

UC Irvine

UC Irvine Previously Published Works

Title

AAV9-Mimetic Peptides and Electroporation Synergistically Enhance Nanoparticle Transport through the Blood–Brain Barrier

Permalink

<https://escholarship.org/uc/item/4nx6c132>

Journal

ACS Biomaterials Science & Engineering, 12(4)

ISSN

2373-9878

Authors

Butkovich, Nina

Xu, Yifei

Song, Yuchen

et al.

Publication Date

2026-04-13

DOI

10.1021/acsbiomaterials.5c01483

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

AAV9-Mimetic Peptides and Electroporation Synergistically Enhance Nanoparticle Transport through the Blood–Brain Barrier

Nina Butkovich, Yifei Xu, Yuchen Song, Lu Wang, Aaron Ramirez, Enya Li, Nikhil Tien Chi Siao, Eric Velazquez-Rivera, Liangzhong Xiang, Xiangmin Xu, and Szu-Wen Wang*



Cite This: *ACS Biomater. Sci. Eng.* 2026, 12, 2201–2214



Read Online

ACCESS |



Metrics & More



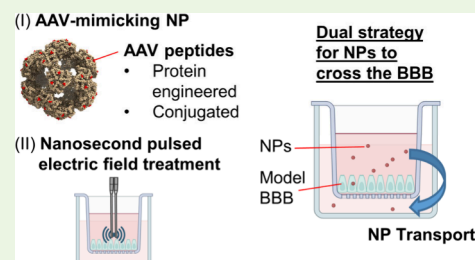
Article Recommendations



Supporting Information

ABSTRACT: Crossing the blood–brain barrier (BBB) remains a major hurdle in neurotherapeutics delivery. However, adeno-associated virus serotype 9 (AAV9) variants can cross from the bloodstream to accumulate in brain tissue, with their tropism facilitated by surface peptide loops. In this work, we examined the feasibility of grafting AAV9-based peptides (AAV.PHP.eB, AAV.X1, and AAV.CPP.16) onto nanoparticles (NPs) to increase transport through brain endothelial cells. We also evaluated the effects of combining the cell-mediated transcytosis strategy with nanosecond pulsed electric fields (nsPEFs). To test the importance of the AAV9-mimetic peptide structural presentation, we chemically conjugated linear peptides or recombinantly inserted peptides into external loops of a protein NP delivery scaffold (E2). E2 shares similar size, symmetry, and nanostructure features to AAV9 but is based on a non-viral source. Computational modeling, particle sizes, and circular dichroism data showed that NPs were folded and intact, even after peptide insertion. NP uptake by brain endothelial cells was 6.2- and 3.4-fold greater (after 4 h) for designs that integrated AAV.X1 or AAV.CPP.16 loop peptides into the NP, relative to chemical conjugation of their respective linear peptides; this highlights the importance of retaining the structural context of the AAV9-derived peptide loops. Using a transwell BBB assay which was optimized to be conducted with nsPEF, we showed that the highest transcellular passage of NPs through the endothelial monolayer was obtained by combining the dual delivery strategies of AAV9 peptide loop incorporation and treatment with nsPEFs. This study also determined the AAV.CPP.16 loop to be the most effective peptide, with E2 NP transport to the basolateral side to be almost twice that of the AAV.X1 peptide in E2 (with nsPEFs). These results support the integration of AAV9-mimetic peptides into biomolecules and drug delivery carriers to facilitate their passage through the BBB.

KEYWORDS: AAV9, blood–brain barrier, nanoparticles, protein nanoparticles, surface peptides



1. BACKGROUND

The blood–brain barrier (BBB) is the primary multicellular, dynamic, and semipermeable structure separating the central nervous system (CNS) from the bloodstream.¹ While its integrity is critical to protect neurons from potentially harmful agents, the BBB conversely restricts the delivery of therapeutics to the CNS for the treatment of crippling disorders such as Alzheimer's disease,^{2,3} with nearly 98% of small molecule drugs and 99.9% of biopharmaceuticals excluded from the brain.^{4–6} Delivery strategies to brain tissue have included lipophilic diffusion, receptor-mediated transcytosis, adsorptive-mediated transcytosis, and physical disruption of the brain endothelial tissue (e.g., focused ultrasound, osmotic pressure due to mannitol, etc.).^{1,7,8} Despite these varied approaches, the transport of drugs past the BBB still remains a challenge. In this work, we investigated the feasibility of transport through endothelial cells of the brain microvasculature using a dual strategy that combined the orthogonal approaches of receptor-mediated uptake and physical disruption, with the aim toward applications in drug delivery. Peptide ligands mimicking those identified from adeno-associated virus serotype 9 (AAV9)

variants were attached onto nanoparticles (NPs) to examine uptake and transport, and the physical disruption method involved transient electroporation using nanosecond pulsed electric fields (nsPEFs).

1.1. Receptor- and Adsorptive-Mediated Transport

Molecules can cross the BBB through a number of transport routes, mediated by receptors, adsorption, paracellular travel, solute carriers, and others, where the combination of these pathways could be involved to different degrees.^{1,2,8} Receptor-mediated transcytosis initiated by the binding of ligands attached to the drug cargo to cell surface receptors (e.g., transferrin (Tf) binding to the transferrin receptor (TfR)) results in subsequent endocytosis and transcellular transport.^{1,8}

Received: August 21, 2025

Revised: February 13, 2026

Accepted: February 16, 2026

Published: March 3, 2026



Various peptide-based strategies have been previously investigated, including peptides targeting TfR and those derived from HIV protein (Tat), which are well studied and have been used in clinical trials.^{9–11} For example, Tat, a cell-penetrating peptide, undergoes adsorptive-mediated transport through cellular membrane interactions leading to internalization.^{5,8,9} Cell-penetrating peptides have been extensively studied for the delivery of macromolecules across cell membranes, including past the BBB, modulated by properties such as size and charge.^{1,9,12} Both receptor- and adsorptive-mediated transport are major potential routes of NP transcytosis past the BBB and are influenced by the surface properties of NPs.⁸ However, neither transferrin nor Tat strategy alone can efficiently cause accumulation in the brain after systemic injection, with typical reported efficiencies of <1% ID/g (injected dose per gram of tissue).^{13,14} Peptides like Tat are also rapidly cleared from the bloodstream within minutes and distributed to other tissues (e.g., liver), with little toward the brain.^{5,13,15}

A number of AAV gene therapies are already approved clinically, and the AAV9 serotype is of particular interest for CNS diseases due to its inherent ability to transduce cells in the brain.^{1,16} AAV9-based capsids with tropism to the brain have been identified, which incorporate non-native peptides within a repeating external capsid loop of highly variable region (HVR) VIII.^{17–20} Included are AAV9 variants AAV.PHP.eB,¹⁹ AAV.X1,²⁰ and AAV.CCP.16,²¹ which exhibit unusually effective accumulation specific to the brain in rodent and nonhuman primate models.^{19–21} For example, AAV.PHP.eB can achieve a relatively high ~10% ID/g accumulation in the brain tissue of C57BL/6 mice for at least 3 days postsystemic injection.²² Each of these capsids is distinguished by short peptide (<10 amino acid) insertions and/or substitutions at positions between amino acids (aa) 588–589 of AAV9 HVR VIII.^{19–21}

These AAV9-based capsids exhibit unique tropism, likely through distinct cell surface receptor recognition capabilities. AAV.PHP.eB has exhibited brain tropism in murine strains that express nonglycosylphosphatidylinositol (GPI)-disrupted, membrane-localized LY6A receptor on brain endothelial cells.^{23–25} AAV.X1 binds to the receptor LRP6, independently of LY6A, for brain endothelial cell accumulation in rodents and nonhuman primates.^{20,26} AAV.CPP.16 results in increased brain endothelial cell transcytosis and neuronal transduction in rodent, nonhuman primate, and human cell line models.^{21,27} Although its unique peptide sequence is based on Bax-inhibiting peptides derived from the Ku70 protein, the transport mechanism is still unconfirmed.²¹ While these peptides have been successful in enhancing transport of AAVs past the BBB, the feasibility of grafting these peptides onto drug molecules or macromolecular systems for drug delivery to the CNS has not yet been investigated.

1.2. Nanosecond Pulsed Electric Fields (nsPEFs)

Another strategy for transport past the BBB includes physical disruption of the barrier, such as focused ultrasound or electroporation.^{8,28} Pulsed electric field (PEF)-facilitated electroporation can enable transient cell membrane pore formation or tight junction disruption to increase penetration of systemic drugs or nanocarriers into the treated tissue.^{28–30} Focally directed by electrodes, the careful control of PEF parameters (including pulse width and voltage) is crucial in affecting the extent of the reversibility of membrane permeability, but potential cell death is often observed within hours of treatment.³¹ This cell toxicity can be minimized by

shortening the pulse width to nanoseconds (i.e., nsPEFs) and decreasing voltage magnitude.^{29,32} nsPEFs can even be used with real-time, high-quality image monitoring during application, which could enable flexibility and responsiveness during a procedure.^{33–35} Although noninvasive, and microsecond-long PEFs have been used to increase molecule or therapeutic transport past the BBB,^{30,36,37} the nsPEF regime has not previously been evaluated in the context of delivery to the CNS.

Prior studies have, however, investigated nsPEFs for cancer treatment.^{38–41} This application is in clinical trials for malignancies such as basal cell carcinoma, with nsPEF treatment considered well-tolerated.^{42,43} The anticancer potential of nsPEFs has in part been attributed to membrane electroporation for increased chemotherapeutic uptake.^{29,40} Furthermore, nsPEFs can reduce neuromuscular stimulation, pain, and heat generation compared to longer PEFs under specific parameters.^{34,44–46} In our current study, we investigated nsPEFs with several AAV-mimicking NPs to increase BBB penetration at short time scales.

To examine the effects of the putative receptor ligand (AAV9 loop peptides) alone, without the virus particle, we needed a similar protein NP without innate, infectious tropism. We used the protein particle E2 to serve as the underlying, nonviral “blank slate” scaffold.^{16,47} Both AAV9 and E2 NPs share several important similarities, including symmetry, size (~25–30 nm), and protein composition of 20 trimeric repeats that in total include 60 protein monomers.^{48,49} For the E2 platform, we can conjugate thiol-terminated peptides to surface lysines of the NP, and the cysteines that reside in the hollow cavity can be functionalized with fluorescent markers, drug molecules, or immune activators.^{50–60} In addition to these conjugation strategies, we also hypothesized that we could incorporate AAV peptide into a repeating external loop on E2 using protein engineering. This approach let us more faithfully replicate amino acid presentation and secondary structures as well as examine our prediction for their functional importance.

This study investigated the effects of combining receptor-mediated transport and physical disruption strategies on uptake and transport past a BBB model comprising brain endothelial cells. We also examined the use of putative transport peptides derived from AAV9 variants with strong tropism to brain tissue, and we tested whether this transport was dependent on the structural context of the peptides (i.e., linear peptide or peptide integrated into a structural loop on the NP surface) by grafting these peptides onto a nonviral protein NP scaffold. These studies required the development of a transwell transport assay, which included determining optimal nsPEF parameters. Our work has important implications for delivering drug cargo past the BBB using AAV9-based peptides and their strategic design.

2. METHODS

2.1. Materials

All materials were purchased from Fisher Scientific unless stated otherwise. Peptides including pHPeB (CGGSGGSDGTLAVPFK), pX1 (CGGSGGSAQGNNTSV), and pCPP (CGGSGGSAQTVS-ALK) were ordered from Genemed Synthesis, and gBlock genes were purchased from IDT DNA. Restriction enzymes *NdeI* and *BamHI* were purchased from New England Biolabs, and miniprep kits were purchased from QIAGEN. Murine brain endothelial cells (bEnd.3) were obtained from ATCC (CRL-2299) and were used between passages 4 to 11. Alexa Fluor 488 C5 maleimide, Alexa Fluor 594 C5 maleimide, and calcein-AM were purchased from Invitrogen, and tris(2-carboxylethyl)phosphine (TCEP) was purchased from Thermo

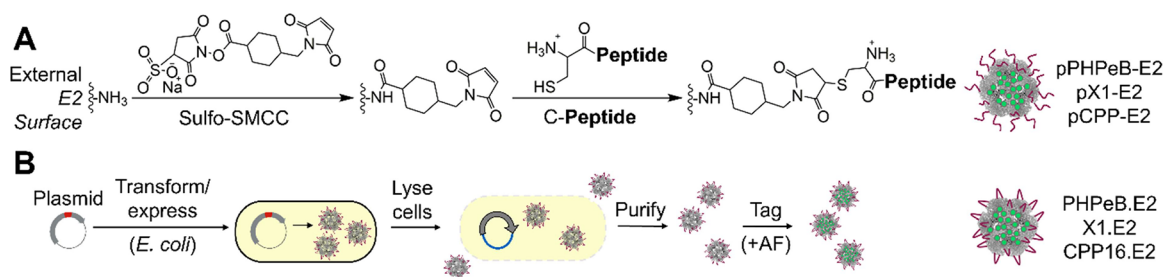


Figure 1. Synthesis of E2 protein NPs. Shown are schemes to synthesize the two types of NPs. (A) Cysteine-terminated peptides (pPHPeB, pX1, or pCPP) were conjugated to E2 NPs through surface lysines. (B) Protein NPs were recombinantly engineered to display unique loops with supportive beta sheets derived from AAV.PHP.eB (PHPeB.E2 NPs), AAV.X1 (X1.E2 NPs), or AAV.CPP.16 (CPP16.E2 NPs). Red and green correlate to peptide ligands and Alexa Fluor (AF), respectively. *Sulfo-SMCC*: sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

