusion assay. From time-lapsed imaging, the mean diffusivities of the peptides were calculated with a custom Matlab script based on the solution to Fick’s First Law, presented as equation (1) shown below.

\[ C(x,t) \propto \text{erfc} \left( \frac{x}{2 \sqrt{D_{\text{eff}} t}} \right) \]  

where \( C \) is the fluorescence intensity of the FAM-labeled peptides, \( x \) is the penetration distance at a given time \( t \), and \( D_{\text{eff}} \) is the effective diffusivity. The diffusivity of each selected peptide was approximately \( 10^{-7} \) cm\(^2\)/s, which was \~10-fold higher than R9 (\( 1.24 \times 10^{-8} \) cm\(^2\)/s) and Angiopep-2 (\( 1.17 \times 10^{-8} \) cm\(^2\)/s) (Figure 10). Selected CX7C peptides demonstrated greater diffusivity than controls, most likely due to their absent or weak electrostatic interactions with the ECM. Matrigel has a net negative charge and the selected CX7C peptides do not possess highly positive charged residues (net charge of 0 to +2, Table 1). The highly positive net-charge of R9 (+9) facilitates efficient cell binding to capillary endothelium, but charge effects can adversely impact exocytosis and perfusion through the brain parenchyma. While work has showed that arginine-rich peptides demonstrate efficient cell uptake, it is unclear whether R9 can cross the BBB and transport through the extracellular space in the brain parenchyma. Also, although Angiopep-2 is able to enter the brain parenchyma, engineered Angiopep-related peptides with +4 and +6 net-charge demonstrated significantly less distribution into the brain parenchyma due to their accumulation with the negatively-charged blood vessels. Another study reported that lactoferrin
protein exhibited hindered diffusivity through the extracellular space due to charge interactions. Lactoferrin has basic amino acids near the N-terminus that bind to negatively charged heparin and heparin sulfate present in the ECM, thereby inhibiting its diffusion through the extracellular space of the brain\textsuperscript{3,22}. These collective findings coupled with our results substantiate that charge-based interactions can impact diffusion through the ECM, which is critical for drug delivery through the extracellular space\textsuperscript{17}.

A 0h

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[Image of gel electrophoresis showing bands at 0h for different peptides]
Figure 10 Diffusion of FAM-CX7C, R9 and Angiopep-2 peptides in Matrigel embedded in a six-channel chamber slide. 6nmol (60µL of 100 µM FAM-conjugated peptide) was added to the one side of the reservoir of the microfluidic chamber slide, meanwhile 60 µL PBS buffer was loaded to the other side reservoir to establish sink conditions. Olympus IX83 fluorescence microscope was calibrated and setup to record the fluorescent images of the whole field of each channel (each peptide) every 30 mins, up to 12h. A, the fluorescent images of each peptide loaded into the reservoir at 0 h. B, the fluorescent images of each peptide migrating through the ECM-embedded channel at 6 h. C, the fluorescent images of each peptide migrating through the ECM-embedded channel at 12 h. Custom Matlab script was written to calculate the mean diffusivity of each peptide through ECM, and data shown in table (bottom).

<table>
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<th>Mean diffusivity (cm²/s)</th>
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Select peptide-presenting M13 phage penetrate the BBB and enter the brain parenchyma *in vivo*

