



Avidity-by-design: spatial control of T7 peptide presentation on polymersomes dictates blood-brain barrier transport fate

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Abstract

Targeting the transferrin receptor (TfR) holds promise for drug delivery across the blood-brain barrier (BBB), but conventional strategies suffer from competition with endogenous ligands and suboptimal trafficking. To overcome these limitations, we engineered a library of T7 peptide-functionalised pH-responsive polymersomes with precise control over ligand density and ligand insertion depth (δ) within a poly(ethylene glycol) (PEG) corona. We generated nanoparticles with δ values ranging from 0.3 (deeply inserted ligands) to 1.0 (fully exposed ligands), enabling systematic evaluation of how spatial ligand presentation influences BBB interactions. In vitro studies revealed that $\delta=1.0$ polymersomes exhibited efficient transcytosis, whereas $\delta=0.6$ polymersomes promoted endothelial retention, a divergence likely linked to differential receptor clustering and trafficking kinetics. Notably, TfR-mediated transport occurred independently of PACSIN2, distinguishing it from tubular transcytosis pathways observed with LRP1-targeted systems. These findings establish avidity-by-design as a strategy to tailor nanocarriers for either BBB penetration or vascular targeting, offering a modular platform for neurological therapeutics.

Keywords Multivalency · pH-sensitive polymersome · Transferrin receptor · Brain endothelium · Transcytosis · PACSIN2

Introduction

The blood-brain barrier (BBB) stands as one of the most sophisticated biological barriers. In this highly selective interface, tightly joined brain endothelial cells (BECs) selectively control the passage of essential nutrients while excluding nearly all macromolecular therapeutics [1]. This exquisite selectivity, while crucial for maintaining neural homeostasis, has rendered the treatment of neurological

disorders particularly challenging. Among strategies developed to circumvent this barrier, receptor-mediated transcytosis (RMT) has emerged as particularly promising, leveraging the BBB's own transport machinery to shuttle therapeutics into the central nervous system (CNS) [2, 3].

The transferrin receptor (TfR), highly expressed on BECs, has become a primary target for RMT approaches [4]. Yet conventional strategies that employ either transferrin itself or anti-TfR antibodies face fundamental limitations: endogenous transferrin competition saturates the binding sites. At the same time, antibody-based approaches often lead to unintended off-target delivery due to the widespread expression of TfR in peripheral tissues, which is one of the key reasons for the limited success of TfR-targeted antibodies in clinical settings [5, 6]. These challenges have urged investigation into alternative targeting moieties that maintain TfR specificity while avoiding these pitfalls, with phage-derived peptides showing promise.

The T7 peptide (HAIYPRH), identified through bio-panning, represents one such alternative [7]. It binds TfR at a site distinct from the transferrin-binding site, thereby

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avoiding competition while maintaining nanomolar affinity [7, 8]. Its small size, compared to antibodies, allows higher grafting densities on nanocarrier surfaces and minimises immunogenicity [9]. Yet, ligand-receptor interactions are governed not only by the affinity of a single ligand-receptor bond. Other parameters, such as ligand density (delivery system valency), orientation, and particularly their three-dimensional positioning relative to the nanoparticle surface, can dramatically influence the avidity of nanocarrier-cellular interactions [10–12].

Multivalency, in particular, has emerged as a double-edged sword. While increasing ligand density can enhance avidity, excessive clustering may generate steric hindrance that blocks productive binding, whereas insufficient presentation may fail to sustain receptor engagement [13, 14]. These considerations have converged into the concept of “avidity-by-design”, *i.e.*, the intentional engineering of multivalent interactions to achieve precise biological outcomes. Perhaps most intriguingly, nanoscale parameters such as ligand density (σ_L) and steric accessibility appear to influence downstream intracellular trafficking pathways, dictating whether delivery systems undergo transcytosis or intracellular retention [15, 16]. A key unanswered question is therefore how the configuration of the cargo avidity can be tuned to favour transcytosis over endocytic retention.

To bridge this gap, we developed a modular pH-responsive polymersome platform permitting exquisite control over both T7 ligand density (σ_L) and insertion depth (δ) within its corona. Our design leverages controlled radical polymerisation to synthesise poly(ethylene glycol)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PEG-*b*-PDPA) block copolymers with defined molecular weights, followed by site-specific T7 conjugation via click chemistry [17–19]. This modular strategy enables us to decouple the effects of ligand accessibility from steric shielding, providing insights into the non-linear correlation between cargo avidity and binding capability [20, 21].

Beyond the mechanistic insight, we demonstrate that tuning σ_L and δ opens a route to distinct therapeutic strategies: nanocarriers with maximally exposed ligands ($\delta \approx 1.0$) may be optimised for efficient brain parenchyma delivery, whereas partially shielded configurations ($\delta \approx 0.6$) could preferentially favour endothelial engagement and functional modulation. This tunability also provides a handle for dissecting the intracellular machinery involved in TfR-mediated trafficking. Notably, our findings suggest that TfR-mediated transcytosis may not rely on Bin/amphiphysin/Rvs (BAR) domain-containing proteins, such as PACSIN2, unlike other receptors in BECs (*i.e.*, LRP1), which have been previously reported to exploit this pathway [13, 22, 23]. Therefore, by dissecting how ligand density and steric presentation shape TfR trafficking, our work establishes a

mechanism-driven framework for BBB-targeted nanocarriers, moving beyond empirical optimisation toward rational, principled design.

Experimental section

Materials

All chemicals and solvents of analytical grade or higher purity, diisopropylaminoethyl methacrylate (DPA, 97% purity), 2-bromoisobutyl bromide (BIBB, 98% purity), diazabicyclo[5.4.0]undec-7-ene (DBU, 98% purity), Copper(I) bromide (CuBr, 98% purity), 2,2'-Bipyridine (Bipy, >99% purity), (+)-sodium L-ascorbate (>98% purity) and Copper (II) sulfate pentahydrate (CuSO₄·5H₂O, >98% purity) were purchased from Sigma-Aldrich. Polymers, including alpha-methoxy-omega-hydroxy poly(ethylene glycol) (CH₃-PEG-OH, 5 kDa, hereafter designated as MeO-PEG) and alpha-azido-omega-hydroxy poly(ethylene glycol) (N₃-PEG-OH; 1.6, 3, and 5 kDa, hereafter denominated as N₃-PEG_{1.6k}, N₃-PEG_{3k}, and N₃-PEG_{5k}) were purchased from Iris Biotech. The T7 peptide with a propargylglycine on the N-terminal (Pra-HAIYPRH, >98% purity) was custom-synthesised by Genscript. Cyanine 5.5 alkyne (alkyne-Cy_{5.5}) was purchased from Lumiprobe. Dialysis membranes (MWCO 1, 3.5 and 8 kDa, Spectra/Por®) and high-performance liquid chromatography (HPLC)-grade solvents, such as acetonitrile (MeCN), tetrafluoroacetic acid (TFA) and tetrahydrofuran (THF), were obtained from Fisher Scientific. Gel permeation chromatography (GPC)-grade THF was prepared by dissolving 250 ppm of 2,6-di-*tert*-butyl-4-methylphenol in HPLC-grade THF. Nuclear magnetic resonance (NMR) solvents were purchased from Eurisotop. For cell culture, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (10,000 U mL⁻¹), and 0.25% trypsin-EDTA were purchased from Thermo Fisher Scientific. Transwell inserts (0.4 µm pore size, 6.5- or 12-mm polyester membrane) were sourced from Corning. The mouse brain endothelial cell line bEnd3 (C0005030) was obtained from AddexBio. The mouse embryonic fibroblast NIH 3T3 (CRL-1658) and the mouse skin melanoma fibroblast B16-F10 (CRL-6475) cell lines were purchased from the American Type Culture Collection (ATCC). Primary antibodies against Zona Occludens (ZO-1, 1:200, rabbit polyclonal, #21773-1-AP) and occludin (1:500, rabbit polyclonal, #27260-1-AP) were acquired from ProteinTech, and claudin-5 (1:500, rabbit monoclonal E8F3D, #49564S) from Cell Signalling. Alexa Fluor 488 goat anti-mouse (Invitrogen #A-11034) was used as the secondary antibody. The enzyme-linked immunosorbent assay (ELISA) kit for quantifying TfR was obtained from