Label	Nanoparticle scaffold	NPs designed and synthesized	References
E2	E2 NPs	Blank-slate NPs	Dalmau <i>et al.</i> (2008)
pPHPeB-E2	E2 NPs	pPHPeB peptide conjugation onto E2 NPs: CGGSGGSDGTLAVPFK	Jang <i>et al.</i> (2022) Martino <i>et al.</i> (2021)
pX1-E2	E2 NPs	pX1 peptide conjugation onto E2 NPs: CGGSGGSAQGNNTSRV	Chen <i>et al.</i> (2023)
pCPP-E2	E2 NPs	pCPP peptide conjugation onto E2 NPs: CGGSGGSAQTVSALK	Yao <i>et al.</i> (2022)
PHPeB.E2	PHPeB.E2 NPs	E2 NPs with 30 aa insertions per monomer from AAV.PHP.eB variant of AAV9: SYGQVATNHQSSDGTLAVPFKAQAQTGWVQN	Jang <i>et al.</i> (2022) Martino <i>et al.</i> (2021)
X1.E2	X1.E2 NPs	E2 NPs with 30 aa loops per monomer from AAV.X1 variant of AAV9: SYGQVATNHQSAQGNNTSRVAQAQTGWVQN	Chen <i>et al.</i> (2023)
CPP16.E2	CPP16.E2 NPs	E2 NPs with 29 aa loops per monomer from AAV.CPP.16 variant of AAV9: SYGQVATNHQSAQTVSALKQAQAQTGWVQN	Yao <i>et al.</i> (2022)

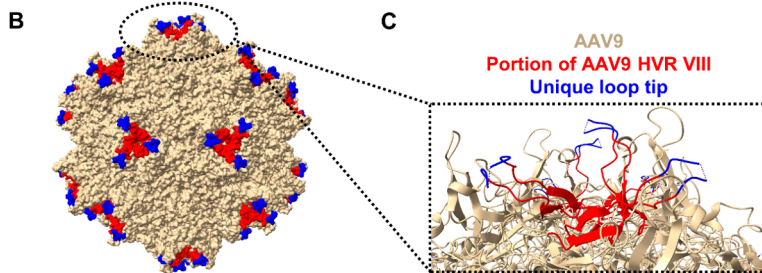


Figure 2. AAV9-mimetic peptide sequences and locations for E2-based NPs. (A) Summary of peptides and NPs. For peptide-conjugated NPs, the sequences of peptides pPHPeB, pX1, and pCPP included the cysteine conjugation site, flexible GS-containing linker, and the unique peptide sequence from AAV.PHP.eB, AAV.X1, or AAV.CPP.16, respectively. All NPs were derived from the D381C E2 variant of the E2 NP, facilitating conjugation of the AF fluorophore. Rows shaded in light green describe peptide-conjugated NPs, while rows shaded in pink are the protein-engineered NPs with AAV9-mimetic peptide loops. Representations of NPs with AF (green) and AAV peptides (red) are shown at the right. (B–C) Structures of AAV.PHP.eB (PDB 7ud4) with highlighted amino acids in blue (aa587–595) of the loop and red (aa576–605) from the flanking region of HVR VIII of AAV9. Although the blue domain shows the unique loop tips for AAV.PHP.eB, it is presumed that peptides from AAV.X1 and AAV.CPP.16 also inhabit this region.

Scientific Pierce. Culture media for bEnd.3 cells was comprised of RPMI 1640 (Corning), heat-inactivated fetal bovine serum (10%, Life Technologies), sodium pyruvate (1 mM, HyClone), penicillin-streptomycin (100 U/mL, Gibco), and L-glutamine (2 mM, Lonza). Transwell inserts with 3.0 μ m pore sizes and their companion 24-well plates were purchased from Falcon. PBS and trypsin-EDTA (0.25%) were purchased from Gibco.

2.2. Computational Modeling of Protein Engineered NPs

Three-dimensional conformations for protein engineered E2 monomers or conjugatable peptides (pPHPeB, pX1, and pCPP) were

predicted using the AlphaFold2 deep learning program, without relaxation and using the standard clustered PDB (PDB100) database.^{61,62} Using ChimeraX software, monomer structures were aligned to each other, E2 (PDB 1b5s), AAV9 (PDB 3ux1), and AAV.PHP.eB (PDB 7ud4), and root-mean-square deviations at 95% residue coverage (RMSD) were calculated.⁶³

2.3. Gene Cloning, Expression, and Purification of Protein NPs

All E2-based protein NPs, including the D381C mutant of E2 (designated as “E2”; baseline scaffold), PHPeB.E2, CPP16.E2, and

X1.E2, were designed to contain one aspartate-to-cysteine modification at the native amino acid site 381 in each of the 60 monomers to enable maleimide-functionalized conjugation of fluorescent Alexa Fluor (AF) molecules (Supporting Information). To construct the E2 variants (PHPeB.E2, CPP16.E2, and X1.E2), blunt-end dsDNA (phosphorylated at the 5' termini) gBlock genes were purchased from IDT DNA, with DNA and amino acid sequences described in Table S1. These gBlock gene sequences included *NdeI* and *BamHI* restriction sites at the N- and C-termini, respectively, and were codon optimized for expression in K12 *E. coli*. Lyophilized gBlock DNA was briefly centrifuged and reconstituted at 10 ng/ μ L in nuclease-free water. Genes were ligated into pJET1.2 using T4 DNA ligase (CloneJET PCR Cloning Kit), transformed into DH5 α *E. coli* cells, and grown on LB agar plates containing 100 μ g/mL ampicillin. Colonies were selected and grown overnight in LB with ampicillin at 37 °C with shaking, followed by miniprep (QIAprep Spin Miniprep Kit) and sequencing. To clone the genes into the pET-11a expression vector, the restriction enzymes *NdeI* and *BamHI* were used, and the vector was transformed into BL21(DE3) *E. coli* cells for expression.

All E2-based protein NPs were expressed and purified using previously described methods.^{50,51} To summarize, BL21(DE3) cells were grown in LB with ampicillin at 37 °C with shaking, and expression was induced by 1 mM IPTG when OD₆₀₀ reached 0.6–0.9. After incubation at 37 °C for 3 h, cells were lysed in a French pressure cell. Lysates were subjected to heat incubation at 70 °C, anion exchange chromatography (HiPrep Q Sepharose anion exchange column, Cytiva), and size-exclusion chromatography (Superose 6 size exclusion column, Cytiva). NPs were stored in phosphate buffer (50 mM potassium phosphate, 100 mM NaCl, pH 7.4) at –80 °C.

NPs (Figure 1) were characterized using dynamic light scattering (DLS), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bicinechoninic acid assay (BCA) to determine NP concentration, and mass spectrometry [Xevo TQ-S (Waters Xevo TQ Absolute with ACQUITY Premier UPLC system) and MALDI-TOF (Bruker UltrafleXtreme MALDI-TOF/TOF)] as previously described.^{50,51,58} For DLS, the volume and intensity percentages were determined.

Circular dichroism (CD) spectroscopy was performed using a Chirascan V100 instrument (Applied Photophysics) with an S&A CW-3000 industrial chiller and QNW TC1 temperature controller (Quantum Northwest). E2 NP variants at concentrations of 0.06 mg/mL in phosphate buffer were scanned in quartz cells with 0.1 cm path length. We measured ellipticity across 200–260 nm at 2 nm/min and 25 °C. Within this wavelength range for all samples, voltage did not exceed the detector's limit (700 V). Spectra were averaged over 3 independent samples. For these samples, the Beta Structure Selection (BeStSel) method^{64,65} secondary structure estimation web server tool was used to approximate the α -helical content of the CD spectra. For each type of NP, we performed a single spectrum analysis using CD inputs of wavelength (ranging from 200 to 260 nm with constant spacing of 1 nm) and corresponding mean residue ellipticity.

2.4. Conjugation of AAV-Mimetic Peptides to Nanoparticles (NPs)

Attachment of peptides to the E2 NPs (Figure 1A) resulted in pPHPeB-E2, pX1-E2, and pCPP-E2, and is described in Figure 2A. Each peptide contained a terminal cysteine to allow conjugation onto lysines of the E2 NP, linker (GGSGGS), and the 8–9 amino acid peptide sequence reported to enhance the ability of AAV9 to cross the BBB from AAV.PHP.eB (DGT LAVPFK),^{25,66} AAV.X1 (AQGNNTSRV),²⁰ and AAV.CPP.16 (AQTVSALK).²¹

The conjugation of sulfo-succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) linker and peptide was performed as previously described.^{50,52,55–58} To summarize, NPs were conjugated to sulfo-SMCC linker for 30 min at room temperature, followed by removal of excess using desalting columns. Peptides reduced with TCEP were incubated with NPs for 2 h at room temperature and overnight at 4 °C. Unreacted peptides were removed overnight at 4 °C using 100 kDa molecular weight cutoff dialysis membranes into a reservoir of phosphate buffer replaced three times.

NPs were characterized with DLS, SDS-PAGE, BCA, CD, and MALDI-TOF for protein engineered NPs or Xevo TQ-S mass spectrometry for peptide-conjugated NPs.⁵¹

2.5. Brain Endothelial Cell (bEnd.3) Assay to Evaluate NP Uptake

The bEnd.3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/mL penicillin-streptomycin, and 2 mM L-glutamine at 37 °C under 5% CO₂ conditions, as described in previous studies.^{67,68} Cells were seeded into 24-well tissue culture-treated plates at 4 $\times$ 10⁴ cells per well, allowed to settle overnight, and incubated with 4 μ g/mL of AF594-tagged NPs or PBS (negative control) for 2, 4, or 24 h. Media was removed, and then cells were washed with PBS and trypsinized with 0.25% Trypsin-EDTA for 7 min at 37 °C to detach cells. Cells were washed 3 times with 200 μ L of PBS containing 1% BSA (FACS buffer), centrifuged at 300 $\times$ g for 5 min at 4 °C, and analyzed in 100 μ L of FACS buffer for cellular uptake of NPs with flow cytometry (ACEA Novocyte, FlowJo) using the PE-TexasRed channel to detect the AlexaFluor 594 attached to NPs. A schematic of the protocol and cell gating strategy is shown in Figure S1.

2.6. Nanosecond Pulsed Electric Field (nsPEF) Instrument

We developed a high-speed electroporation platform consisting of two main components: a 3D positioning system and an electroporation system (Figures S2A to S2B). For the former, we integrated a two-axis microscope stage to perform scanning along the X- and Y-axes (MLS203, Thorlabs, USA). A custom-designed 24-well plate holder, fabricated by 3D printing, was used to enable automated scanning across all wells. Z-axis scanning was achieved using a linear stepper stage (FSL40, FUYU Technology, China). Limit switches were incorporated to maintain the electrode at the correct working position 1 mm above the layer of bEnd.3 cells. The electroporation system employed a custom-built nanosecond pulse generator capable of delivering adjustable pulses with amplitudes ranging from 0 to 2 kV, pulse widths (PW) from 100 ns to 1 ms, and repetition frequencies between 1 Hz and 1 MHz. These parameters fully meet the requirements for reversible cell electroporation.^{33,34} Pulses were delivered through clinical electroporation electrodes (20400103, AngioDynamics). A 3D-printed spacer was used to maintain a fixed electrode gap of 1 mm, matching the depth of the transwell inserts. The electrodes were mounted on a 3D-printed holder attached to the Z-axis linear stage, allowing precise positioning.

Prior to all assays using nsPEFs, the device was calibrated to ensure 1 mm probe positioning above the cell layer (Z-axis) and well centering (X- and Y-axes). Electrode positions were kept constant between all conditions and independent experiments. Each electroporation treatment lasted ~20 s per well or insert, minimizing cell exposure to ambient temperature and CO₂ levels to under 12 min before returning to the cell incubator maintained at 37 °C and 5% CO₂. For each well or insert, any nsPEF treatment consisted of 400 pulses and utilized a frequency of 20 Hz.

2.7. In Vitro Cell Assays Using nsPEFs

2.7.1. nsPEF Parameters. To establish conditions that enabled maximum cell viability after nsPEF treatment, 4 $\times$ 10⁴ bEnd.3 cells per well were seeded into 24-well tissue culture-treated plates and cultured overnight at 37 °C and 5% CO₂. nsPEFs were applied using 1000 V or 2000 V with 100 ns or 500 ns PW. Controls included cells not treated with nsPEFs (positive control), no calcein-AM cell control, or lysing buffer [20% (w/v) sodium dodecyl sulfate in 50: 50 dimethylformamide: water] treated cells before calcein-AM incubation (negative control). After 1 or 24 h, media was removed, and cells were incubated with 200 μ L of 10 μ M calcein-AM (or PBS control) for 30 min at 37 °C. Wells were washed three times with PBS, and cells were incubated with media for 30 min at 37 °C. Fluorescent images were obtained using an Olympus IX5a microscope with a QICAM Fast camera and a FITC filter. Wide-field images were taken using a Keyence BZ-X810 microscope with a FITC filter. To quantify relative levels of cell viability per well, media was removed, and 120 μ L of lysis buffer was added per well and incubated for 5 min. Following vendor protocols,

relative fluorescent intensities for calcein were measured on a plate reader (SpectraMax M3) with excitation and emission of 490 and 515 nm, respectively. The readings for the negative control (lysed samples) were averaged and subtracted as the background. Samples were normalized to maximum cell growth (no nsPEFs, positive control) for each set of conditions.