From the prior studies, Pep-3 and Pep-9 presenting phage were the most promising clones for BBB penetration and ECM diffusion *in vitro*, and they were subsequently tested for their ability to penetrate the BBB and reach the brain parenchyma *in vivo*. Pep-3, Pep-9 and negative control M13 were injected into healthy Balb/C mice, and 30 minutes post-injection, mice were sacrificed, perfused, and their brains were harvested. To determine if select phage distributed into the brain parenchyma, capillary depletion was done on the harvested brains to isolate brain parenchyma from the blood vessels, and amount of phage in the parenchyma, blood vessel, and blood serum was quantified by standard phage titering. Pep-3 and Pep-9 had significantly higher accumulation in the brain parenchyma than control, with brain parenchyma/serum ratios of 0.28, 0.26 and 0.10 µl/g, respectively (*Figure 11A*). The brain capillary/serum ratio was 1.44 and 0.90 µl/g for Pep-3 and Pep-9 presenting M13 phages respectively, which were both significantly higher than NC-clone (0.65µl/g) (*Figure 11B*). The clones exhibited similar distribution in the blood 30 minutes after injection, which suggests they have similar circulation half-life. As a result, the difference of uptake suggests that select clones mediate BBB transport into the parenchyma. The amount calculated in the brain parenchyma/serum ratio (µl/g) and brain capillary/serum ratio (µl/g) for Pep-3 and Pep-9 were on the same order of magnitude to reported values from other BBB transport studies. BBB permeable peptide-nesfatin demonstrated a parenchyma/serum ratio ~1.05 µl/g and capillary/serum ratio ~0.51 µl/g\(^91\). In other studies, epinephrine-mediated delivery of beta glucuronidase had ~1.04 and 1.08 µl/g\(^92\) in the parenchyma and capillary, respectively, and sulfamidase enzyme had the values ~0.87 and 0.17 µl/g\(^93\), respectively. Epinephrine and sulfamidase both shuttle across the BBB through mannose 6-phosphate/insulin-like growth factor 2 receptor-mediated transport\(^91-93\). Our select M13 clones traverse the BBB and enter the brain
parenchyma compared to control, but the kinetics of uptake require further optimization. M13 phage demonstrates a short systemic circulation half-life in vivo, and by increasing its half-life, accumulation into the parenchyma is expected to increase. While the amount of M13 penetrating into the brain parenchyma can improve, it is important to note that these select peptides can ferry this large macromolecule (molecular weight of M13 phage ~16.4 MDa, with dimensions of ~900 nm length, 6-7 nm diameter) across the BBB.
Figure 11 Distribution of select phage clones into the brain parenchyma *in vivo*. 6-8 weeks old Balb/c mice were injected intravenously with either Pep-3, Pep-9, or negative control (NC) presenting M13 phage clones and were allowed to circulate in vivo for 30 min. Afterwards, mice were euthanized and perfused, and the whole brain was harvested and prepared by capillary depletion to differentiate the brain parenchyma and brain capillaries. The distribution of phage clones in blood serum, brain parenchyma and capillaries was quantified by standard phage titering. A, the ratio of brain parenchyma to blood serum (µL/g) was calculated accounting for the individual brain weight, which is shown in bar plot, with mean and standard deviation calculated from five mice (P < 0.05 for each clone). B, The ratio of brain capillaries/blood serum was shown in bar plot, which was calculated from data of five mice in each group (P value <0.05).

Conclusions

Treatment for neurological illnesses remains poor due in part of the inability of therapeutics to distribute throughout the compartments of the brain to reach the diseased site. As a result, drug delivery remains a major challenge to successful treatment of CNS diseases. The majority of therapeutics are unable to traverse either the BBB or blood-cerebrospinal fluid barrier and
effectively diffuse through the surrounding extracellular space to reach the brain parenchyma. Current brain delivery strategies about BBB transport mainly focus on transiently opening the BBB using focused ultrasound\(^9^4\) and hyperosmotic agents\(^9^5\), on bypassing the barriers through local delivery\(^9^6\), or receptor-mediated transport. These studies address either transport across the BBB or circumventing the BBB to study diffusive transport through the ECS. However, there has been no strategy that explicitly addresses delivery of molecules through both barriers of the BBB and the extracellular space into the brain parenchyma, which is critical to achieve therapeutic concentrations throughout the brain.