FineTest. Syndapin2-pmCherryC1 was a gift from Christien Merrifield (Addgene plasmid #27681), and lentivirus packaging was provided by GenScript. All other biochemical reagents, including protease inhibitor cocktail, bovine serum albumin (BSA), and Triton-X100, were purchased from Sigma-Aldrich unless otherwise noted.

Synthesis of PEG-*b*-PDPA block copolymers

The PEG-*b*-PDPA block copolymers were synthesised following the method described by Ruiz-Pérez et al. with minor modifications [24]. The synthesis involved the use of PEG-based macroinitiators (*i.e.*, MeO-PEG blocks of 5 kDa or N₃-PEG blocks of 1.6, 3, or 5 kDa) followed by the polymerisation of DPA through atom transfer radical polymerisation (ATRP) (Figure S1). In brief, MeO-PEG and N₃-PEG derivatives (0.5 mmol, 1 equiv.) were dried under vacuum at 70°C for 30 min and placed under an argon atmosphere. The polymers were dissolved in anhydrous DCM (20 mL) and cooled in an ice bath. To this solution, DBU (2.5 mmol, 5 equiv.) was added. Separately, BIBB (2.5 mmol, 5 equiv.) was dissolved in anhydrous DCM (3 mL) and added dropwise to the reaction mixture over 15 min. The reaction was stirred at room temperature (RT) for 72 h. The resulting PEG-Br macroinitiator was purified by dialysis (MWCO: 1 kDa) against a 1:1 (v/v) N,N-Dimethylformamide (DMF)/water mixture (3 exchanges) followed by exhaustive dialysis against water. The product was isolated by lyophilisation. Successful esterification was confirmed by proton nuclear magnetic resonance (¹H-NMR). PEG-Br macroinitiator (0.4 mmol, 1 equiv.) was dissolved in degassed ethanol (12 mL) under an argon atmosphere. To this solution, DPA (46 mmol, 115 equiv.) and bipyridine (0.8 mmol, 2 equiv.) were added. The mixture was purged with argon for 10 min and equilibrated at 30°C. Polymerisation was initiated by adding Cu(I)Br (0.4 mmol, 1 equiv.) under inert conditions. The reaction was stirred at 30°C for 24 h, with monomer conversion monitored by GPC. To terminate the reaction, the flask was exposed to air to oxidise the residual copper catalyst. The crude mixture was passed through a neutral alumina column and eluted with ethanol to remove copper complexes. The purified solution was dialysed (MWCO:

3.5 kDa) against DCM/methanol (1:1 v/v; 3 exchanges), followed by methanol (2 exchanges) and water. The final PEG-*b*-PDPA block copolymers were isolated by lyophilisation and characterised by ¹H-NMR and GPC (Table 1, Figures S2–S4).

Conjugation of PEG-*b*-PDPA block copolymers

The N₃-PEG-*b*-PDPA copolymers derivatives were conjugated to propargylglycine-bearing T7 peptide (Pra-HAIIY-PRH) or alkyne-Cy_{5.5} via copper(I)-catalysed azide–alkyne cycloaddition (CuAAC, Figure S5), as previously described [14], with minor modifications. Briefly, N₃-PEG-*b*-PDPA (1 equiv.) was dissolved in degassed THF, followed by the addition of Pra-T7 or alkyne-Cy_{5.5} (1.3 equiv.), sodium ascorbate (5 equiv.), and copper (II) sulfate pentahydrate (1 equiv.). The reaction mixture was diluted with degassed water and THF to achieve a final solvent ratio of 1:9 (v/v) water:THF and a polymer concentration of 12 mg mL^{−1}. The reaction was stirred under an argon atmosphere at RT for 72 h. After completion, water was removed using anhydrous sodium sulfate, and the crude product was purified by column chromatography (neutral alumina, THF eluent). The solvent was evaporated under vacuum to yield the conjugated polymer. The degree of functionalisation was quantified by reverse-phase (RP)-HPLC (Section "Determination of T7 in conjugated block-copolymers").

Polymer characterisation

¹H-NMR spectroscopy

For sample preparation, 5 mg of the polymer was solubilised in 0.6 mL of 99.8% deuterated chloroform containing 1% (v/v) tetramethylsilane (TMS). ¹H-NMR spectra were acquired through 32 scans on a Bruker AVANCE NEO 400 MHz spectrometer, with chemical shifts reported in parts per million (ppm) (Figures S2–S3).

Table 1 Molecular weight characterisation of PEG-*b*-PDPA block copolymers

Polymer code	Polymer	Mn (g/mol) ^a	Mn (g/mol) ^b	Mw (g/mol) ^b	PDI ^b	H _{ratio}
MeO-PEG _{5k}	MeO-PEG ₁₁₇ -PDPA ₈₈	24072	24147	28902	1.20	85%
N ₃ -PEG _{5k}	N ₃ -PEG ₁₁₂ -PDPA ₁₂₃	31318	31877	38679	1.21	89%
N ₃ -PEG _{3k}	N ₃ -PEG ₆₅ -PDPA ₁₀₃	24990	25392	29426	1.16	91%
N ₃ -PEG _{1.6k}	N ₃ -PEG ₃₅ -PDPA ₁₁₀	25161	25134	27806	1.11	95%

Mn, number-average molecular weight; Mw, weight-average molecular weight; PDI, polydispersity index; H_{ratio}, calculated as the mass percentage of the hydrophobic PDPA block relative to the total polymer mass

^aDetermined by ¹H-NMR

^bDetermined by GPC

Gel permeation chromatography

For GPC analysis, 5 mg of polymer sample was solubilised in 1 mL of GPC-grade THF, and the solution was filtered through a 0.22 μm PTFE syringe filter. Samples were run at 30°C with a flow rate of 1 mL min⁻¹ using GPC-grade THF as the eluent. The experiments were performed on an Agilent 1260 Infinity II instrument equipped with a PLgel 5 μm MIXED-C 300 $\times$ 7.5 mm column, using a triple-detector system comprising a dual-angle light scattering detector, a refractive index detector, and a viscometer. Data analysis employed the triple detection method to determine the number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity index (PDI).