2.7.2. Transwell Assays for NP Transport Across a BBB Monolayer. The schematic summarizing the protocol for transwell assays is shown in Figure S2C. Porous transwell inserts in 24-well plates [tissue culture-treated inserts with 3.0 μm pore sizes for 24 well plates (Falcon)] were first soaked for at least 30 min by adding cell culture media into both apical and basolateral chambers. To seed inserts, 2×10^4 bEnd.3 cells were added per insert and cultured in 5% CO_2 at 37 $^\circ\text{C}$ until confluency was reached (10 days). Confluency was defined as transendothelial electrical resistance (TEER) value stabilized at $>30 \Omega\cdot\text{cm}^2$ for cell monolayers (after insert background subtracted) (EVOM-2 V/Ohm meter), which is consistent with TEER values in literature for bEnd.3 cells.^{68–72} As a control, inserts in wells without cells were prepared equivalently. Media was replaced in both chambers every 2–3 days, maintaining 200 and 700 μL volumes in the apical and basolateral chambers, respectively.

nsPEFs were applied at 1000 V using 100 ns PW, conditions shown to minimize cell death (see Results and Discussion). Immediately afterward, 90 μL of PBS or AF488-tagged NPs were added to apical chambers (for a final concentration of 150 $\mu\text{g}/\text{mL}$ [$\sim 0.1 \mu\text{M}$] of NPs containing 3 μM of AF488 in the apical side). At times of 0, 0.5, 2, 4, 8, 24, and 48 h, 70 μL of media was sampled from the basolateral chamber, and an equivalent volume of fresh media was replaced.

To consider nonspecific adsorption of NPs to surfaces, maximal transport of NPs was defined as the concentration of NPs recovered from the basolateral side of a transwell without cells.⁷¹ Fluorescence at ex/em of 490/525 nm to detect AF488 was measured for basolateral samples at each time point, in agreement with vendor recommendations. The transport percentage was defined as the basolateral concentration of NPs that passed the monolayer of bEnd.3 cells, divided by the maximal transport (without cells).

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 8. All particle characterizations were repeated for $n \geq 3$ independent samples to determine mean $\pm$ standard deviation (S.D.) values. *In vitro* NP uptake studies and transwell assays were performed with $n \geq 3$ and $n \geq 2$ independent experiments, respectively, and plotted as mean $\pm$ SD or mean $\pm$ standard error of the mean (S.E.M.), respectively, with $n \geq 2$ technical replicates. Quantification of cell viability following nsPEF treatment was determined for $N = 6$ replicates and plotted as mean $\pm$ SD. TEER values were reported as mean $\pm$ SEM for $n \geq 2$ independent experiments with $n \geq 2$ technical replicates. All data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

3. RESULTS AND DISCUSSION

3.1. Protein NPs with AAV9-Mimetic Peptides Were Designed and Computationally Evaluated

Our overall goal of this investigation was to examine the feasibility of incorporating the peptide loops of AAV9 variants onto a model drug delivery NP as a strategy to facilitate molecular transport past the BBB. We focused on reported peptide sequences from a repeating loop from HVR VIII of AAV9 variants: AAV.PHP.eB,^{25,66} AAV.X1,^{20,26} and AAV.CPP.16.²¹ To study the effects of these peptide sequences, we chemically conjugated flexible peptides onto the surface of a model nanoparticle scaffold (E2) (Figure 1A), yielding pPHPeB-E2, pX1-E2, and pCPP-E2 (Figure 2A). However, because these peptides exist in the context of a surface loop on AAV9, and it was likely that better transport responses would be obtained when peptides were structurally constrained, we therefore also incorporated the peptides into loops of E2 by

protein engineering, yielding PHPeB.E2, X1.E2, and CPP16.E2 NPs (Figures 1B and 2A).

3.1.1. Peptide Sequences and Domains. AAV-mimetic peptides sequences were derived from AAV.PHP.eB, AAV.X1, and AAV.CPP.16 variants of AAV9. The AAV.PHP.eB peptide (DGT LAVPFK) was chosen for its ability to increase CNS transduction by >40 -fold compared to native AAV9, with improved targeting of neurons in C57BL/6 mice for gene transduction following systemic intravenous injection.^{18,19} By comparison, AAV.X1 peptide (AQGN NTRSV) led to 65–70% transduction of brain endothelial cells compared to only 1% by AAV9 for an eGFP reporter after systemic injection.²⁰ In BALB/c mice, unlike AAV.PHP.eB, AAV.CPP.16 peptide (AQTVSALK) led to 7-fold greater brain tropism over AAV9, while also targeting the brain after IV injection in nonhuman primates, evaluated by the expression of human aromatic L-amino acid decarboxylase.²¹ These defining sequences were therefore incorporated into pPHPeB, pX1, and pCPP (Figure 2A). The three peptides were designed to be chemically conjugated to the NP and were expected to be linear and flexible.

We hypothesized that the structural presentation and context of these AAV-derived peptides would be important to increase transport past the BBB. To test this, we used protein engineering to recombinantly incorporate the peptide sequences into a 60-repeating loop on the E2 NP scaffold. The crystal structure of AAV.PHP.eB shows that the peptide is flanked by interacting antiparallel beta-strands within the HVR VIII region of AAV9 (Figure 2, red); the resulting external bent-tip conformation of the AAV.PHP.eB peptide loop (Figure 2, blue) was reported to be important for improved CNS tropism due to binding to its receptor LY6A.⁶⁶ In fact, to design AAV1 to transduce brain endothelial cells and localize to the brain, the beta-sheet (Figure 2, red) flanking the DGT LAVPFK peptide (Figure 2, blue) in AAV9 was also necessary.²⁵ While other portions of the AAV9 capsid may assist CNS tropism, the HVR VIII region is most clearly implicated, so we focused on transferring only this domain to our nonviral NPs (Figure 2C).^{20,73} Therefore, in their designs, our nanoparticles PHPeB.E2, X1.E2, and CPP16.E2 included the loop tip sequences DGT LAVPFK, AQGN NTRSV, and AQTVSALK, respectively (Figure 2A–B, blue), together with the flanking amino acids from the AAV9 HVR VIII region (Figure 2A–B, red).^{19–21}

To recombinantly incorporate these AAV9 regions into the E2 NP, we identified a repeating loop region on E2 that could potentially accommodate the AAV-mimetic, BBB-penetrating peptide loops (Figure S3). We chose the aa280/281 site as the insertion location on E2 because these amino acids are on an external loop on the E2 NPs (Figure S3B), and insertion at this location creates trimers of AAV-derived loops for similar geometric configuration as in the parent AAV.PHP.eB, AAV.X1, or AAV.CPP.16 capsids (Figure S3A). Based on analysis of the PDB protein structures of AAV9 and E2 NPs, the distances between these loop sites on an AAV9 or E2 NP trimer were close, approximately 2.4 and 3.1 nm, respectively. Of note, while pPHPeB, pX1, or pCPP conjugation to E2 NPs could allow multiple peptides conjugated per NP monomer (with each NP formed from a 60-mer assembly), the genetically engineered AAV-inspired E2 NPs have only one loop insertion per monomer, thus yielding 60 peptides per NP.

3.1.2. Computational Prediction of Structure for Protein NPs with Peptides. Our prior studies demonstrated that chemical conjugation of peptides to E2 NPs does not affect NP integrity.^{50,55–58} However, we anticipated that the relatively

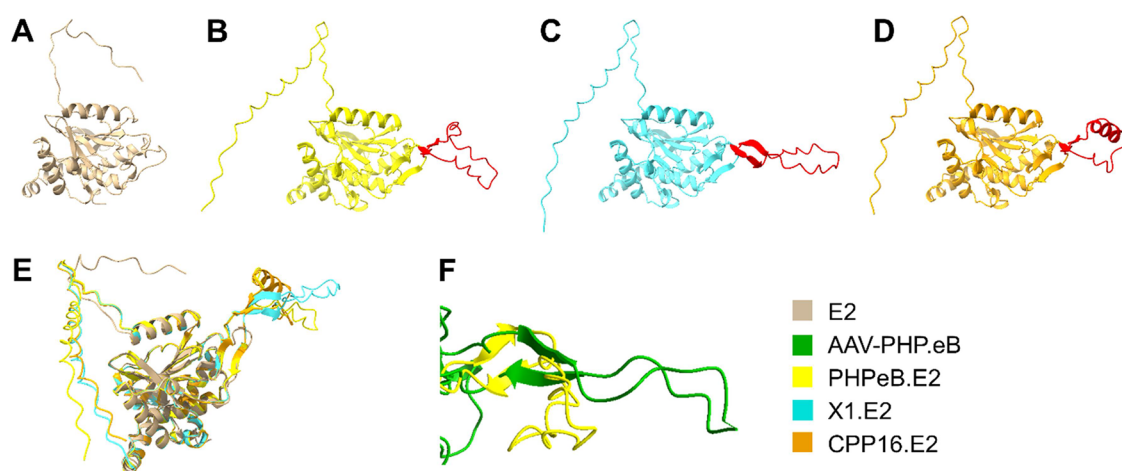


Figure 3. Monomers of engineered E2 NPs display AAV-derived peptide loops. Shown are protein folding for individual monomers of (A) E2 (PDB 1b5s structure) and AlphaFold-predicted monomers of (B) PHPeB.E2, (C) X1.E2, and (D) CPP16.E2, with AAV-mimicking peptide loops in red. Images show E2 (tan), AAV.PHP.eB (green), PHPeB.E2 (yellow), X1.E2 (teal), or CPP16.E2 (orange). (E) Overlay of AlphaFold-predicted engineered monomers with the crystal structure of an E2 monomer (PDB 1b5s). (F) Overlay of the loop region prediction for PHPeB.E2 (yellow) and the crystal structure of AAV.PHP.eB (PDB 7ud4; green).

large ~ 30 amino acid recombinant insertions into E2 monomers ($\sim 10\%$ increase in molecular weight) to fabricate PHPeB.E2, X1.E2, or CPP16.E2 NPs (Figure 2A), could destabilize the folding of E2 monomers and their 60-mer dodecahedral assemblies.⁴⁹ To predict whether these NPs would maintain stable protein folding, we used AlphaFold to computationally examine each type of NP (Figure 3A–D).

We found that despite these insertions into the E2 framework, the proposed monomers were predicted to maintain their folded E2 structure (Figure 3E) based on RMSD analysis.⁷⁴ Compared to the published experimental protein structure of native E2 (PDB 1b5s), RMSD values were 0.77 Å, 0.74 Å, or 0.77 Å, for PHPeB.E2, X1.E2, and CPP16.E2 (excluding the inserted loop region), respectively (Figure 3E), which are comparable to the 0.78 Å RMSD value for the E2 structure predicted by AlphaFold (control). The <1 Å deviations indicate high fidelity of PHPeB.E2, X1.E2, or CPP16.E2 to the native E2 structure.^{62,74,75} Furthermore, the AAV-mimicking loops were predicted to reside on the external surface of the NPs (Figures S3B and S4), as needed for interactions with endothelial cells. The set of 5 monomers in Figure S4 is repeated a total of 12 times, composing each 60-mer NP.

These predictions also showed that the supportive β -sheet grafted from the HVR VIII of AAV9 would be maintained in PHPeB.E2, X1.E2, CPP16.E2 NPs (Figures 2B–C, red; 3F; and S4E, arrow), anchoring the variable loop regions within similar geometric distances. This AlphaFold result was in contrast to the predicted structures of the peptides pPHPeB, pX1, and pCPP, which showed that peptides without the E2 NP scaffold were flexible and unfolded, even when the amino acids from AAV antiparallel beta strands were included in the model. Because our computational results not only suggested high fidelity to the backbone of native E2 but also maintained secondary protein folding structure adopted from AAV9, we expected that the PHPeB.E2, X1.E2, and CPP16.E2 NP assemblies could retain the aspects of the parent AAV NPs needed for BBB transport.

3.2. E2 That Displayed AAV9-Mimetic Peptides Assembled into NPs

Using the strategies of chemical conjugation or protein engineering shown in Figure 1, we synthesized and characterized

the NPs (E2, pPHPeB-E2, pX1-E2, pCPP-E2, PHPeB.E2, X1.E2, and CPP16.E2). DLS analysis showed that all NPs had diameters near 30 nm, which is consistent with the size of an assembled 60-mer E2 NP. All particles, regardless of synthesis strategy, were also monodisperse with minimal aggregation (Figures 4A–B and S5).^{51,76} Analysis of volume percentage (Figure 4A) and intensity percentage (Figure S5) from DLS showed that the vast majority of sample formed NPs with diameter ~ 30 nm rather than aggregates. Although aggregation was observed for X1.E2, apparent from particulates with measured diameter of hundreds of nanometers (Figure S5), this constituted a minority of the sample (Figure 4A). DLS measurements also showed PDI values of ~ 0.2 (Figure 4B), further demonstrating a high degree of monodispersity.