Here, for the first time, we used a combinatorial approach to identify peptides that transport across the BBB and diffuse through ECM *in vitro* and *in vivo*. We address this challenge by using phage display with next-generation sequencing to identify peptide-presenting phage that transport across the BBB and diffuse through the ECM. Through *in vitro* biopanning, peptides were identified that functionally penetrate the BBB and ECM. While prior strategies have used rational design (Angiopep family\(^7^8\)) and phage display *in vitro*\(^9^7\) and *in vivo*\(^9^8\) biopanning to screen BBB-targeting peptides that were mainly identified by Sanger sequencing, they did not effectively account for the necessity for peptides to transport through the extracellular space of the brain microenvironment after crossing the BBB. Here, we demonstrated that our selected CX7C peptides, in particular Pep-3 and Pep-9, exhibited greater transport across the BBB than the cell-penetrating peptide R9 and comparable with Angiopep-2, which has been clinically tested to improve drug delivery for brain-associated cancers\(^7^9,8^0,8^1\). Importantly, our identified CX7C peptides showed improved diffusivity through extracellular matrix than R9 and Angiopep-2. Angiopep-2 has been shown to reach the brain parenchyma\(^7^4\) but demonstrates around 10-fold slower diffusion through ECM than our peptides. The selected peptides have less positive net charge than Angiopep-2 and
could account for improved diffusion through the negatively charged ECM; improved diffusivity will improve solute transport through the extracellular space. In \textit{in vivo} studies, Pep-3 and Pep-9 presenting M13 phage clones intravenously administered in healthy mice were able to cross the BBB, extravasate, and enter the brain parenchyma. Pep-3 and Pep-9 presenting phage exhibited brain parenchyma/blood serum ratios comparable to the other BBB permeable macromolecules that underwent receptor-mediated BBB transport. While further work is needed to elucidate the mechanism of BBB transport and ECS transport \textit{in vivo}, these collective findings suggest that Pep-3 and Pep-9 can specifically bind to the BBB and mediate transport into the brain parenchyma. Since these peptides facilitate transport of the “large” M13 phage across the BBB and into the parenchyma, these brain penetrating peptides are an attractive and broad platform that can potentially transport and deliver previously BBB impermeable drugs, including macromolecules such as enzymes, antibodies, and nanoparticles, across the BBB and through extracellular space to treat diseases of the CNS.
Methods

**CX7C-presenting M13 phage biopanning against hCMEC/D3 cells**

A M13 phage CX7C library was used to pan against established BBB model hCMEC/D3 *in vitro* to identify potential brain-penetrating peptides. hCMEC/D3 cells (CELLutions Biosystems Inc.) were cultured in EndoGRO medium (Millipore, #SCME004) on Collagen I (Fisher Scientific, #44310001) coated Culture Ware (150 µg/ml, coated for at least 1 h at 37ºC)\(^9\). Besides collagen I coating on cell culture surface, hCMEC/D3 cells produce extracellular matrix components for adhesion and support and can partially provide a mimic of the brain ECS. To establish the cell monolayer on the transwell plate setup, hCMEC/D3 cells (passage 27-30) were seeded on a pre-coated collagen I (100 µg/ml for 3 h) coated 12-well transwell plate (Corning,#3401) at density of 1.5 - 2.0 *10^5* cells/cm\(^2\) for 12-15 days\(^35\). Transepithelial electrical resistance measurements and permeability assay with small tracer molecule sodium fluorescein were used to monitor the tightness of hCMEC/D3 monolayer. Prior to biopanning, hCMEC/D3 cells were incubated in fetal bovine serum depleted medium for 1 h at 37ºC, and then 10\(^{11}\) plaque forming units (pfu) of M13 phage CX7C library (NEB, #E8120S) was added to the donor compartment of the transwell and incubated for 1 h at 37ºC. After the “pulse”, the donor and receiver compartment volumes were collected, the surface of hCMEC/D3 cells was washed for 3 times with phosphate buffered saline (PBS). The transwell inserts were then transferred to a new 12-well plate with replenished fresh medium and incubated for 1 h at 37ºC. After, the eluate from receiver compartment was collected and amplified in XL-1 Blue *E. coli* (Fisher scientific #50-125-053) for the subsequent round of biopanning. In total, the biopanning was done for three rounds, and each round was done in duplicate. The phage library DNA from the amplified eluates were isolated and prepared for next generation sequencing (NGS) (see below). In addition, for analysis of fast growing phage, the
original, naïve M13 CX7C library was also amplified in XL-1 Blue *E. coli* for three rounds without any selection and the pooled library DNA was prepared for NGS.