Determination of T7 in conjugated block-copolymers

The conjugation of T7 to block-copolymers was characterised by RP-HPLC. In brief, T7-conjugated block copolymer and its N₃-functionalised counterpart were dissolved at 1 mg mL⁻¹ in 0.1 M acetate buffer, pH 4. After complete dissolution, the polymer solutions were filtered through a 0.22 μm PTFE syringe filter, and 50 μL of the filtered solution was injected into a PLRP-S 300 Å, 4.6 $\times$ 250 mm, 10 μm (PL1512-5101, Agilent) column. HPLC-MS grade MeCN acidified with 0.05% (v/v) TFA (a-MeCN) and ultrapure water acidified with 0.065% (v/v) TFA (a-H₂O) were fluxed as mobile phases at 1 mL min⁻¹, linearly increasing the amount of a-MeCN in the mixture from 5% to 65% in 25 min. The column was then purged for 2 min at 95% a-MeCN and reconditioned to 5% a-MeCN for 8 min. T7 peptide was quantified from the fluorescence of the aromatic amino acid tyrosine (Ex 276 nm, Em: 310 nm), as shown in Figure S6. Free T7 eluted at ~8.9 min while the T7-polymer conjugates eluted between 15.5 and 19 min. The degree of conjugation, D_{conj} (%), represents the molar ratio of conjugated T7 peptide to total polymer chains, calculated as follows:

$$D_{\text{conj}} (\%) = \frac{n_{\text{conj}} M_0}{m - n_{\text{conj}} M_{\text{T7}}} \cdot 100, \quad (1)$$

Where n_{conj} is the detected moles of conjugated peptide, M_0 is the molecular weight of the N₃-polymer, m is the injected mass, and M_{T7} is the molecular weight of the T7 peptide.

The residual free peptide (%T7_{free}) represents the molar ratio of unconjugated (free) T7 peptide to total T7 peptide (conjugated + free), calculated as:

$$\%T7_{\text{free}} = \frac{n_{\text{free}}}{n_{\text{conj}} + n_{\text{free}}} \cdot 100, \quad (2)$$

Where n_{free} is the detected moles of free peptide.

T7-conjugated polymersomes

The PEG-*b*-PDPA block copolymers were self-assembled into polymersomes using a solvent-switch method [13]. Briefly, a mixture of pristine MeO-PEG_{5k}, 10 mol% Cy_{5.5}-PEG_{3k}, and 2-10 mol% T7-PEG_{1.6k}, T7-PEG_{3k}, or T7-PEG_{5k} was dissolved in a mixture of THF, methanol, and dimethyl sulfoxide (DMSO) (8:1:1) to a final polymer concentration of 20 mg mL⁻¹. Pristine polymersomes, *i.e.*, lacking the T7 peptide, were prepared using 90 and 10 mol% of MeO-PEG_{5k} and Cy_{5.5}-PEG_{3k}, respectively. For self-assembly, 2.3 mL of Milli-Q water (70% v/v) was injected into 1 mL of polymer mix solution (30% v/v) at a flow rate of 0.1 mL min⁻¹ under magnetic stirring (500 rpm) using a syringe pump (New Era Pump Systems, Inc.). The polymersome dispersion was dialysed (MWCO: 8 kDa) against phosphate buffer saline (PBS) for 24 h, with 3-4 buffer exchanges. The resulting polymersomes were purified by centrifugation at 1000 $\times$ g, followed by filtration through a 0.22 μm polyethersulfone syringe filter, before quantification by HPLC. Sterile polymersome dispersions were stored at 4°C and protected from light exposure until further use.

Polymersome characterisation

Dynamic light scattering (DLS)

Polymersome size distribution and surface charge were assessed by DLS using a Zetasizer Ultra instrument (Malvern Panalytical, UK) operating with a 633 nm He-Ne laser and detecting scattered light at a 173° backscatter angle. Before analysis, polymersome dispersions in PBS (pH 7.4) were diluted 1:9 in water (adjusted to pH 7.4). Measurements were performed in triplicate at RT, within a disposable folded capillary cell. Size distribution measurements were done first, followed by zeta potential analysis. Intensity-weighted distributions were measured by the instrument, and number-weighted distributions were subsequently derived by Zetasizer software via its standard Mie-theory-based transformation, employing refractive indices of 1.59 for the polymersomes and 1.33 for the dispersant (water), while assuming spherical, optically homogeneous particles. PDI provided a measure of size heterogeneity, with values below 0.2 indicating a monodisperse population. To ensure data quality, a fluorescent filter was used to prevent interference with the sample's fluorescence.

Transmission electron microscopy (TEM)

Polymersome samples (1 mg mL⁻¹ in PBS, pH 7.4) were adsorbed onto 300-mesh carbon film grids (EM Resolution) that had been pre-treated with glow discharge (30 sec,

15–20 mA) to enhance their hydrophilicity. After 1 minute of adsorption, excess liquid was blotted, and the grids were negatively stained with Uranyless for 30 seconds. The excess of staining solution was blotted and air-dried overnight at RT. Morphology was analysed by TEM (JEOL JEM-1010, Japan) operated at 100 kV, with images acquired using a CCD Megaview 1k × 1k camera.

Calculation of ligand insertion parameters

For each family of formulations, the ligand insertion parameter δ [10] was defined as the ratio between the length of the peptide-conjugated PEG-tether and that of the pristine MeO-PEG_{5k} brush. The resulting values were $\delta=1$ for T7-PEG_{5k}, $\delta=0.6$ for T7-PEG_{3k}, and $\delta=0.3$ for T7-PEG_{1.6k}.

Estimation of ligand surface density

The aggregation number (N_{agg}), *i.e.*, the number of block copolymer molecules forming an individual polymersome, was estimated from a semiempirical structural model based on DLS size data. The average number of ligands per polymersome (N_L) was then calculated for each diameter within the size distribution, as: $N_L = 0.5 \cdot X_L \cdot N_{\text{agg}}$ where X_L is the actual molar fraction of peptide-conjugated polymer molecules introduced into the self-assembly polymer solution. This calculation assumed that only half of the peptide-conjugated polymer molecules integrated within the membrane are oriented with the ligand facing outwards. After averaging N_L over the size distribution, the ligand surface density (σ_L) was calculated as:

$$\sigma_L = \frac{N_L}{4\pi R^2} \quad (3)$$

where R is the average radius of each polymersome formulation. The resulting σ_L and N_L values for all formulations are summarised in Table S1.