For the peptide-conjugated NPs (pPHPeB-E2, pX1-E2, and pCPP-E2), SDS-PAGE analysis confirmed that NP monomers showed small increases in molecular weights after conjugation, demonstrated by the smear effect on the SDS-PAGE gel, consistent with the attachment of a range of peptides per monomer (Figure 4C). The conjugation of each peptide was expected to increase monomer molecular weight by 1.5 kDa, 1.5 kDa, or 1.3 kDa for pPHPeB-E2, pX1-E2, and pCPP-E2, respectively, analogous to conjugated peptides from our previous studies with CSIINFEKL (1.3 kDa) and other antigen peptides, which resulted in similar smears on SDS-PAGE gels with their functional presence confirmed by immunological studies.^{50,55–57,77} The ~ 56 kDa band observable on SDS-PAGE (Figure 4C) is consistent with the use of sulfo-SMCC with E2, as previously demonstrated.^{55,57,58} Using mass spectrometry,⁵¹ we determined that 1.7 ± 0.5 pPHPeB peptides, 1.6 ± 0.1 pX1 peptides, or 1.6 ± 0.1 pCPP peptides on average were conjugated per E2 monomer, constituting approximately 102 pPHPeB, 93 pX1, or 99 pCPP peptides displayed per particle.

Mass spectrometry of E2, PHPeB.E2, X1.E2, or CPP16.E2 (Figure S6) showed monomer sizes of 28172 ± 6 Da; 31378 ± 1 Da; 31367 ± 4 Da; and 31241 ± 1 Da, values within $<0.3\%$ from the theoretical molecular weights of 28105 Da; 31291 Da; 31290 Da; and 31161 Da, respectively (Figure S6). As anticipated, each NP had only one size of monomer. SDS-PAGE further supported these expected sizes (Figure 4D). Due

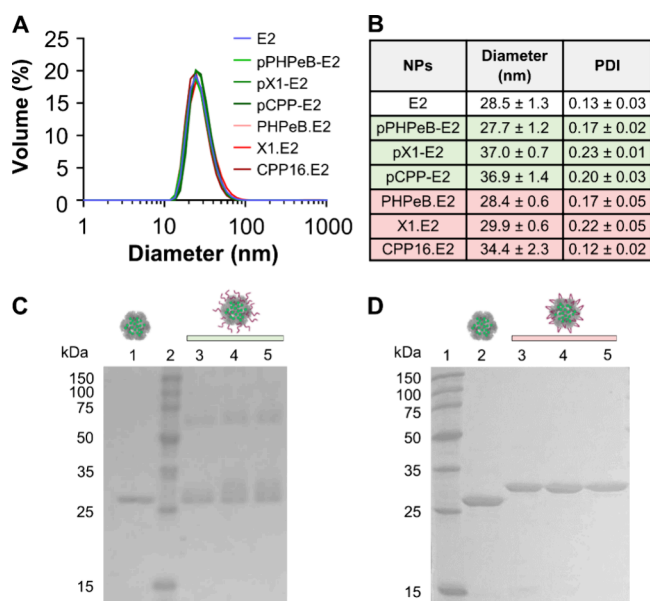


Figure 4. Nanoparticles were synthesized and characterized. (A) Representative hydrodynamic diameter DLS measurements for volume percentage of E2 (blue), pPHPeB-E2 (light green), pX1-E2 (green), pCPP-E2 (dark green), PHPeB.E2 (pink), X1.E2 (red), and CPP16.E2 (dark red). (B) Hydrodynamic diameters and PDI of E2-based NPs, as determined by DLS ($n = 3$ independently synthesized batches of each type of NP). Shaded in green and red describe designs that utilized the chemical conjugation of peptide or genetically engineered NPs inspired by AAV9-based capsids with high brain tropism, respectively. $Mean \pm S.D.$ (C) SDS-PAGE of fluorescently tagged NPs conjugated with linear peptides, and control. Lanes: (1) E2 control; (2) molecular weight ladder; (3) pPHPeB-E2; (4) pX1-E2; (5) pCPP-E2. (D) SDS-PAGE of fluorescently tagged NPs engineered with peptide loops, and control. Lanes: (1) molecular weight ladder; (2) E2 control; (3) PHPeB.E2; (4) X1.E2; (5) CPP16.E2.

to the geometrical symmetry, each NP has 60 AAV-mimetic peptides.

All protein NPs maintained a degree of structural folding that was sufficient for 60-mer macromolecular assembly, but with lower α -helical content after recombinant peptide loop insertions. E2, PHPeB.E2, X1.E2, and CPP16.E2 (Figure 5) or flexible peptide-conjugated NPs like pCPP-E2 and pX1-E2 (Figure S7) had CD spectra with minima at 208 and 220 nm, associated with expected protein folding and structures containing α -helices, and data are consistent with our prior studies for E2-based NPs.^{49,51,54,60,78} The closer alignment of

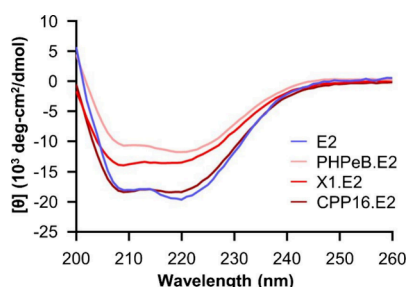


Figure 5. Circular dichroism analysis of protein engineered NPs. Shown are the molar ellipticity of E2 (blue), PHPeB.E2 (pink), X1.E2 (red), or CPP16.E2 (dark red) versus wavelength at 25 °C. The average of $n \geq 3$ independent samples is shown for each NP type.

peptide-conjugated NPs (Figure S7) to E2 NPs suggests that the conjugation process did not disrupt NP folding as much as recombinant integration of the longer loop insertions into the NP scaffold (Figure 5). NP integrity would be beneficial to deliver cargo to the tissue of interest with less premature degradation, which could compromise BBB targeting efficiency or lead to aggregation. These results are overall consistent with the computational modeling results in Figure 3, which predicted close alignment of the structures between the engineered variants and the E2 PDB structure, excluding the AAV-mimetic loop insertions. In agreement with CD spectra (Figure 5), the α -helical content of E2 was estimated to be greatest by using the BeStSel method, which was applied to estimate secondary structures:^{64,65} $42.3 \pm 4.4\%$ for E2, $19.6 \pm 6.4\%$ for PHPeB.E2, $24.0 \pm 1.9\%$ for X1.E2, and $29.3 \pm 16.0\%$ for CPP16.E2. Although these estimations for the amount of α -helical folding in PHPeB.E2, X1.E2, and CPP16.E2 are lower than those for E2, DLS showed that this minimally affected NP assembly, as all engineered NP variants successfully formed NP structures of highly uniform size (Figure 4A and S5).

While E2 NPs have been recombinantly engineered previously for a multitude of purposes, our current designs are relatively unique. To date, we and others have generated E2-based NP constructs with N-terminal additions (such as SpyTag-E2 for bioluminescence investigations⁵³ and SpyTag-E2 or antigen-fused E2 for vaccine applications^{77,79–84}); examined N-terminal truncation of E2 for pH-sensitive disassembly;^{49,78} and developed a number of E2 variants with point mutations.^{51,76} However, the larger-scale insertion of a relatively long chain of amino acids into the internal sequence of E2 monomers (29–30 aa) had not been previously described, likely due to complications that can arise from such a design, including improper protein folding during expression.^{85,86} The successful creation and 60-mer assembly of a correctly folded PHPeB.E2, X1.E2, and CPP16.E2 created through ~ 30 -mer insertions between aa280/281 of E2 NPs, which still exhibited uniform and assembled NP structures, demonstrates the robustness of the E2 scaffold and its promise toward further applications.

3.3. X1.E2 and CPP16.E2 NPs Experienced Increased Uptake by Brain Endothelial Cells Relative to NP Alone or Peptide-Conjugated NPs

We quantified the uptake of the different NP variants by bEnd.3 brain endothelial cells over time (Figures 6, S8, and S9). Significantly, after 4 h, the X1.E2 and CPP16.E2 NPs were taken up by more cells than either the E2 scaffold (with no peptide) or the peptide-conjugated NPs (Figure 6). Specifically, X1.E2 and CPP16.E2 were taken up 2.4- and 2.3-fold higher than the E2 control, and 6.2- and 3.4-fold higher than pX1-E2 and pCPP-E2, respectively. We note that the degree of uptake of peptide-conjugated NPs was the same regardless of the peptide surface densities examined (Figure S8). By 24 h, differences between protein engineered NP groups were not as pronounced, although levels were still significantly higher than their respective flexible peptide-conjugated NPs (Figure S9). The PHPeB.E2 NPs were taken up at similar levels to the E2 control for each incubation time tested, suggesting no difference due to the PHPeB peptide.

These results are consistent with previously reported AAV9 variant distribution trends *in vivo*. Because AAV.PHP.eB does not efficiently localize to the brain in BALB/c mice, the strain from which bEnd.3 cells were derived,²⁴ we did not expect

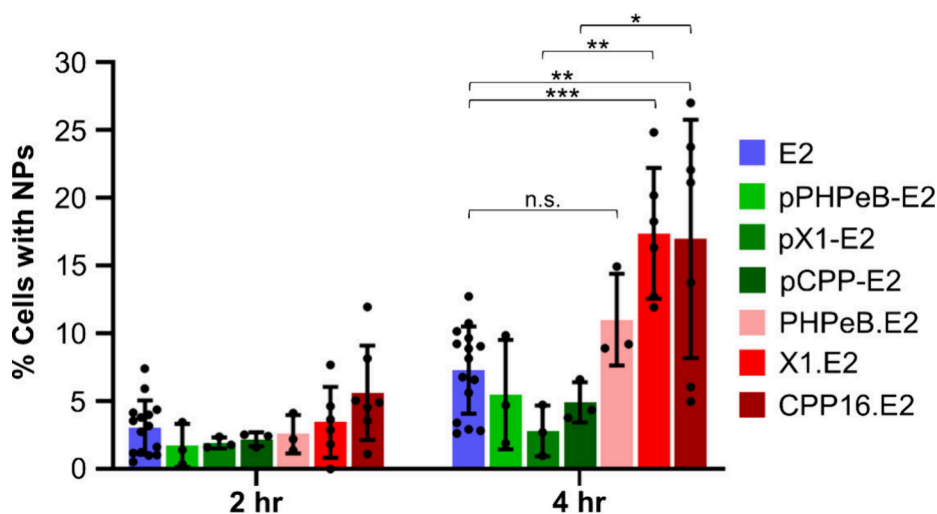


Figure 6. Uptake of AAV-mimetic nanoparticles by murine brain endothelial (bEnd.3) cells. Shown are the percentages of bEnd.3 cells containing fluorescently tagged NPs (E2, pPHPeB-E2, pX1-E2, pCPP-E2, PHPeB.E2, X1.E2, and CPP16.E2) following incubation for 2 or 4 h. Mean $\pm$ S.D., with $n \geq 3$ independent experiments. Statistics: 1-way ANOVA, Tukey's test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. = no significance.

improved uptake of pPHPeB-E2 or PHPeB.E2 NPs. The ineffectiveness of AAV.PHP.eB to enter brain tissue in certain mouse strains, including BALB/c, is attributed to the lack of LY6A expression on brain endothelial cells.²³ Conversely, both AAV.X1 and AAV.CPP.16 efficiently localize to the brain following systemic injection into BALB/c mice.^{20,21} Thus, this suggests that X1.E2 and CPP16.E2 NPs adopted some degree of receptor-mediated recognition by the brain endothelial cells, unlike the PHPeB.E2 NPs. For X1.E2, the responsible putative receptor is likely to be the LRP6 receptor and not LY6A, as AAV.X1 tropism across species has been attributed to the former.^{20,26} By contrast, our data also support that flexible peptide-conjugated NPs like pX1-E2 and pCPP-E2 may have lower affinity to these receptors or block the specific interactions needed for receptor-mediated endocytosis and intracellular trafficking.^{87,88} In addition to receptor-mediated uptake, NPs could also be taken up by adsorptive-mediated processes to varying degrees;⁸ the lower uptake of the flexible peptide-conjugated NPs compared to E2 could be due to differential NP surface properties, which could alter the receptor and cell membrane interactions with the NPs and affect intracellular trafficking and uptake.^{87,88} In fact, the CPP16.E2 NP could likely be taken up by an adsorptive-mediated process, as a specific receptor interaction with the peptide in AAV9.CPP.16 has not been definitively identified.²¹ Since CD demonstrated that the peptide-conjugated NPs had more similar assembly to E2 than protein engineered NPs (Figures 5 and S7), the low uptake by bEnd.3 cells was not caused by lack of NP folding.