**Phage DNA sample preparation and next generation sequencing (NGS) analysis**

20 µL (phage concentration ~1.0*10⁸ pfu/µL, which is equivalent to phage DNA concentration 5 ng/µL) of each amplified eluate from biopanning and naive library were incubated at 100°C for 15 min then cooled down at room temperature. Library DNA preparation protocol (Illumina 16S metagenomic sequencing library protocol) was followed according to manufacturer’s recommendations to prepare the M13 phage library DNA. Briefly, two-step PCR was performed to prepare the library DNA amplicon. The first step PCR was to amplify the random region of the M13 library DNA. The primers were designed as 5’ TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGC AAG CTG ATA AAC CGA TAC A 3’ (forward primer) and 5’ GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTT GTC GTC TTT CCA GAC GTT AG 3’ (reverse primer). The second step PCR was to add the barcodes to the first step PCR product. Here for the second step PCR, the PCR primers were provided with Nextera XT Index kit (FC-131-1001, Illumina). Two steps of PCR sample preparation, including PCR conditions, PCR product clean up, and library DNA pooling, were performed according to the instructions provided in the protocol (16S-, Illumina). Two replicates of eluates from the every round of biopanning were prepared for the library DNA and pooled to run by Illumina MiSeq on two different batches.

**Bioinformatics analysis of the top 20 peptide sequences**

The NGS data from two replicates of MiSeq run were analyzed on Stampede, a supercomputer run on Texas Advanced Computing Center at UT-Austin. First, to confirm the sequence quality and trim the low-quality sequence reads, fastqc and fastx_toolkit were used on the dataset. Customized Perl and Bash scripts were written to filter out sequences of insertless M13 phage DNA.
Customized Python and bash scripts were used to translate DNA sequences into CX7C peptides and count the distribution (i.e. frequency) of CX7C peptides. Meanwhile, additional online tools (Clustal Omega, Emboss transeq and Gibbs Cluster server) were also used to confirm the quality of DNA sequence reads, translate DNA sequences to peptide sequences, as well as calculate the motifs shown in each round of biopanning. After filtering the low-quality and insertless M13 phage DNA sequences, the 20 most frequent CX7C peptides from the third round of biopanning were selected for further enrichment analysis. The motifs were calculated from the 20 most abundant peptide sequences from each round of biopanning and the amplification of M13 phage CX7C naive library to find out the consensus related to BBB transport. Physiochemical properties were also calculated to the 20 most frequent peptides\textsuperscript{100}. Protein Calculator v3.4 (The Scripps Research Institute) was used to calculate the net charge and grand average of hydropathy (GRAVY), where the more positive score is indicative of the hydrophobicity of the sequence.