Cell culture conditions

bEnd3, NIH 3T3, and B16-F10 cell lines were cultured in complete medium, composed of DMEM high glucose (4.5 g L⁻¹ D-glucose) supplemented with 10% (v/v) FBS and 100 U mL⁻¹ penicillin-streptomycin (1% v/v). Cells were maintained in a humidified incubator at 37°C under 5% CO₂ and were routinely passaged every 3–4 days upon reaching 80–90% confluence. All experiments were conducted using cells between passages 8 and 20 to ensure phenotypic stability. For the subculture, cells were washed twice with PBS lacking calcium and magnesium, then incubated with 0.25% (v/v) trypsin-EDTA solution for 3 min at 37°C.

Trypsinisation was stopped by adding complete culture medium. The cell suspension was centrifuged at 1000 × g for 5 min; the supernatant was then aspirated, and the pellet was resuspended in fresh complete medium. Cells were typically seeded at a density of 50,000 cells cm⁻², unless otherwise stated. All cell lines were routinely tested for mycoplasma contamination.

TfR expression analysis

The relative expression levels of mouse TfR in bEnd3 cells were quantified using a sandwich ELISA protocol. Two fibroblast cell lines, NIH 3T3 and B16-F10, were used as comparative samples. In brief, cells were seeded in rat tail collagen I-coated 6-well plates and cultured for 72 h in complete medium under standard conditions (37°C, 5% CO₂). Upon reaching confluency, cells were washed twice with ice-cold PBS and lysed using Cell Lysis Buffer containing 1:10 protease inhibitor cocktail. Lysates were clarified by centrifugation at 13,000 × g for 10 min at 4°C, and supernatants were aliquoted and stored at -80°C until analysis. Total protein concentration was determined in triplicate using the Quick Start™ Bradford Protein Assay (Bio-Rad) with BSA standards. For ELISA-based TfR levels quantification, recombinant TfR standards and diluted cell lysates were loaded in duplicate onto antibody-coated plates, with bEnd3 lysates loaded at 1 µg of total protein and fibroblast lysates at 5 µg or 10 µg of total protein for B16-F10 and NIH 3T3 cells, respectively, to account for expected expression differences. Following sequential incubations with detection antibody (1 h, RT), horseradish peroxidase conjugate (30 min at RT, light-protected), and TMB substrate (15 min), reactions were stopped with Stop Solution. Absorbance was measured at 450 nm in a microplate reader. Raw TfR concentrations (ng mL⁻¹) were derived from the standard curve and normalised to total protein loaded to calculate relative expression levels (ng TfR µg⁻¹ of total protein).

Metabolic activity assay

The effects of T7-functionalised polymersomes on the metabolic activity of bEnd3 cells were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, which measures mitochondrial dehydrogenase activity in viable cells. bEnd3 cells were seeded in 96-well plates and allowed to adhere for 24 h before polymersome treatment. Cells were exposed to serially diluted polymersome formulations (concentration range: 3–100 µM) in complete medium for an additional 24 h under standard culture conditions. Untreated cells were maintained in fresh medium as positive controls for cell viability. For toxicity evaluation, only pristine polymersomes (unmodified) and

those conjugated with the highest T7 ligand density were assayed, as these represent the extremes of potential biological impact. Following treatment, the medium was carefully aspirated and replaced with 100 μL of MTT working solution (0.5 mg mL^{-1} in PBS). After 2 h of incubation at 37°C, the MTT solution was removed without disturbing the formed formazan crystals. The crystals were solubilised in 50 μL of anhydrous DMSO with gentle agitation to ensure complete dissolution. Absorbance was measured at 570 nm using a microplate reader, with 630 nm as the reference wavelength to correct for nonspecific absorption. Cell viability was normalised to untreated control cells and reported as mean $\pm$ standard deviation (SD).

In vitro BBB targeting and internalisation

The binding and internalisation of T7-conjugated polymersomes were evaluated using confluent monolayers of bEnd3 cells cultured in collagen I-coated 96-well plates. Cells were allowed to form tight monolayers over a 3-day incubation period before experimentation. For uptake studies, the culture medium was replaced with polymersome dispersions prepared in phenol red-free complete medium at a concentration of 1.25×10^9 polymersomes per mL, and plates were maintained at 37°C under 5% CO_2 atmosphere. Supernatant samples were collected at 5 min of exposure by carefully withdrawing 100 μL aliquots from each well, which were transferred to black opaque 96-well plates. The decrease in supernatant fluorescence intensity (measured at Ex: 666 nm and Em: 731 nm) served as an indicator of nanoparticle association with cells, with time-zero measurements representing the initial concentration of polymersomes. After collection, cells were washed 3 times with PBS to remove unbound particles, then stained with Hoechst 33342 (1 $\mu\text{g mL}^{-1}$ in PBS) for 20 min at 37°C to enable cell number quantification by nuclear fluorescence.

For comprehensive cell uptake analysis, the number of polymersomes (N_p) associated with cells (*i.e.*, bound or uptaken) was determined by subtracting the N_p remaining in the supernatant from the initial polymersome dose. A calibration curve of known polymersome concentrations was used to convert fluorescence values into absolute particle numbers. Specific binding of T7-functionalised polymersomes was determined by subtracting nonspecific binding measured using pristine polymersomes (no T7 ligand) as a control. Finally, the number of bound/internalised T7-polymersomes was normalised to the initial polymersome dose administered and displayed as the percentage of the initial dose (% ID) per 10^5 cells. Eight replicates were performed per condition across three independent biological experiments.

In vitro BBB model

To establish an in vitro BBB model, bEnd3 cells were seeded at a density of 50,000 cells cm^{-2} onto rat tail collagen I and fibronectin-coated transwell inserts (0.1 mg mL^{-1} in PBS). To enhance cell adhesion and monolayer integrity, transwell inserts underwent an additional coating step with bovine plasma fibronectin solution (30 $\mu\text{g mL}^{-1}$) for 2 h at 37°C. Cells were cultured in complete medium. At 3 days in vitro, the medium was renewed, and the basolateral medium was replaced with serum-free culture medium. Cells were cultured under these conditions for an additional 3 days to achieve complete confluency and facilitate tight junction expression. Barrier integrity was assessed by measuring trans-endothelial electrical resistance (TEER) using an EVOM3 volt-ohmmeter (World Precision Instruments Ltd) equipped with STX2 electrodes. Before measurements, electrodes were sterilised in ethanol, thoroughly rinsed, and calibrated for accuracy. Due to TEER's temperature sensitivity, plates were equilibrated to measurement conditions before recording. During assessment, electrodes were held perpendicular to the monolayer, ensuring complete submersion without disturbing the cell membrane. TEER values ($\Omega \cdot \text{cm}^2$) were calculated by subtracting the resistance of blank (cell-free) inserts from experimental measurements and multiplying by the transwell surface area. This approach ensured reproducible quantification of endothelial barrier tightness, validating the model's suitability for permeability and transport studies.