Using a transwell assay to model transport through the BBB (Figure S2C), we observed that E2, X1.E2, and CPP16.E2 NPs passed through the bEnd.3 cell monolayers at similar amounts (Figure S10). Although bEnd.3 cells in transwell assays have been reported to form tight junctions,⁷¹ capillary intercellular junctional complexes by themselves do not necessarily prevent the paracellular transport of nanometer-sized molecules.⁸⁹ Therefore, the comparable increase in transport over time of E2, X1.E2, and CPP16.E2 into the basolateral chamber (Figure S10) could be at least partially caused by paracellular transport. Our data also suggest that despite greater cellular uptake after 4 h (Figure 6), X1.E2 and CPP16.E2 accumulated within these cells rather than committing to transcytosis and that a strategy is

needed to promote the continued movement of NPs through the cells. Therefore, we examined the combination of peptide-mediated transport with nsPEFs to increase permeation past the BBB model.

3.4. Determination of nsPEF Parameters in Brain Endothelial Cell Assays

To minimize cell death that can result from nsPEFs,³¹ we first established suitable parameters for nsPEFs. This technique can cause reversible or irreversible electroporation, with the latter often used for tumor ablation.²⁸ Since we aimed to evaluate our NPs with an intact but permeabilized BBB model, representative of minimally invasive treatment, we used nsPEFs within the nonlethal regime, correlating to reversible electroporation.⁹⁰

For all nsPEF studies, we designed an instrument equipped with clinical-grade electric probes for alignment above the layer of bEnd.3 cells (Figure S2). The nsPEF instrument included a two-axis microscope stage, a linear stepper stage, and clinical electroporation electrodes. For a range of voltages and pulse widths (PWs), we assessed cell viability using a number of metrics, including quantification of calcein-AM viability signal within a well of cells (Figure 7), wide-field imaging (Figure S11), and fluorescent microscopy (Figure S12).

For all assays, we uniformly and consistently observed no cell death only for the lowest tested nsPEF settings (1000 V: 100 ns PW). Through live cell quantification 1 h post-nsPEF, we observed no decrease in viability, which was comparable to non-nsPEFs treated wells (Figure 7). In fact, the average viability fluorescence for the 1000 V: 100 ns PW condition was slightly higher than that for no-nsPEF treatment, possibly due to increased permeability of dye molecules.⁹⁰ As voltage and pulse width were increased, the average percent viability tended to decrease; while these values were not statistically different than the no-nsPEF condition, images of the wells suggested that these decreases indeed correlated to cell death (Figures S11 and S12).

Imaging studies also showed that the 1000 V: 100 ns PW condition consistently enabled complete cell viability (Figures S11 and S12). In contrast, the highest settings of 2000 V: 500 ns PW caused cell death at the center of the wells (the location of the electric probes) after 1 or 24 h. Previous studies have also demonstrated the greatest density of death events after nsPEFs within a few hours, similarly stabilizing within 24 h.³¹

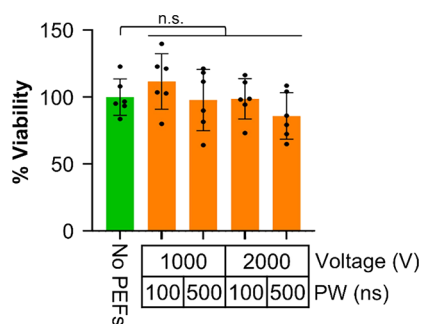


Figure 7. Quantification of viability dye in nsPEF-treated bEnd.3 cells. Shown is percent viability 1 h after or without nsPEFs. Percent viability is defined as the relative fluorescent intensities of calcein-AM signal over background, relative to that of the no-nsPEFs control. The nsPEF parameters are indicated. PW: pulse width. Mean $\pm$ S.D. Statistics: 1-way ANOVA, Tukey's test. n.s. = no significance between no-nsPEFs control and each nsPEF treatment.

Intermediate conditions such as 2000 V: 100 ns PW and 1000 V: 500 ns PW sometimes caused cell death after 1 h. Because the lowest settings of 100 V: 100 ns PW were never observed to cause cell death by any tested metric (Figures 7, S11, and S12), these settings were used in the transwell BBB assay.

3.5. NPs with BBB-Penetrating AAV Peptide Loops, Combined with nsPEFs, Most Effectively Crossed the BBB, with CPP16.E2 Yielding the Highest Amount of Passage

We anticipated that by combining both transport strategies, NPs would differentially cross a nsPEF-permeabilized BBB model, dependent upon AAV mimicry. We determined the transport of NPs past the BBB model using an established brain endothelial cell transwell assay, with the formation of a confluent monolayer of cells confirmed by TEER measurements. Specifically, TEER values prior to NP incubations were similar between cell inserts ($53.1 \pm 2.2 \Omega \cdot \text{cm}^2$ for E2, $49.6 \pm 1.2 \Omega \cdot \text{cm}^2$ for X1.E2, and $51.5 \pm 1.7 \Omega \cdot \text{cm}^2$ for CPP.E2), consistent with previous reports for this model.^{68,71,72} Since TEER values were comparable across all conditions, we expected that differences in transport would be due to nsPEFs and types of NPs, rather than variations in leakiness for each insert. Additionally, cellular uptake of empty E2 NPs was previously shown to not result in cell death *in vitro*,³⁹ so we expected neither nsPEFs (Figures 7, S11, and S12) nor the NPs to decrease bEnd.3 viability.

We observed that nsPEF treatment prior to NP incubation increased the transport of NPs to the basolateral side of the transwell, especially CPP16.E2 NPs, within 8 h (Figures 8, S13A, and S13B). The nsPEF + CPP16.E2 group transported 2.0- and 1.9-fold more than nsPEFs + E2 (no peptide), after 2 and 4 h, respectively. By contrast, X1.E2 NPs were transported at statistically similar amounts to E2 NPs, and nsPEF + CPP16.E2 transport was nearly twice (~ 1.7 -fold) that of nsPEFs + X1.E2 over the time periods tested (Figures 8 and S13). These data show that the AAV.CPP.16 peptide, integrated as a loop in E2, was the most effective peptide tested to transport the NPs through the endothelial monolayer.

Our results therefore support the use of the two-pronged strategy for the transport of nanocarriers past a model BBB, with synergistic results from the combination of brain-penetrating AAV mimicry with nsPEFs. We determined multiple important design features for using these AAV-derived, brain-targeting peptides. For increased bEnd.3 uptake, the proper structural context of these loop peptides within folded engineered NPs, rather than conjugated linear peptides, is required (Figure 6).

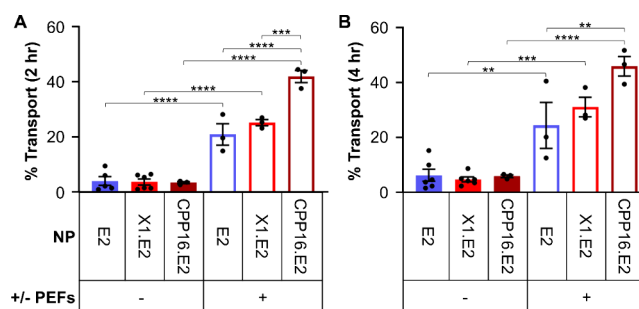


Figure 8. Nanosecond pulsed electric field (nsPEF) treatment increased transport of NPs with AAV-mimetic peptides. Shown are transport percentages of fluorescently tagged NPs after (A) 2 h or (B) 4 h across the BBB. Transport percentage was defined as the concentration of NPs in the basolateral chamber which had traveled past a monolayer of bEnd.3 cells, normalized to the maximum value control (i.e., analogous conditions but without cells). Blue, red, and dark red indicate cell incubations with E2, X1.E2, or CPP16.E2 NPs, respectively. Filled or bordered bars indicate transport without or with nsPEF treatment, respectively. Mean $\pm$ S.E.M., with $n \geq 3$ independent experiments and $n \geq 2$ technical replicates per experiment. Statistics: 1-way ANOVA, Tukey's test. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Additionally, the choice of the AAV mimicry peptide is another factor, with consistency in behavior observed in our *in vitro* assays (Figures 8, S9, and S13A-B) to the results of their parent AAV9 capsids *in vivo*.^{20,21,25} In particular, nsPEF + CPP16.E2 NPs transported most effectively through the brain endothelial cell monolayer compared to the groups of nsPEF + E2 and nsPEF + X1.E2 NPs over all times examined, with statistical significance between groups observed up to 8 h. These trends can be anticipated; while AAV.CPP.16 can cross the BBB in BALB/c mice,²¹ AAV.X1 localizes to the brain endothelial cells following systemic injection.²⁰ Because AAVs can utilize receptor-mediated endocytosis to be taken up by cells,^{24,91} our AAV-mimicking E2 NPs may have adopted this property, although adsorptive-mediated transport also likely plays a role.^{9,12} We speculate that the peptide sequence defining CPP16.E2 from E2 (Figure 2A and Table S1) can facilitate interactions with a cognitive receptor or cellular membranes for increased transport. The AAV.CPP.16 cognate receptor(s) and its intracellular trafficking mechanism are still unknown though speculated to be related to the Ku70 protein,²¹ but considering that this AAV9 variant translates well between rodents, nonhuman primates, and human cell lines, this capsid has been of particular interest for further study.²⁷

Our cell uptake and transwell results suggest possible application of the two-pronged BBB transport strategy for therapeutic delivery to the brain. For instance, lipid NPs (LNPs) are commonly explored and combined with Tat or TfR targeting strategies with low brain targeting efficiency,^{9,10} and our results indicate that LNPs displaying AAV-peptides with nsPEF could be another avenue to explore. In addition, nsPEFs offer a tunable option for tissue permeabilization with temporal and spatial control^{29,31,34,37} but have not been applied to delivery through the BBB. Since the nsPEF parameters in our studies were not observed to decrease cell viability, the increased transport of NPs with nsPEFs was likely due to reversible permeabilization^{28,29} of the BBB cell monolayer.

Protein-engineered NPs could be interacting with cell surface receptors and cell membranes for greater receptor- and adsorptive-mediated transport, although partial explanation by other mechanisms such as paracellular diffusion cannot be

discounted. While bEnd.3 monocultures are widely used as a BBB model and do form tight junctions,^{71,92,93} other systems using primary cells, cocultures with multiple cell types (such as astrocytes), cells that form tighter junctions like immortalized murine brain microvascular endothelial cells, and microfluidic organ-on-a-chip approaches could be explored.^{70,92,94–96} The use of less leaky models reduces paracellular diffusion of NPs,⁹² yielding results that may better model BBB transport. While cognate receptor identity is unclear for the AAV.CPP.16 peptide, we would anticipate efficient transport of CPP16.E2 in higher complexity models using human cell lines, as AAV.CPP.16 could cross a human spheroid coculture model more than AAV9.²¹ By contrast, we would expect less translatability for PHPeB.E2 because the AAV.PHP.B family of capsids, utilizing LY6A, does not translate to efficient BBB crossing following IV injection in nonhuman primates, unlike AAV.CPP.16.^{21,97}

Additionally, future studies toward translation could examine the transport of drugs, either directly or packaged into NPs, for facilitated delivery to the brain using the AAV9-mimetic peptides as a mediator. For example, we previously conjugated guest molecules to the internal surface of NPs at cysteine sites (60 available per NP assembly), which have included fluorescent dyes (as in this current work),⁵¹ RNA and DNA,⁵⁰ and anticancer drug molecules⁵⁹ into E2-based NPs. We thus expect that we could attach other small molecule drugs using similar or alternative strategies. The conjugation of drugs could be performed using linkers,^{59,60} recombinant engineering,^{79,82,98} or SpyTag-SpyCatcher technology.^{53,77} These drugs could include treatments for CNS pathologies for such as cancer (e.g., glioblastoma) or neurodegenerative disorders.⁹⁹ More broadly, however, our AAV9-mimetic peptide studies were performed to examine the future feasibility of grafting these peptides onto NP carriers or therapeutics for delivery to the brain and the constraints under which the strategy can be applied. For example, one possibility could be to attach these peptides to antibody-based treatments that have been developed for Alzheimer's disease to increase their transport past the BBB.^{100,101} The choice of delivery strategy would depend upon many factors, including size constraints, desired dose, and possible impact on BBB targeting efficiency.

The synergy of AAV-mimicking NPs with nsPEFs could be explained by a number of possible mechanisms. Nanosecond PEFs may temporarily cause pore formation within cell membranes as a result of electric field-induced movement of charged molecules^{28,29} or through disruption of tight junctions between brain endothelial cells.³⁰ While still debated, there is evidence that intracellular organelles such as endosomes, as well as the outer cytoplasmic membrane, can become permeabilized.^{29,102} In a previous study, a majority of nonencapsulated DNA cargo trafficked through endosomal compartments following electroporation, similar to the intracellular trafficking of viruses such as AAVs.^{102,103} Thus, the permeabilization of endosomes or other intracellular vesicles could be especially relevant, as these bodies may traffic NPs after receptor- or adsorptive-mediated endocytosis and during transcytosis,¹⁰⁴ for which our different types of NPs experienced varying efficiencies.