**Cloning CX7C peptide-presenting M13 phages and their scrambled controls**

From the enrichment analysis of the 20 most frequent peptides from the third round of panning, the top 11 frequent peptides shown significant enrichment from the three rounds biopanning. Therefore, complementary DNA oligonucleotides were designed for the 11 most frequent peptides and their respective scrambled controls. Meanwhile, oligonucleotides were also designed for the motif sequences shown in 20 most frequent peptides in the third round of screening. Oligonucleotides were also designed encoding for positive control BBB shuttle peptides PC-1 and PC-2. One of the non-enriched peptides NC-1 was selected as the negative control, and oligonucleotides encoding for the peptide were designed for this control. All the DNA oligonucleotides (synthesized by IDT) were dissolved in DNase free and RNase free H$_2$O to a stock concentration of 100µM and subsequently diluted to 1 µM as the working concentration.
Complimentary oligonucleotides encoding for the respective peptides were annealed starting at 95°C for 2 min and cooled down to room temperature in 1 h using a heat-block plate (ThermoFisher Scientific, model #2001). Annealed oligonucleotides were phosphorylated using T4 polynucleotide kinase (NEB) following manufacturer’s recommendations. M13KE phage cloning vector (NEB) was double digested with Kpn I and Eag I high fidelity restriction enzymes (NEB) at room temperature for 2 h. Double digested M13KE phage vector was ligated with phosphorylated oligos at 16 °C overnight with T4 ligase (NEB). XL-1 Blue chemically competent cells (Fisher Scientific #50-125-053) were transformed with ligated DNA by the heat shock transformation and overlaid on solid agar plates for plaque formation. The resulting phage plaques were isolated, and phage DNA was isolated and purified. Sanger sequencing confirmed the identity and correct insertion of the peptide sequence in M13KE. The correctly sequenced peptide-presenting phage clones were amplified in XL-1 Blue E. coli in sufficient quantities for subsequent in vitro and in vivo experiments.

Transcytosis of CX7C peptide presenting M13 phage against hCMEC/D3 cells

hCMEC/D3 cells were seeded on the 12-well transwell plate at passage 27-30 following the same procedures described in biopanning. $10^9$-$10^{10}$ pfu of each M13 clone (input) was incubated with hCMEC/D3 cells. The eluate output in the receiver compartment of the transwell system from second hour incubation after “pulse” assay was collected and titered by standard double layer plaque assay. The total titer of phage (pfu) in the eluate was calculated for each M13 clone. The ratio of output to input phage (i.e. ratio of phage clone that transcytoses the BBB model) was calculated to obtain the efficiency of BBB shuttling for each M13 clone and control, and samples were run in triplicate. To compare the sequence specificity of M13 clones, selected BBB-shuttling clones and their respective scrambled controls were run using the same transcytosis assay as
described earlier. In addition, temperature-dependent transcytosis assays were run using the same setup at 4°C and 37°C.

**Intracellular tracking of fluorescently-labeled M13 phage**

Select peptide-presenting M13 phage (Pep-1, Pep-2, Pep-3, Pep-4, Pep-5, Pep-8, and Pep-9), motif-sequence clone, negative and positive control M13 clones were conjugated with Alexa Fluor 488 5-sulfodichlorophenol (SDP)-ester, (ThermoFisher Scientific, #A30052) at a ratio of $1.0 \times 10^{10} - 10^{11}$ pfu/50 µg dye. The fluorescent dye labeling was done shaking on a rocker for 1 h at room temperature. The labeling reactions were then dialyzed overnight with a dialysis cassette with a molecular weight cutoff of 3500-5000 Da (Spectrum Labs, #G235029, MWCO: 3.5k-5k). Dialyzed M13 clones were precipitated with 1/6 volume of 20% polyethylene glycol (PEG) and 2.5 M NaCl overnight at 4°C. After centrifugation, the resulting phage pellets were washed with PBS and then reconstituted in PBS. For tracking studies, hCMEC/D3 cells were cultured in 8-well chamber slide (ThermoFisher Scientific #154534) at a seeding density of $1.0 - 2.0 \times 10^4$ cells/cm². For each clone, $10^8 - 10^9$ pfu of each fluorescent-labeled M13 phage was incubated with confluent hCMEC/D3 cells for 1 h at 37°C. After, the cell culture medium with unbound phage clones was removed, cells were washed with PBS three times, and fresh cell culture medium was added to chamber prior to 2D particle tracking.