In vitro BBB transcytosis

Before conducting polymersome transport studies, the functional integrity of the in vitro BBB model was further validated through quantitative apparent permeability (P_{app}) measurements using fluorescent dextran tracers. Two molecular weight markers were employed to assess paracellular permeability: 3–5 kDa fluorescein isothiocyanate (FITC)-dextran for small molecule transport, and 65–85 kDa tetramethylrhodamine isothiocyanate (TRITC)-dextran for macromolecular barrier function. The permeability assay was performed by applying 100 μL of dextran solution (1 mg mL^{-1} in phenol red-free DMEM) to the apical chamber of pre-equilibrated transwell inserts, with 800 μL of fresh medium in the basolateral compartment to establish a physiological sink condition. To ensure temporal resolution of transport kinetics, 100 μL samples were collected from the basolateral chamber at precisely timed intervals (15, 30, 45, 60, and 90 min) and transferred to black 96-well plates for subsequent fluorometric analysis. Following each sampling time point, an equal volume of fresh medium was added to maintain constant hydrostatic pressure and

prevent volume-associated artefacts. Fluorescence intensity measurements were performed using a Spark Multimode microplate reader with excitation/emission wavelengths of 485/535 nm for FITC and 557/576 nm for TRITC, respectively. Background correction was applied using blank samples from control inserts without dextran. All experiments were conducted in triplicate at 37°C under a 5% CO₂ humidified atmosphere to maintain physiological conditions. Fluorescence intensity measurements were translated into concentration values based on the corresponding standard curves, which were then used to determine the P_{app} values using the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{V}{AC_0}, \quad (4)$$

where dQ/dt represents the transport rate calculated as the gradient of mass over time, V the receptor volume (cm³), A the total membrane surface area (cm²), and C_0 the initial donor concentration.

To ensure valid interpretation of polymersome transcytosis, only cell monolayers demonstrating appropriate barrier integrity were used for subsequent polymersome transport studies. The transcytosis capability of T7-conjugated polymersomes across the in vitro BBB was quantitatively assessed by measuring their P_{app} coefficient, as described for dextran tracers. The polymersome concentration loaded per Transwell was $6.25 \times 10^9 N_p \text{ mL}^{-1}$.

Immunocytochemistry (ICC) for tight junction protein expression

ICC staining was performed on confluent bEnd3 cell monolayers grown on transwell insert membranes to assess the localisation and expression of tight junction proteins. Briefly, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at RT, followed by three washes with PBS to remove residual fixative. Permeabilisation was conducted using 0.1% (v/v) Triton X-100 in PBS for 10 min at RT. Non-specific binding sites were blocked with 10% (v/v) normal goat serum in PBS for 10 min at RT. Primary antibodies against ZO-1, occludin, and claudin-5 were diluted in 1% (w/v) BSA and incubated overnight at 4°C. Cells were washed thrice in PBS and incubated with species-matched Alexa Fluor 488 secondary antibodies for 1 h at RT, protected from light. Nuclei were counterstained with Hoechst 33342 (1 µg mL⁻¹ in PBS) for 10 min, followed by three final PBS washes. Samples were mounted in coverslips using Fluoromount reagent and allowed to cure overnight in the dark before imaging.

Lentiviral transduction for stable expression of mCherry-PACSIN2 in bEnd3

The Syndapin2-pmCherryC1 fusion construct was custom-cloned into lentiviral particles by GenScript's Lentivirus service, which included vector packaging and titre quantification. The pre-packaged lentivirus particles were used directly for transduction. For transduction, bEnd3 cells were seeded at a density of 15,000 cells cm⁻² in collagen I-coated 6-well plates and allowed to adhere overnight under standard culture conditions. Before transduction, cells were pre-treated for 30 min with complete growth medium supplemented with 5 µg mL⁻¹ Polybrene to reduce electrostatic repulsion between lentiviral particles and cell membranes. Lentiviral particles were then added at a multiplicity of infection (MOI) of 3, corresponding to approximately 432,000 infectious units per well. To enhance transduction efficiency, plates were briefly centrifuged at 800 × g for 30 sec at RT immediately following viral addition, promoting close contact between viral particles and target cells. Cells were maintained in lentivirus-containing medium for 24 h under standard incubation conditions. After this period, the supernatant was carefully aspirated, and cells were gently washed once with PBS to remove residual lentiviral particles. Fresh complete medium was added, and the cells were cultured for an additional 48 h to allow for robust transgene expression. To select stably transduced populations, cells were treated with 2 µg mL⁻¹ puromycin for 48 h, with medium replacement every 24 h. Successful transduction and stable expression of mCherry-PACSIN2 were confirmed by fluorescence microscopy, with the mCherry signal serving as a visual reporter of PACSIN2 fusion protein expression.

Confocal microscopy and imaging

For live-cell imaging, mCherry-PACSIN2-expressing bEnd3 cells were cultured on collagen I-coated µ-Slide 18-well chamber slides (IBIDI) for three days to ensure monolayer formation. T7-functionalised PEG-PDPA polymersomes were diluted to a final concentration of 200 µg mL⁻¹ in pre-warmed complete medium and added to the cells immediately before imaging. Imaging was performed using a Zeiss LSM 800 confocal microscope equipped with diode lasers at 405, 488, 561, and 633 nm. Sequential scanning was employed to minimise fluorophore cross-talk and spectral bleed-through. For live-cell acquisition, a stage-top incubation chamber maintained at 37°C with 5% CO₂ was equilibrated for at least 1 h before imaging. High-resolution images were acquired using 63× and 100× oil-immersion objectives under both live and fixed-cell conditions.

Statistical analysis

Quantitative data were analysed to determine the statistical significance of differences between experimental groups and controls. Normality of data distributions was assessed using the Shapiro-Wilk test ($\alpha=0.05$). For normally distributed data, ordinary one-way analysis of variance (ANOVA) was performed, followed by Dunnett's multiple comparison post hoc test. Non-normally distributed data were analysed using the Kruskal-Wallis test, with uncorrected Dunn's test for post hoc testing. All data are presented as mean $\pm$ SD unless otherwise indicated. Statistical significance levels are denoted as follows: * $p<0.05$, ** $p<0.01$.

Results and discussion

Multivalent T7-polymersomes for controlled BBB targeting

The foundation of our study lies in the rational design of BBB-targeted nanocarriers that leverage both the biological specificity of TfR-mediated BBB transport and the physicochemical advantages of multivalent nanocarriers for phenotypic targeting. TfR has been reported to be highly expressed on BECs and is therefore commonly explored as a target for drug delivery to the brain. However, using

natural transferrin as a targeting ligand has been challenging due to competition from endogenous transferrin in the bloodstream [25]. As an alternative, the synthetic peptide T7, identified through biopanning, selectively binds to TfR at a distinct site, thereby avoiding this competition [7]. By conjugating the T7 peptide to pH-responsive PEG-*b*-PDPA copolymers, we designed a library of polymersomes with precisely defined T7 peptide densities (σ_L), thereby establishing a framework for BBB phenotypic targeting. Furthermore, we systematically varied the PEG length (1.6, 3, or 5 kDa), thereby tuning the T7 insertion ratio (δ) within the polymersome brush and modulating the balance between ligand-receptor attraction and steric repulsion. This approach mimics biological recognition systems, in which both binding energy and spatial confinement govern specificity – a principle we refer to as avidity-by-design.