4. CONCLUSIONS

In this study, we examined the feasibility of decorating NPs with AAV-mimetic peptides in combination with nsPEFs as a two-pronged strategy for BBB transport. To rationally design NPs,

we used E2 NPs, which share similar icosahedral symmetry and trimeric repeats to AAV9-based variants that can cross the BBB *in vivo*. We designed NPs to mimic AAV.PHP.eB, AAV.X1, and AAV.CPP.16, the latter able to cross the BBB in the most diverse mouse models. Using these capsids' unique peptide sequences, we evaluated the NP conjugation of nonlooped peptides (pPHPeB-E2, pX1-E2, or pCPP-E2) or the protein engineering of peptide loops (PHPeB.E2, X1.E2, or CPP16.E2). We computationally modeled the engineered protein variants, predicting that all designs would properly fold. *In vitro* assays utilizing murine brain endothelial cells demonstrated the increased ability of X1.E2 and CPP16.E2 NPs to be taken up by these cells, improving performance over flexible peptide-conjugated NPs and demonstrating the need to keep the AAV9-based peptide loops within their structural context. Furthermore, the most efficient transient transport of NPs past the BBB combined both strategies of receptor-mediated transcytosis and physical disruption: NPs designed to mimic brain-penetrating AAV.CPP.16 by peptide loop decoration (CPP16.E2), together with nsPEF treatment. Overall, our results highlight that these AAV9-mimetic peptides warrant further investigation toward facilitating molecular delivery to the brain for CNS therapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsbmaterials.5c01483>.

Additional experimental methods, table of DNA and amino acid sequences, schematics for flow cytometry and nsPEF protocols, models of E2 nanoparticles with AAV-mimetic peptides, and other supporting data including DLS data, mass spectrometry, circular dichroism, NP uptake in cells, parameters of nsPEF, and transport of NPs past *in vitro* BBB cell model (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Szu-Wen Wang – Department of Chemical and Biomolecular Engineering, University of California, Irvine, California 92697, United States; Department of Biomedical Engineering, Center for Neural Circuit Mapping, Chao Family Comprehensive Cancer Center, and Institute for Immunology, University of California, Irvine, California 92697, United States; orcid.org/0000-0001-7398-0220; Email: wangsw@uci.edu

Authors

Nina Butkovich – Department of Chemical and Biomolecular Engineering, University of California, Irvine, California 92697, United States

Yifei Xu – Department of Biomedical Engineering, University of California, Irvine, California 92697, United States

Yuchen Song – Department of Biomedical Engineering and Beckman Laser Institute and Medical Clinic, University of California, Irvine, California 92697, United States

Lu Wang – Department of Chemical and Biomolecular Engineering, University of California, Irvine, California 92697, United States

Aaron Ramirez – Department of Chemical and Biomolecular Engineering, University of California, Irvine, California 92697, United States

Enya Li — Department of Chemical and Biomolecular Engineering, University of California, Irvine, California 92697, United States

Nikhil Tien Chi Siao — Department of Biomedical Engineering, University of California, Irvine, California 92697, United States

Eric Velazquez-Rivera — Department of Anatomy and Neurobiology, University of California, Irvine, California 92697, United States

Liangzhong Xiang — Department of Biomedical Engineering and Beckman Laser Institute and Medical Clinic, University of California, Irvine, California 92697, United States; Department of Radiological Sciences, University of California Irvine, Orange, California 92686, United States

Xiangmin Xu — Department of Biomedical Engineering, Department of Anatomy and Neurobiology, Center for Neural Circuit Mapping, Department of Computer Science, and Department of Microbiology and Molecular Genetics, University of California, Irvine, California 92697, United States

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsbiomaterials.5c01483>

Author Contributions

All authors have approved the final version of the manuscript. N.B.: conceptualization, data curation, formal analysis, investigation, methodology, writing — original draft, writing — review and editing; Y.X.: data curation, investigation, methodology, writing — review and editing, software, validation; Y.S.: data curation, investigation, methodology, writing — review and editing, software, validation; L.W.: data curation, investigation; A.R.: conceptualization; E.L.: investigation; N.T.C.S.: data curation, investigation, methodology, software, validation; E.V.-R.: conceptualization, writing — review and editing; L.X.: conceptualization, resources, supervision, writing — review and editing; X.X.: conceptualization, funding acquisition, methodology, project administration, supervision, writing — review and editing; S.-W.W.: conceptualization, funding acquisition, methodology, project administration, supervision, writing — original draft, writing — review and editing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a grant from the UCI Center for Neurotherapeutics (to S.W.W. and X.X.) and the National Institutes of Health (NIH) T32 training grant (T32AG081185) fellowship (to N.B.). We gratefully acknowledge additional funding from the UCI Center for Neural Circuit Mapping (CNCM). Flow cytometry and mass spectrometry were carried out at the UCI Institute for Immunology Flow Cytometry Facility and the UCI Mass Spectrometry Facility, respectively. Discussions with Prof. Philip Felgner, Dr. Jiin Felgner, and Dr. Alexis Bouin were greatly appreciated. We gratefully acknowledge Prof. Matthew Inlay, who generously shared a EVOM-2 V/Ohm meter for TEER measurements. We thank Jocelyn (Tzulung) Lin for her assistance in passaging and maintaining the bEnd.3 cells, and Eshana Bethur, Dr. Rweetuparna Guha, and Prof. Stacy Copp for their training and use of the CD spectrometer. Molecular graphics were made using ChimeraX, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with

support from the National Institutes of Health (R01GM129325) and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases. We acknowledge the AlphaFold2 and ColabFold teams for generously allowing community use of their state-of-the-art computational programs.^{61,62} Portions of figures were generated using BioRender.

ABBREVIATIONS

AAV: adeno-associated virus; AF: Alexa Fluor; ANOVA: analysis of variance; BBB: blood–brain barrier; BCA: bicinchoninic acid assay; CD: circular dichroism; CNS: central nervous system; DLS: dynamic light scattering; GPI: glycosylphosphatidylinositol; HVR: highly variable region; LNP: lipid nanoparticle; LRP6: low-density-lipoprotein-receptor-related-protein 6; LY6A: lymphocyte antigen 6 complex, locus A; PEF: pulsed electric field; PW: pulse width; NP: nanoparticle; nsPEF: nanosecond PEF; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sulfo-SMCC: sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate; TCEP: tris-(2-carboxylethyl)phosphine; TEER: transendothelial/transepithelial electrical resistance; RMSD: root-mean-square deviation at 95% residue coverage

REFERENCES

- (1) Wu, D.; Chen, Q.; Chen, X.; Han, F.; Chen, Z.; Wang, Y. The blood-brain barrier: structure, regulation, and drug delivery. *Signal Transduct Target Ther* **2023**, *8* (1), 217.
- (2) Abbott, N. J.; Patabendige, A. A.; Dolman, D. E.; Yusof, S. R.; Begley, D. J. Structure and function of the blood-brain barrier. *Neurobiol Dis* **2010**, *37* (1), 13–25.
- (3) Ackerman, S. Discovering the Brain. <http://www.nap.edu/catalog/1785.html>.
- (4) Pardridge, W. M. The blood-brain barrier: bottleneck in brain drug development. *NeuroRx* **2005**, *2* (1), 3–14.
- (5) Zhou, X.; Smith, Q. R.; Liu, X. Brain penetrating peptides and peptide-drug conjugates to overcome the blood-brain barrier and target CNS diseases. *Wiley Interdiscip Rev. Nanomed Nanobiotechnol* **2021**, *13* (4), No. e1695.
- (6) Terstappen, G. C.; Meyer, A. H.; Bell, R. D.; Zhang, W. Strategies for delivering therapeutics across the blood-brain barrier. *Nat. Rev. Drug Discov* **2021**, *20* (5), 362–383.
- (7) Fischell, J. M.; Fishman, P. S. A Multifaceted Approach to Optimizing AAV Delivery to the Brain for the Treatment of Neurodegenerative Diseases. *Front Neurosci* **2021**, *15*, 747726.
- (8) Hersh, A. M.; Alomari, S.; Tyler, B. M. Crossing the Blood-Brain Barrier: Advances in Nanoparticle Technology for Drug Delivery in Neuro-Oncology. *Int. J. Mol. Sci.* **2022**, *23* (8), 4153.
- (9) Ghorai, S. M.; Deep, A.; Magoo, D.; Gupta, C.; Gupta, N. Cell-Penetrating and Targeted Peptides Delivery Systems as Potential Pharmaceutical Carriers for Enhanced Delivery across the Blood-Brain Barrier (BBB). *Pharmaceutics* **2023**, *15* (7), 1999.
- (10) Komin, A.; Russell, L. M.; Hristova, K. A.; Searson, P. C. Peptide-based strategies for enhanced cell uptake, transcellular transport, and circulation: Mechanisms and challenges. *Adv. Drug Deliv Rev.* **2017**, *110–111*, 52–64.
- (11) Hill, M. D.; Martin, R. H.; Mikulis, D.; Wong, J. H.; Silver, F. L.; terBrugge, K. G.; Milot, G.; Clark, W. M.; MacDonald, R. L.; Kelly, M. E.; Boulton, M.; Fleetwood, I.; McDougall, C.; Gunnarsson, T.; Chow, M.; Lum, C.; Dodd, R.; Poublanc, J.; Krings, T.; Demchuk, A. M.; Goyal, M.; Anderson, R.; Bishop, J.; Garman, D.; Tymianski, M. Safety and efficacy of NA-1 in patients with iatrogenic stroke after endovascular aneurysm repair (ENACT): a phase 2, randomised, double-blind, placebo-controlled trial. *Lancet Neurology* **2012**, *11* (11), 942–950.