Wide-field imaging for single-particle tracking (SPT) was performed using an Olympus IX71 inverted microscope equipped with a 60× 1.2 N.A. water objective (UPLSAPO 60XW, Olympus). All imaging was conducted at 37°C using a temperature-controlled stage (Stable Z System, Bioptechs). Wide-field excitation was provided by a metal halide lamp with a 480/40 nm BP excitation filter. Emission was collected by a Scientific CMOS camera (ORCA-Flash4.0) through 510 nm LP dichroic mirror and 535/50 BP filter. The pixel size is equivalent to 107 nm.
Fluorescent images of labeled phage were acquired at 20 frames per second for a total of 600 frames. The analysis of the acquired image series was performed as described previously\textsuperscript{101,102} to obtain trajectories. The SPT software was a gift from Prof. Keith Lidke at the University of New Mexico. The trajectories were analyzed using a mean-squared displacement (MSD)-based trajectory classification algorithm\textsuperscript{62} to extract the diffusion coefficient ($D$) and identify the sub-trajectories exhibiting active transport.

**Cellular uptake of M13 clones in hCMEC/D3 cells**

hCMEC/D3 cells (passage 27-30) were seeded in a 12-well plate (Corning #3513) at a seeding density of 4.0*10^4/cm^2 and cultured for 5 days. Each M13 clone (~10^9 pfu) was added to the confluent hCMEC/D3 cells and incubated at 37 °C for 1 h. Then, culture medium was removed, stripping buffer (0.2% BSA in basal endothelial culture medium, pH 3.5 adjusted by HCl) was added to remove cell-surface bound M13 phages\textsuperscript{103}. hCMEC cells were then washed with PBS three times, and cells were lysed with RIPA buffer (ThermoFisher Scientific, #89900) to collect M13 clones internalized in hCMEC/D3 cells. M13 phage from cell lysates were quantified by double-layer agar plaque assay.

**Transcytosis of CX7C peptides against hCMEC/D3**

Selected CX7C peptides and controls were synthesized and fluorescently labeled with 5’carboxyfluorescein (5-FAM) at the N-terminus of each peptide (Lifetein). Transcytosis of peptides were performed against confluent hCMEC/D3 cells cultured in the 24-well transwell plate (Corning #3413) as described earlier. Upon formation of tight, continuous hCMEC/D3 on day 12 – 15, cells were incubated in fetal bovine serum-free medium for 1 h. Then 100 µM of each peptide (concentration dependent assay determined by flow cytometry, data not shown) was prepared in 100 µL basal culture medium and added to donor compartment, and 600 µL medium
was replenished the receiver compartment. At 5, 10, 15, 30, 45, 60, 90 and 120 mins, each transwell insert was temporarily transferred to the neighboring empty wells, and the fluorescence intensity in the receiver compartment (denoted as output) was read using a plate reader (Infinite M200, Tecan)\textsuperscript{104}. The ratio of output to input was calculated to compare the transcytosis efficiency of the CX7C and controls. To compare the energy dependent effect of transcytosis, the assay was run at 37ºC and 4ºC for each peptide.

**Diffusion of selected peptides through Matrigel in a transwell assay**

Matrigel (Corning #354230) was used as an *in vitro* mimic of the ECM with 2 mm thickness coating in 24-well transwell insert\textsuperscript{105}. Matrigel consists of soluble basement membrane proteins derived from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM components\textsuperscript{83,84}. Matrigel-formed ECM barrier has a pore size around 0.14 μm\textsuperscript{85}, which has been frequently used for diffusion study of solutes including nanoparticles\textsuperscript{85–87}. Matrigel was added to the transwell and allowed to gel at 37ºC for 30 min. Upon gelation, 200 μL of 100 μM CX7C and control peptides were added to the donor compartment, 1000 μL fresh medium was replenished to the receiver compartment to maintain sink conditions. The fluorescence intensity was measured in the basolateral compartment using the plate reader every 5 min for the first 1 h and every 1 h up to 6 h\textsuperscript{104}. The output to input (ratio of fluorescence in the receiver compartment to fluorescence of initial 20 nmol peptide added to donor compartment) and diffusion coefficient was calculated to compare the diffusivity of each peptide in Matrigel.