To this end, we synthesised PEG-*b*-PDPA block copolymers via ATRP polymerisation to achieve excellent control over the polymerisation process, a critical factor for obtaining reproducible nanocarrier behaviour. We confirmed the success of the synthesis through a combination of $^1\text{H-NMR}$ and GPC analysis. Structural validation by $^1\text{H-NMR}$ showed characteristic peaks for both PEG (3.7–3.3 ppm) and PDPA blocks (3.9, 3.0, and 2.6 ppm), and integral values consistent with the desired degree of polymerisation (Figure S2 and S3). GPC analysis revealed monomodal distributions with well-defined molecular weights and a $\text{PDI}\leq 1.2$ (Table 1). Across all block copolymers, the hydrophobic PDPA block accounts for more than 80% of the total polymer mass, a well-established prerequisite for the spontaneous formation of stable bilayer vesicle membranes [26, 27] (Table 1).

We conjugated a T7 peptide to azide-terminated copolymers via CuAAC, achieving $D_{\text{conj}}\geq 55\%$ across all polymer batches (Table 2). To perform this quantification, we applied RP-HPLC so that the conjugated T7 peptide could be entirely separated from residual unconjugated peptide, as shown for the three polymers in Figure S6. This analysis was crucial for ensuring precise control of the T7 peptide density on the surface of polymersomes.

By mixing different ratios of pristine (*i.e.*, unconjugated) and conjugated copolymers, we generated a library of polymersomes via a bottom-up 'solvent switch' protocol, with variable σ_L for each insertion ratio δ studied (0.3, 0.6, and 1.0) (Fig. 1a–c and Table S1). An alternative strategy not explored in this study would be to present T7 on a longer linker than the surrounding PEG corona. While such a design could further reduce steric hindrance and enhance receptor engagement, greater ligand exposure may also increase susceptibility to protein corona masking and enzymatic degradation in biological fluids. For this reason, the present work focused on systematically varying ligand insertion depth (δ) within a fixed PEG corona length.

Table 2 Molecular weights and conjugation efficiency of targeted PEG-*b*-PDPA block copolymers

Polymer code	Polymer	R-alkyne	Mn (g/mol) ^a	$D_{\text{conj}}(\%)^b$	%T7 _{free} ^c
T7-PEG _{5k}	T7-PEG ₁₁₂ -PDPA ₁₂₃	Pra-HAIY-PRH	32306	55 $\pm$ 2.4	16 $\pm$ 2.8
T7-PEG _{3k}	T7-PEG ₆₅ -PDPA ₁₀₃	Pra-HAIY-PRH	25978	66 $\pm$ 2.0	26 $\pm$ 1.6
T7-PEG _{1.6k}	T7-PEG ₃₅ -PDPA ₁₁₀	Pra-HAIY-PRH	26149	64 $\pm$ 2.0	15 $\pm$ 1.8
Cy _{5.5} -PEG _{3k}	Cy _{5.5} -PEG ₆₅ -PDPA ₁₀₃	Alkyne-Cy _{5.5}	25646	ND ^d	ND ^d

^aMn (number-average molecular weight) determined by summing the Mn of the polymer, as measured by $^1\text{H-NMR}$, and the molecular weight (Mw) of the R-alkyne molecule

^bDegree of conjugation, calculated as the molar ratio of conjugated T7 peptide to total polymer chains (Eq. 1 in section "Determination of T7 in conjugated block-copolymers"), as determined by HPLC

^cMolar ratio of unconjugated T7 peptide to total T7 peptide (Eq. 2 in section "Determination of T7 in conjugated block-copolymers"), as determined by HPLC. Values ^b and ^c are calculated with respect to different denominators and are, therefore, not directly complementary

^dND = Not Determined

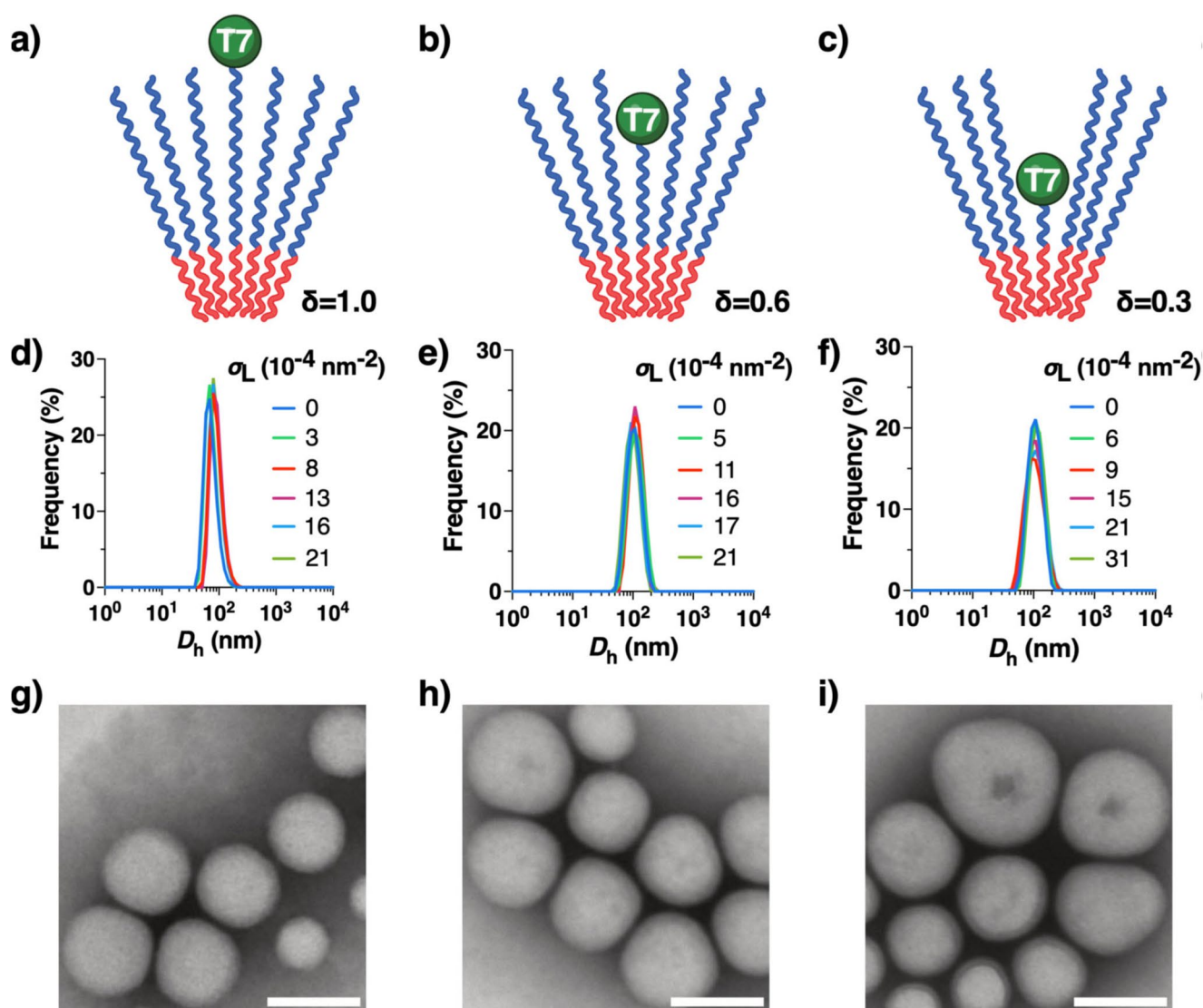


Fig. 1 Characterisation of T7-functionalised polymersomes with varying ligand insertion ratios ($\delta = 1.0$, 0.6 , and 0.3). **a–c** Schematic representation of polymersomes with $\delta = 1.0$ (**a**), 0.6 (**b**), and 0.3 (**c**). **d–f** Size distribution by number, showing hydrodynamic diameter (D_h)