- (12) Zhang, H.; Zhang, Y.; Zhang, C.; Yu, H.; Ma, Y.; Li, Z.; Shi, N. Recent Advances of Cell-Penetrating Peptides and Their Application as Vectors for Delivery of Peptide and Protein-Based Cargo Molecules. *Pharmaceutics* **2023**, *15* (8), 2093.
- (13) Sarko, D.; Beijer, B.; Boy, R. G.; Nothelfer, E. M.; Leotta, K.; Eisenhut, M.; Altmann, A.; Haberkorn, U.; Mier, W. The Pharmacokinetics of Cell-Penetrating Peptides. *Mol. Pharmaceut* **2010**, *7* (6), 2224–2231.
- (14) Johnsen, K. B.; Moos, T. Revisiting nanoparticle technology for blood-brain barrier transport: Unfolding at the endothelial gate improves the fate of transferrin receptor-targeted liposomes. *J. Controlled Release* **2016**, *222*, 32–46.
- (15) Stalmans, S.; Bracke, N.; Wynendaele, E.; Gevaert, B.; Peremans, K.; Burvenich, C.; Polis, I.; De Spiegeleer, B. Cell-Penetrating Peptides Selectively Cross the Blood-Brain Barrier In Vivo. *PLoS One* **2015**, *10* (10), No. e0139652.
- (16) Issa, S. S.; Shaimardanova, A. A.; Solovyeva, V. V.; Rizvanov, A. A. Various AAV Serotypes and Their Applications in Gene Therapy: An Overview. *Cells* **2023**, *12* (5), 785.
- (17) Ravindra Kumar, S.; Miles, T. F.; Chen, X.; Brown, D.; Dobрева, T.; Huang, Q.; Ding, X.; Luo, Y.; Einarsson, P. H.; Greenbaum, A.; Jang, M. J.; Deverman, B. E.; Gradinaru, V. Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types. *Nat. Methods* **2020**, *17* (5), 541–550.
- (18) Deverman, B. E.; Pravdo, P. L.; Simpson, B. P.; Kumar, S. R.; Chan, K. Y.; Banerjee, A.; Wu, W. L.; Yang, B.; Huber, N.; Pasca, S. P.; Gradinaru, V. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat. Biotechnol.* **2016**, *34* (2), 204–9.
- (19) Chan, K. Y.; Jang, M. J.; Yoo, B. B.; Greenbaum, A.; Ravi, N.; Wu, W. L.; Sanchez-Guardado, L.; Lois, C.; Mazmanian, S. K.; Deverman, B. E.; Gradinaru, V. Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nat. Neurosci* **2017**, *20* (8), 1172–1179.
- (20) Chen, X. H.; Wolfe, D. A.; Bindu, D. S.; Zhang, M. Y.; Taskin, N.; Goertsen, D.; Shay, T. F.; Sullivan, E. E.; Huang, S. F.; Kumar, S. R.; Arokiaj, C. M.; Plattner, V. M.; Campos, L. J.; Mich, J. K.; Monet, D.; Ngo, V.; Ding, X. Z.; Omstead, V.; Weed, N.; Bishaw, Y.; Gore, B. B.; Lein, E. S.; Akrami, A.; Miller, C.; Levi, B. P.; Keller, A.; Ting, J. T.; Fox, A. S.; Eroglu, C.; Gradinaru, V. Functional gene delivery to and across brain vasculature of systemic AAVs with endothelial-specific tropism in rodents and broad tropism in primates. *Nat. Commun.* **2023**, *14* (1), 3345.
- (21) Yao, Y.; Wang, J.; Liu, Y.; Qu, Y.; Wang, K.; Zhang, Y.; Chang, Y.; Yang, Z.; Wan, J.; Liu, J.; Nakashima, H.; Lawler, S. E.; Chiocca, E. A.; Cho, C. F.; Bei, F. Variants of the adeno-associated virus serotype 9 with enhanced penetration of the blood-brain barrier in rodents and primates. *Nat. Biomed Eng.* **2022**, *6* (11), 1257–1271.
- (22) Wang, H.; Li, R.; Sadekar, S.; Kamath, A. V.; Shen, B.-Q. A novel approach to quantitate biodistribution and transduction of adeno-associated virus gene therapy using radiolabeled AAV vectors in mice. *Molecular Therapy - Methods & Clinical Development* **2024**, *32* (3), 101326.
- (23) Batista, A. R.; King, O. D.; Reardon, C. P.; Davis, C.; Shankaracharya, Philip, V.; Gray-Edwards, H.; Aronin, N.; Lutz, C.; Landers, J.; Sena-Esteves, M. Ly6a Differential Expression in Blood-Brain Barrier Is Responsible for Strain Specific Central Nervous System Transduction Profile of AAV-PHP.B. *Hum. Gene Ther.* **2020**, *31* (1–2), 90–102.
- (24) Hordeaux, J.; Yuan, Y.; Clark, P. M.; Wang, Q.; Martino, R. A.; Sims, J. J.; Bell, P.; Raymond, A.; Stanford, W. L.; Wilson, J. M. B Transport across the Blood-Brain Barrier. *Mol. Ther* **2019**, *27* (5), 912–921.
- (25) Martino, R. A.; Fluck, E. C.; Murphy, J.; Wang, Q.; Hoff, H.; Pumroy, R. A.; Lee, C. Y.; Sims, J. J.; Roy, S.; Moiseenkova-Bell, V. Y.; Wilson, J. M. Context-Specific Function of the Engineered Peptide Domain of PHP.B. *J. Virol* **2021**, *95* (20), No. e0116421.
- (26) Shay, T. F.; Jang, S.; Brittain, T. J.; Chen, X. H.; Walker, B.; Tebbutt, C.; Fan, Y. J.; Wolfe, D. A.; Arokiaj, C. M.; Sullivan, E. E.; Ding, X. Z.; Wang, T. Y.; Lei, Y. P.; Chuapoco, M. R.; Chou, T. F.; Gradinaru, V. Human cell surface-AAV interactomes identify LRP6 as blood-brain barrier transcytosis receptor and immune cytokine IL3 as AAV9 binder. *Nat. Commun.* **2024**, *15* (1), 7853.
- (27) Sangster, M. L.; Bishop, M. M.; Yao, Y.; Feitor, J. F.; Shahriar, S.; Miller, M. E.; Chekuri, A. K.; Budnik, B.; Bei, F.; Grishchuk, Y. A blood-brain barrier-penetrant AAV gene therapy improves neurological function in symptomatic mucopolidosis IV mice. *Mol. Ther Methods Clin Dev* **2024**, *32* (2), 101269.
- (28) Fang, Z.; Chen, L.; Moser, M. A. J.; Zhang, W.; Qin, Z.; Zhang, B. Electroporation-Based Therapy for Brain Tumors: A Review. *J. Biomech Eng.* **2021**, *143* (10), 100802.
- (29) Ruiz-Fernandez, A. R.; Campos, L.; Gutierrez-Maldonado, S. E.; Nunez, G.; Villanelo, F.; Perez-Acle, T. Nanosecond Pulsed Electric Field (nsPEF): Opening the Biotechnological Pandora's Box. *Int. J. Mol. Sci.* **2022**, *23* (11), 6158.
- (30) Lorenzo, M. F.; Campelo, S. N.; Arroyo, J. P.; Aycock, K. N.; Hinckley, J.; Arena, C. B.; Rossmeisl, J. H.; Davalos, R. V. An Investigation for Large Volume, Focal Blood-Brain Barrier Disruption with High-Frequency Pulsed Electric Fields. *Pharmaceutics (Basel)* **2021**, *14* (12), 1333.
- (31) Carr, L.; Golzio, M.; Orlacchio, R.; Alberola, G.; Kolosnjaj-Tabi, J.; Leveque, P.; Arnaud-Cormos, D.; Rols, M. P. A nanosecond pulsed electric field (nsPEF) can affect membrane permeabilization and cellular viability in a 3D spheroids tumor model. *Bioelectrochemistry* **2021**, *141*, 107839.
- (32) Mercadal, B.; Beitel-White, N.; Aycock, K. N.; Castellvi, Q.; Davalos, R. V.; Ivorra, A. Dynamics of Cell Death After Conventional IRE and H-FIRE Treatments. *Ann. Biomed Eng.* **2020**, *48* (5), 1451–1462.
- (33) Jiang, Z. R.; Xu, Y. F.; Sun, L. S.; Srinivasan, S.; Wu, Q. J.; Xiang, L. Z.; Lei, R. Enhanced Electroacoustic Tomography with Supervised Learning for Real-time Electroporation Monitoring. *Precis Radiat Oncol* **2024**, *8* (3), 110–118.
- (34) Xu, Y.; Sun, L.; Wang, S.; Yan, Y.; Pandey, P.; Novickij, V.; Xiang, L. Electroacoustic tomography for real-time visualization of electrical field dynamics in deep tissue during electroporation. *Communications Engineering* **2023**, *2* (1), 75.
- (35) Pakhomov, A. G.; Gudvangen, E.; Mangalanathan, U.; Kondratiev, O.; Redondo, L.; Semenov, I. Next generation CANCAN focusing for remote stimulation by nanosecond electric pulses. *Bioelectrochemistry* **2023**, *152*, 108437.
- (36) Cooper, I.; Last, D.; Ravid, O.; Rand, D.; Matsree, E.; Omes, L.; Shemesh, C.; Liberman, M.; Zach, L.; Furman, O.; Daniels, D.; Liraz-Zaltsman, S.; Mardor, Y.; Sharabi, S. BBB opening by low pulsed electric fields, depicted by delayed-contrast MRI, enables efficient delivery of therapeutic doxorubicin doses into mice brains. *Fluids and Barriers of the Cns* **2023**, *20* (1), 67.
- (37) Sharabi, S.; Last, D.; Daniels, D.; Fabian, I. D.; Atrakchi, D.; Bresler, Y.; Liraz-Zaltsman, S.; Cooper, I.; Mardor, Y. Non-Invasive Low Pulsed Electrical Fields for Inducing BBB Disruption in Mice-Feasibility Demonstration. *Pharmaceutics* **2021**, *13* (2), 169.
- (38) Oshin, E. A.; Minhas, Z.; Biancatelli, R. M. L. C.; Catravas, J. D.; Heller, R.; Guo, S. Q.; Jiang, C. Q. Synergistic effects of nanosecond pulsed plasma and electric field on inactivation of pancreatic cancer cells in vitro. *Sci. Rep.* **2024**, *14* (1), 885.
- (39) Bardet, S. M.; Carr, L.; Soueid, M.; Arnaud-Cormos, D.; Leveque, P.; O'Connor, R. P. Multiphoton imaging reveals that nanosecond pulsed electric fields collapse tumor and normal vascular perfusion in human glioblastoma xenografts. *Sci. Rep.* **2016**, *6*, 34443.
- (40) Szlasa, W.; Michel, O.; Sauer, N.; Novickij, V.; Lewandowski, D.; Kasperkiewicz, P.; Tarek, M.; Saczko, J.; Kulbacka, J. Nanosecond pulsed electric field suppresses growth and reduces multi-drug resistance effect in pancreatic cancer. *Sci. Rep* **2023**, *13* (1), 351.
- (41) Szlasa, W.; Sauer, N.; Baczyńska, D.; Zietek, M.; Haczkiwicz-Lesniak, K.; Karpinski, P.; Fleszar, M.; Fortuna, P.; Kulus, M. J.; Piotrowska, A.; Kmiecik, A.; Baranska, A.; Michel, O.; Novickij, V.; Tarek, M.; Kasperkiewicz, P.; Dziegiel, P.; Podhorska-Okolow, M.; Saczko, J.; Kulbacka, J. Pulsed electric field induces exocytosis and

overexpression of MAGE antigens in melanoma. *Sci. Rep.* **2024**, *14* (1), 12546.

(42) Munavalli, G. S.; Zelickson, B. D.; Selim, M. M.; Kilmer, S. L.; Rohrer, T. E.; Newman, J.; Jauregui, L.; Knappe, W. A.; Ebberts, E.; Uecker, D.; Nuccitelli, R. Safety and Efficacy of Nanosecond Pulsed Electric Field Treatment of Sebaceous Gland Hyperplasia. *Dermatol Surg* **2020**, *46* (6), 803–809.

(43) Nuccitelli, R.; Wood, R.; Kreis, M.; Athos, B.; Huynh, J.; Lui, K.; Nuccitelli, P.; Epstein, E. H., Jr. First-in-human trial of nano-electroablation therapy for basal cell carcinoma: proof of method. *Exp Dermatol* **2014**, *23* (2), 135–7.

(44) Cvetkoska, A.; Macek-Lebar, A.; Trdina, P.; Miklavcic, D.; Rebersek, M. Muscle contractions and pain sensation accompanying high-frequency electroporation pulses. *Sci. Rep.* **2022**, *12* (1), 8019.

(45) Gudvangen, E.; Kim, V.; Novickij, V.; Battista, F.; Pakhomov, A. G. Electroporation and cell killing by milli- to nanosecond pulses and avoiding neuromuscular stimulation in cancer ablation. *Sci. Rep.* **2022**, *12* (1), 1763.

(46) Kim, V.; Gudvangen, E.; Kondratiev, O.; Redondo, L.; Xiao, S.; Pakhomov, A. G. Peculiarities of Neurostimulation by Intense Nanosecond Pulsed Electric Fields: How to Avoid Firing in Peripheral Nerve Fibers. *International Journal of Molecular Sciences* **2021**, *22* (13), 7051.

(47) Neek, M.; Kim, T. I.; Wang, S. W. Protein-based nanoparticles in cancer vaccine development. *Nanomedicine* **2019**, *15* (1), 164–174.

(48) Stagg, S. M.; Yoshioka, C.; Davulcu, O.; Chapman, M. S. Cryo-electron Microscopy of Adeno-associated Virus. *Chem. Rev.* **2022**, *122* (17), 14018–14054.

(49) Dalmau, M.; Lim, S.; Wang, S. W. Design of a pH-dependent molecular switch in a caged protein platform. *Nano Lett.* **2009**, *9* (1), 160–6.

(50) Butkovich, N.; Tucker, J. A.; Ramirez, A.; Li, E.; Meli, V. S.; Nelson, E. L.; Wang, S. W. Nanoparticle vaccines can be designed to induce pDC support of mDCs for increased antigen display. *Biomater Sci.* **2023**, *11* (2), 596–610.

(51) Dalmau, M.; Lim, S.; Chen, H. C.; Ruiz, C.; Wang, S. W. Thermostability and molecular encapsulation within an engineered caged protein scaffold. *Biotechnol. Bioeng.* **2008**, *101* (4), 654–64.

(52) Li, E.; Butkovich, N.; Tucker, J. A.; Nelson, E. L.; Wang, S. W. Evaluating Anti-tumor Immune Responses of Protein Nanoparticle-Based Cancer Vaccines. *Methods Mol. Biol.* **2023**, *2671*, 321–333.

(53) Li, E.; Brennan, C. K.; Ramirez, A.; Tucker, J. A.; Butkovich, N.; Meli, V. S.; Ionkina, A. A.; Nelson, E. L.; Prescher, J. A.; Wang, S. W. Macromolecular assembly of bioluminescent protein nanoparticles for enhanced imaging. *Mater. Today Bio* **2022**, *17*, 100455.

(54) Molino, N.; et al. Complement activation and cell uptake responses towards polymer-functionalized protein nanocapsules. *Biomacromolecules* **2012**, *13*, 974–981.

(55) Molino, N. M.; Anderson, A. K.; Nelson, E. L.; Wang, S. W. Biomimetic protein nanoparticles facilitate enhanced dendritic cell activation and cross-presentation. *ACS Nano* **2013**, *7* (11), 9743–52.

(56) Molino, N. M.; Neek, M.; Tucker, J. A.; Nelson, E. L.; Wang, S. W. Viral-mimicking protein nanoparticle vaccine for eliciting anti-tumor responses. *Biomaterials* **2016**, *86*, 83–91.

(57) Neek, M.; Tucker, J. A.; Butkovich, N.; Nelson, E. L.; Wang, S. W. An Antigen-Delivery Protein Nanoparticle Combined with Anti-PD-1 Checkpoint Inhibitor Has Curative Efficacy in an Aggressive Melanoma Model. *Adv. Ther.* **2020**, *3* (12), 2000122.

(58) Neek, M.; Tucker, J. A.; Kim, T. I.; Molino, N. M.; Nelson, E. L.; Wang, S. W. Co-delivery of human cancer-testis antigens with adjuvant in protein nanoparticles induces higher cell-mediated immune responses. *Biomaterials* **2018**, *156*, 194–203.

(59) Ren, D.; Kratz, F.; Wang, S. W. Protein nanocapsules containing doxorubicin as a pH-responsive delivery system. *Small* **2011**, *7* (8), 1051–60.

(60) Ren, D.; Kratz, F.; Wang, S. W. Engineered drug-protein nanoparticle complexes for folate receptor targeting. *Biochem Eng. J.* **2014**, *89*, 33–41.

(61) Mirdita, M.; Schütze, K.; Moriwaki, Y.; Heo, L.; Ovchinnikov, S.; Steinegger, M. ColabFold: making protein folding accessible to all. *Nat. Methods* **2022**, *19* (6), 679.

(62) Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Zidek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S. A. A.; Ballard, A. J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A. W.; Kavukcuoglu, K.; Kohli, P.; Hassabis, D. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596* (7873), 583–589.