**Diffusion of selected peptides through Matrigel in a gel-embedded multi-channel chamber slide**

For diffusion through microchannel μ-Slide 0.4 VI chamber (ibidi) was loaded with 30 μL Matrigel in each channel of the chamber in a cold room and allowed to gel following the
manufacturer’s recommendations. After gelation, the basal medium was added to hydrate the gel for 10 min. 60 µL of 100 µM of each peptide was added to the inlet reservoir of the chamber, and 60 µL of basal medium was added to the other reservoir to create sink conditions. Olympus IX83 fluorescence microscope was set-up to image migration of fluorescent peptides in the Matrigel filled channel by recording fluorescent images every 30 min up to 12 h with Hamamatsu ORCA-flash 4.0 camera and 4*UPLSAPO objective using the GFP filter. Custom MATLAB scripts were written to analyze the fluorescent images. The following equation was used to calculate the diffusion coefficient of each peptide in the Matrigel:

\[ C(x,t) \propto \text{erfc} \left( \frac{x}{\sqrt{D_{eff}t}} \right). \]

**Distribution of selected M13 phage clones in the brain**

An *in vivo* study to determine accumulation of select M13 phage clones in the brain was performed in accordance with an animal protocol approved by the IACUC committee at The University of Texas at Austin. Six to eight weeks old female Balb/c mice were injected by tail vein injection with 100 µL of Pep-3, Pep-9 presenting phages or negative control M13 clone at a concentration \(\sim 4.8 - 6.3 \times 10^7\) pfu/µL (n=5 for each group). Thirty minutes post-injection, mice were humanely euthanized by CO2 inhalation, and 300 - 400 µL blood was collected by cardiac puncture and stored in heparin blood collection tube (BD #365985). Mice were then perfused via transcardiac perfusion with a syringe pump at a flow rate of 4 mL/min, for a total of 10 ml PBS for each mouse. Brains were dissected and stored in 2ml ice-cold physiological buffer with complete protease inhibitor cocktail (Roche, #5892791001). To isolate the brain parenchyma from the brain capillary, capillary depletion was performed following the method by Triguero et al. Briefly, harveted brains were homogenized and dextran solution was added to the brain homogenate to obtain a final 13% dextran concentration. Then, the brain parenchyma supernatant
was separated from the brain capillary pellet using dextran density centrifugation at 5400g at 4°C for 15 min\textsuperscript{107}. The blood, brain supernatant, and capillary samples after perfusion and capillary depetion were stored at -80°C until use. The amount of Pep-3, Pep-9, and negative control-presenting M13 phage in the blood, brain parenchyma supernatant, and blood vessel (i.e. capillary) were quantified by double-layer plaque assay\textsuperscript{109}. From these values, the ratio of phage in brain parenchyma/blood serum (in µL/g) and brain capillaries/blood serum (in µL/g) were calculated to compare the BBB shuttling efficiency and brain accumulation of M13 clones.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgement**

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**Supporting information**

Attached supporting materials

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Figure S1 Enrichment of top 20 frequent peptides among the three rounds of biopanning of second replicate (repertoire 2)
Figure S2  Frequency distribution of top 20 frequent peptides in the original, naïve CX7C phage library
Figure S3  Active transport of peptide displayed M13 phages in hCMEC/D3 cells after 1h uptake
Figure S4 Intracellular movements of M13 clones. (A) Representative trajectories from five peptide-presenting M13 phages in the hCMEC/D3 cells. A series of images of the fluorescently-labeled M13 phage particles were projected on the bottom of the trajectory box. These five trajectories, a trajectory exhibits two active transport sub-trajectories lasting for 1s and 5s respectively (by visual inspection); b-e represent passive diffusion. (B) MSD curves of trajectory segments that were classified as passive diffusion. The $D$ value represents the mean and standard deviation of the derived diffusion coefficients. (C) MSD curves of trajectory segments that were classified as active transport. The $V$ value represents the mean and standard deviation of the derived velocities.