profiles. **g–i** Representative TEM images confirming morphological integrity across formulations ($\delta = 1.0$, 0.6 , and 0.3 , respectively). Scale bars: 100 nm

Nevertheless, comparing the δ -modulation strategy with an extended-linker design represents a valuable direction for future optimisation of targeted nanocarriers under physiologically relevant conditions.

DLS analysis shows the monotonically decreasing auto-correlation functions with flat baselines at long lag times (Figure S7a–c), confirming that the dispersions are unimodal and colloidally stable. Moreover, the size distributions indicate minimal variability in the hydrodynamic diameters (D_h) across the different formulations, with an average D_h of 80 ± 7 nm (Fig. 1d–f). In addition, the ζ -potential measurements indicate a neutral surface charge conferred by the dense PEG brush, which is essential to minimise non-specific protein adsorption and thus enhance bioavailability

in *in vivo* applications (Figure S7g–i). Finally, we confirmed the expected spherical vesicular morphology by TEM imaging (Fig. 1g–i), which revealed that the geometric sizes were consistent with the DLS distributions.

T7-Polymersomes exhibit selective BEC binding and efficient internalisation

To validate TfR targeting, we first quantified TfR expression in bEnd3 cells, confirming that, consistent with their brain endothelial origin, these cells exhibited ~ 7.5 -fold and ~ 4.5 -fold higher TfR levels than NIH 3T3 and B16-F10 fibroblast cells, respectively ($p < 0.01$, Fig. 2a). Before functional studies, we confirmed the biocompatibility of the

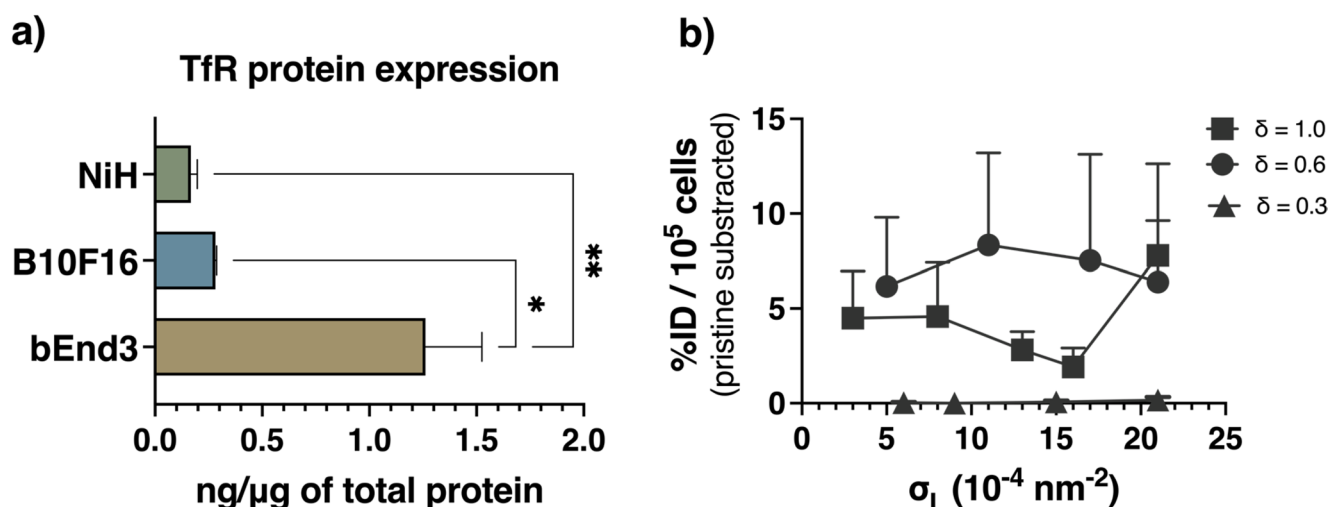


Fig. 2 Transferrin receptor (TfR) expression and nanoparticle binding as a function of ligand insertion ratio (δ). **a** Quantification of TfR protein levels by ELISA in three cell lines: NIH 3T3 fibroblasts, B16-F10 melanoma cells, and brain endothelial bEnd3 cells. Data are expressed as ng TfR per μ g total protein (mean $\pm$ SD, $n=2$) (** $p < 0.01$, one-way ANOVA). **b** Binding of T7-functionalised PEG-*b*-PDPA polymersomes

to bEnd3 cells, measured 5 minutes after incubation. The percentage of internalised and membrane-bound nanoparticles (% of injected dose (ID) per 10^5 cells), normalised by the pristine formulations, is plotted versus ligand surface density σ_L (10^{-4} nm^{-2}). Three insertion depths were tested: $\delta = 1.0$ (squares), $\delta = 0.6$ (circles), and $\delta = 0.3$ (triangles). (mean $\pm$ SEM, $N=3$ for $\delta = 1.0$ and 0.6 , $N=1$ for $\delta = 0.3$)

polymersome formulation groups using the MTT assay. No significant reduction in metabolic activity was observed in bEnd3 cells after 24 h of exposure to T7-polymersomes (up to $100 \mu\text{g mL}^{-1}$; $p > 0.05$ vs. untreated controls (Figure S8), confirming the safety profile of the platform for therapeutic applications.