(63) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Meng, E. C.; Couch, G. S.; Croll, T. I.; Morris, J. H.; Ferrin, T. E. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci.* **2021**, *30* (1), 70–82.

(64) Kardos, J.; Nyiri, M. P.; Moussong, E.; Wien, F.; Molnar, T.; Murvai, N.; Toth, V.; Vadaszi, H.; Kun, J.; Jamme, F.; Micsonai, A. Guide to the structural characterization of protein aggregates and amyloid fibrils by CD spectroscopy. *Protein Sci.* **2025**, *34* (3), No. e70066.

(65) Micsonai, A.; Wien, F.; Bulyaki, E.; Kun, J.; Moussong, E.; Lee, Y. H.; Goto, Y.; Refregiers, M.; Kardos, J. BeStSel: a web server for accurate protein secondary structure prediction and fold recognition from the circular dichroism spectra. *Nucleic Acids Res.* **2018**, *46* (W1), W315–W322.

(66) Jang, S.; Shen, H. K.; Ding, X.; Miles, T. F.; Gradinaru, V. Structural basis of receptor usage by the engineered capsid AAV-PHP.eB. *Mol. Ther. Methods Clin Dev* **2022**, *26*, 343–354.

(67) Zhou, Y.; Khan, H.; Hoi, M. P. M.; Cheang, W. S. Piceatannol Protects Brain Endothelial Cell Line (bEnd.3) against Lipopolysaccharide-Induced Inflammation and Oxidative Stress. *Molecules* **2022**, *27* (4), 1206.

(68) Rabanel, J. M.; Piec, P. A.; Landri, S.; Patten, S. A.; Ramassamy, C. Transport of PEGylated-PLA nanoparticles across a blood brain barrier model, entry into neuronal cells and brain bioavailability. *J. Controlled Release* **2020**, *328*, 679–695.

(69) Booth, R.; Kim, H. Characterization of a microfluidic in vitro model of the blood-brain barrier (muBBB). *Lab Chip* **2012**, *12* (10), 1784–92.

(70) Srinivasan, B.; Kolli, A. R.; Esch, M. B.; Abaci, H. E.; Shuler, M. L.; Hickman, J. J. TEER measurement techniques for in vitro barrier model systems. *J. Lab Autom* **2015**, *20* (2), 107–26.

(71) Aday, S.; Li, W.; Karp, J. M.; Joshi, N. An in vitro Blood-brain Barrier Model to Study the Penetration of Nanoparticles. *Bio Protoc* **2022**, *12* (4), No. e4334.

(72) Tylawsky, D. E.; Kiguchi, H.; Vaynshteyn, J.; Gerwin, J.; Shah, J.; Islam, T.; Boyer, J. A.; Boue, D. R.; Snuderl, M.; Greenblatt, M. B.; Shamay, Y.; Raju, G. P.; Heller, D. A. P-selectin-targeted nanocarriers induce active crossing of the blood-brain barrier via caveolin-1-dependent transcytosis. *Nat. Mater.* **2023**, *22* (3), 391–399.

(73) Xu, G.; Zhang, R.; Li, H.; Yin, K.; Ma, X.; Lou, Z. Structural basis for the neurotropic AAV9 and the engineered AAVPHP.eB recognition with cellular receptors. *Mol. Ther. Methods Clin Dev* **2022**, *26*, 52–60.

(74) Watson, J. L.; Juergens, D.; Bennett, N. R.; Trippie, B. L.; Yim, J.; Eisenach, H. E.; Ahern, W.; Borst, A. J.; Ragotte, R. J.; Milles, L. F.; Wicky, B. I. M.; Hanikel, N.; Pellock, S. J.; Courbet, A.; Scheffer, W.; Wang, J.; Venkatesh, P.; Sappington, I.; Torres, S. V.; Lauko, A.; De Bortoli, V.; Mathieu, E.; Ovchinnikov, S.; Barzilay, R.; Jaakkola, T. S.; DiMaio, F.; Baek, M.; Baker, D. De novo design of protein structure and function with RFdiffusion. *Nature* **2023**, *620* (7976), 1089–1100.

(75) Shay, T. F.; Sullivan, E. E.; Ding, X.; Chen, X.; Ravindra Kumar, S.; Goertsen, D.; Brown, D.; Crosby, A.; Vielmetter, J.; Bosors, M.; Wolfe, D. A.; Lam, A. W.; Gradinaru, V. Primate-conserved carbonic anhydrase IV and murine-restricted LY6C1 enable blood-brain barrier crossing by engineered viral vectors. *Sci. Adv.* **2023**, *9* (16), No. eadg6618.

(76) Ren, D.; Dalmau, M.; Randall, A.; Shindel, M. M.; Baldi, P.; Wang, S. W. Biomimetic Design of Protein Nanomaterials for

Hydrophobic Molecular Transport. *Adv. Funct. Mater.* **2012**, *22* (15), 3170–3180.

(77) Ramirez, A.; Felgner, J.; Jain, A.; Jan, S.; Albin, T. J.; Badten, A. J.; Gregory, A. E.; Nakajima, R.; Jasinskas, A.; Felgner, P. L.; Burkhardt, A. M.; Davies, D. H.; Wang, S. W. Engineering Protein Nanoparticles Functionalized with an Immunodominant *Coxiella burnetii* Antigen to Generate a Q Fever Vaccine. *Bioconjug. Chem.* **2023**, *34* (9), 1653–1666.

(78) Dalmau, M.; Lim, S.; Wang, S. W. pH-triggered disassembly in a caged protein complex. *Biomacromolecules* **2009**, *10* (12), 3199–206.

(79) He, L.; de Val, N.; Morris, C. D.; Vora, N.; Thinnies, T. C.; Kong, L.; Azadnia, P.; Sok, D.; Zhou, B.; Burton, D. R.; Wilson, I. A.; Nemazee, D.; Ward, A. B.; Zhu, J. Presenting native-like trimeric HIV-1 antigens with self-assembling nanoparticles. *Nat. Commun.* **2016**, *7*, 12041.

(80) Domingo, G. J.; Caivano, A.; Sartorius, R.; Barba, P.; Backstrom, M.; Piatier-Tonneau, D.; Guardiola, J.; De Berardinis, P.; Perham, R. N. Induction of specific T-helper and cytolytic responses to epitopes displayed on a virus-like protein scaffold derived from the pyruvate dehydrogenase multienzyme complex. *Vaccine* **2003**, *21* (13–14), 1502–9.

(81) Domingo, G. J.; Orru, S.; Perham, R. N. Multiple display of peptides and proteins on a macromolecular scaffold derived from a multienzyme complex. *J. Mol. Biol.* **2001**, *305* (2), 259–267.

(82) He, L.; Chaudhary, A.; Lin, X.; Sou, C.; Alkutkar, T.; Kumar, S.; Ngo, T.; Kosviner, E.; Ozorowski, G.; Stanfield, R. L.; Ward, A. B.; Wilson, I. A.; Zhu, J. Single-component multilayered self-assembling nanoparticles presenting rationally designed glycoprotein trimers as Ebola virus vaccines. *Nat. Commun.* **2022**, *13* (1), 6343.

(83) Caivano, A.; Doria-Rose, N. A.; Buelow, B.; Sartorius, R.; Trovato, M.; D'Apice, L.; Domingo, G. J.; Sutton, W. F.; Haigwood, N. L.; De Berardinis, P. HIV-1 Gag p17 presented as virus-like particles on the E2 scaffold from *Geobacillus stearothermophilus* induces sustained humoral and cellular immune responses in the absence of IFN γ production by CD4⁺T cells. *Virology* **2010**, *407* (2), 296–305.

(84) Jaworski, J. P.; Krebs, S. J.; Trovato, M.; Kovarik, D. N.; Brower, Z.; Sutton, W. F.; Waagmeester, G.; Sartorius, R.; D'Apice, L.; Caivano, A.; Doria-Rose, N. A.; Malherbe, D.; Montefiori, D. C.; Barnett, S.; De Berardinis, P.; Haigwood, N. L. Co-immunization with multimeric scaffolds and DNA rapidly induces potent autologous HIV-1 neutralizing antibodies and CD8⁺ T cells. *PLoS One* **2012**, *7* (2), No. e31464.

(85) Yang, H.; Liu, L.; Xu, F. The promises and challenges of fusion constructs in protein biochemistry and enzymology. *Appl. Microbiol. Biotechnol.* **2016**, *100* (19), 8273–81.

(86) Rosano, G. L.; Ceccarelli, E. A. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol* **2014**, *5*, 172.

(87) Fan, K.; Jia, X.; Zhou, M.; Wang, K.; Conde, J.; He, J.; Tian, J.; Yan, X. Ferritin Nanocarrier Traverses the Blood Brain Barrier and Kills Glioma. *ACS Nano* **2018**, *12* (5), 4105–4115.

(88) Goulatis, L. I.; Shusta, E. V. Protein engineering approaches for regulating blood-brain barrier transcytosis. *Curr. Opin Struct Biol.* **2017**, *45*, 109–115.

(89) De Bock, M.; Van Haver, V.; Vandenbroucke, R. E.; Decrock, E.; Wang, N.; Leybaert, L. Into rather unexplored terrain-transcellular transport across the blood-brain barrier. *Glia* **2016**, *64* (7), 1097–123.

(90) Bonakdar, M.; Wasson, E. M.; Lee, Y. W.; Davalos, R. V. Electroporation of Brain Endothelial Cells on Chip toward Permeabilizing the Blood-Brain Barrier. *Biophys. J.* **2016**, *110* (2), 503–513.

(91) Liu, D.; Zhu, M. Y.; Zhang, Y. Q.; Diao, Y. Crossing the blood-brain barrier with AAV vectors. *Metabolic Brain Disease* **2021**, *36* (1), 45–52.

(92) Bors, L. A.; Erdo, F. Overcoming the Blood-Brain Barrier. Challenges and Tricks for CNS Drug Delivery. *Sci. Pharm.* **2019**, *87* (1), 6.

(93) Guarnieri, D.; Muscetti, O.; Netti, P. A. A Method for Evaluating Nanoparticle Transport Through the Blood-Brain Barrier In Vitro. *Drug Delivery System* **2014**, *1141*, 185–199.

(94) Idris, F.; Hanna Muharram, S.; Zaini, Z.; Diah, S. Establishment of murine in vitro blood-brain barrier models using immortalized cell lines: co-cultures of brain endothelial cells, astrocytes, and neurons. *bioRxiv* **2018**, 1.

(95) Falanga, A. P.; Pitingolo, G.; Celentano, M.; Cosentino, A.; Melone, P.; Vecchione, R.; Guarnieri, D.; Netti, P. A. Shuttle-mediated nanoparticle transport across an in vitro brain endothelium under flow conditions. *Biotechnol. Bioeng.* **2017**, *114* (5), 1087–1095.

(96) Burek, M.; Salvador, E.; Forster, C. Y. Generation of an immortalized murine brain microvascular endothelial cell line as an in vitro blood brain barrier model. *J. Vis Exp* **2012**, *66*, No. e4022.

(97) Matsuzaki, Y.; Konno, A.; Mochizuki, R.; Shinohara, Y.; Nitta, K.; Okada, Y.; Hirai, H. Intravenous administration of the adeno-associated virus-PHP.B capsid fails to upregulate transduction efficiency in the marmoset brain. *Neurosci. Lett.* **2018**, *665*, 182–188.

(98) Zhang, Y. N.; Paynter, J.; Antanasijevic, A.; Allen, J. D.; Eldad, M.; Lee, Y. Z.; Copps, J.; Newby, M. L.; He, L.; Chavez, D.; Frost, P.; Goodroe, A.; Dutton, J.; Lanford, R.; Chen, C.; Wilson, I. A.; Crispin, M.; Ward, A. B.; Zhu, J. Single-component multilayered self-assembling protein nanoparticles presenting glycan-trimmed uncleaved prefusion optimized envelope trimmers as HIV-1 vaccine candidates. *Nat. Commun.* **2023**, *14* (1), 1985.

(99) Wohlfart, S.; Gelperina, S.; Kreuter, J. Transport of drugs across the blood-brain barrier by nanoparticles. *J. Controlled Release* **2012**, *161* (2), 264–73.

(100) Cummings, J. Anti-Amyloid Monoclonal Antibodies are Transformative Treatments that Redefine Alzheimer's Disease Therapeutics. *Drugs* **2023**, *83* (7), 569–576.

(101) Zhang, Y.; Chen, H.; Li, R.; Sterling, K.; Song, W. Amyloid beta-based therapy for Alzheimer's disease: challenges, successes and future. *Signal Transduct Target Ther* **2023**, *8* (1), 248.

(102) Rosazza, C.; Deschout, H.; Buntz, A.; Braeckmans, K.; Rols, M. P.; Zumbusch, A. Endocytosis and Endosomal Trafficking of DNA After Gene Electroporation In Vitro. *Mol. Ther Nucleic Acids* **2016**, *5* (2), No. e286.

(103) Naso, M. F.; Tomkowicz, B.; Perry, W. L., 3rd; Strohl, W. R. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs* **2017**, *31* (4), 317–334.

(104) Barar, J.; Rafi, M. A.; Pourseif, M. M.; Omid, Y. Blood-brain barrier transport machineries and targeted therapy of brain diseases. *Bioimpacts* **2016**, *6* (4), 225–248.