For a specific receptor, both the type of ligand and its spatial characteristics (*e.g.*, density, arrangement, and multivalency) are considered crucial factors in modulating receptor binding and behaviour, and consequently, biological function [28, 29]. The most-studied spatial characteristic is σ_L . A higher σ_L indicates a more compact inter-ligand distribution on the nanoparticle surface, which can induce targeted receptor clustering and alter cellular responses [30–32]. However, higher σ_L can also lead to steric hindrance from closely packed ligands, disrupting ligand-receptor interactions. Another important spatial characteristic is ligand accessibility and orientation. Proper ligand presentation is essential for effective receptor engagement, as misoriented or buried ligands may fail to bind efficiently, reducing targeting efficacy. The choice of linker chemistry and conjugation methods is critical for ensuring optimal ligand exposure and binding affinity. Additionally, the spatial arrangement of multiple ligand types on a single nanoparticle (homo- and hetero-multivalent displays) can enable synergistic receptor activation, allowing for more precise control over downstream signalling pathways. These spatial multi-characteristics of ligands on a nanoparticle synergistically affect overall targeting efficacy and should therefore be considered to maximise target delivery efficiency. To investigate how the spatial presentation of T7 ligands affects polymersome

interactions with BECs, we measured the binding of T7-functionalised PEG-*b*-PDPA polymersomes to bEnd3 cells as a function of δ and σ_L . Binding was assessed five minutes after incubation and expressed as a percentage of the initial dose (%ID) per 10^5 cells, normalised to pristine (non-functionalised) formulations (Fig. 2b). When the ligands were fully exposed above the PEG corona ($\delta = 1.0$, squares), binding oscillated between 2% and 8% ID across all densities tested, indicating that exposed ligands are accessible for receptor engagement. However, the variability among replicates and the absence of a direct dependence on σ_L suggest potential receptor saturation or limitations on the number of effective binding events within the short incubation time. At intermediate insertion ratio ($\delta = 0.6$, circles), the ligands are partially embedded within the PEG brush. Binding levels were slightly higher and more uniform across all σ_L than those observed for $\delta = 1.0$. This suggests that partial insertion likely reflects a critical balance between sufficient ligand accessibility for TfR engagement and enough PEG shielding to reduce steric interference, potentially improving ligand orientation. The lack of increase in %ID with increasing σ_L supports the interpretation that T7 ligand exposure, rather than density alone, governs productive binding interactions in this regime. To further explore this weak σ_L dependence in binding, we estimated the average spacing between T7 ligands (Table S1) and compared it to the estimated average TfR spacing on bEnd3 cells ($\approx 68 \text{ nm}$). Across all three insertion ratios, the T7 ligand spacings range from 63 nm at the lowest density level down to 19 nm at the highest density, bridging from just below to well below the average baseline inter-receptor distance. Because TfR can diffuse

laterally within the fluid plasma membrane to form high-density domains, it is progressively recruited to the polymersome contact zone, facilitating multivalent engagement upon initial docking. Consequently, further increasing the σ_L within this tightly packed regime does not proportionally enhance binding, as the system has already reached a binding plateau (Fig. 2b). Lower ligand densities (spacings > 63 nm) would likely reveal a more pronounced dependence on σ_L ; however, such formulations approach the technical limits for reliable ligand quantification. In contrast to $\delta=1.0$ and 0.6, when ligands were deeply inserted within the PEG corona ($\delta=0.3$, triangles), their accessibility to the extracellular environment would be minimal. Accordingly, binding was lowest in this condition and also did not increase with higher σ_L . These results are consistent with extensive steric hindrance by the PEG chains, which can prevent efficient interaction with cell surface receptors. Given their inability to specifically engage with cells, $\delta=0.3$ formulations were excluded from further biological characterisation to focus on functionally active variants ($\delta=1.0$ and 0.6). Collectively, these findings show that the spatial presentation of targeting ligands, as defined by δ , is a crucial determinant of nanoparticle-cell interactions. Only partially or fully exposed ligands translate into enhanced binding.

Ligand exposure dictates transcytosis versus endocytic retention

Having established that ligand insertion depth critically influences binding efficiency, we next evaluated whether these binding patterns translated into functional transcytosis across the brain endothelial barrier or into intracellular retention within BECs' cytoplasm. Before evaluating polymersome transcytosis, we first characterised our in vitro BBB model using three complementary metrics: (i) ICC analysis confirmed robust expression of tight junction proteins (ZO-1, occludin and claudin-5) at the cell membrane, demonstrating proper intercellular junction formation (Fig. 3a-c); (ii) transepithelial electrical resistance measurements consistently indicated functional barrier integrity (Fig. 3d); and (iii) permeability assays using 3-5 and 65-85 kDa dextrans revealed minimal paracellular leakage (Fig. 3e), further validating the model's integrity for transcytosis studies.

With the model validated, we quantified polymersome transcytosis to determine how ligand presentation influences BBB trafficking efficiency. Polymersomes with fully exposed ligands ($\delta=1.0$) exhibited efficient transcytosis at σ_L above $7 \times 10^{-4} \text{ nm}^{-2}$, surpassing the baseline permeability of pristine (non-targeted) nanoparticles (Fig. 3f). This suggests that maximal σ_L facilitates subsequent receptor-mediated trafficking pathways required for transcellular transport. A significantly higher rate of transcytosis was observed at the

highest σ_L . In contrast, polymersomes with partially inserted ligands ($\delta=0.6$) only achieved detectable transcytosis at the highest σ_L tested ($>17 \times 10^{-4} \text{ nm}^{-2}$) (Fig. 3g), demonstrating that while partial PEG embedding supports binding by optimising ligand orientation (Fig. 2b), it may simultaneously restrict receptor clustering or signalling dynamics required to initiate efficient transcytosis. Together, our results show that while both polymersome batches bound BECs effectively, only those with exposed ligands achieved transcytosis. We attribute this to differential receptor clustering dynamics: partially-immersed ligands likely promote stable receptor clustering and subsequent lysosomal sorting (*i.e.*, endocytic retention), whereas exposed ligands enable transient interactions compatible with transcellular sorting.

Building on our previous demonstration of PACSIN2's essential role in tubular carrier formation for fast transcytosis of LRP1 ligands across BECs [13, 22], we investigated whether PACSIN2 similarly contributes to TfR-targeted polymersome transcytosis. PACSIN2 is a F-BAR (FERCIP4 homology–Bin–Amphiphysin–Rvs) domain protein that senses and/or induces positive membrane curvature to promote tubular carrier formation [33]. Its molecular architecture comprises both an F-BAR domain for membrane interaction and an SH3 domain that binds to dynamin-2 and the WASP/Scar family proteins, thereby regulating actin dynamics and vesicle formation [34]. Given its established function in membrane tubulation and dynamin-2 recruitment for vesicle fission, PACSIN-2 could theoretically facilitate T7 trafficking. While recent studies have shown that monovalent anti-TfR antibodies undergo transcytosis via tubular structures in bEnd3 cells [35], the specific molecular machinery involved remains undefined. To examine the potential role of PACSIN2 in T7-polymersome trafficking, we employed bEnd3 cells stably expressing mCherry-tagged PACSIN2 to qualitatively analyse potential colocalisation between PACSIN2 and internalised T7-polymersomes using high-resolution confocal microscopy. For this, we focused our analysis on the T7 ($\sigma_L=21$, $\delta=1.0$) PEG-*b*-PDPA polymersome formulation, which demonstrated high apparent permeability in our transport assays (Fig. 3f). Live-cell imaging revealed initial nanoparticle-cell surface interactions within 15 minutes (Supplementary Movie S1), though significant internalisation occurred only after 1 h (Fig. 4a-c). Yet, no co-localisation of T7-polymersomes and PACSIN2-positive tubules was observed at any time point examined. These findings stand in stark contrast to our previous observations with LRP1-targeted systems [13], suggesting two important conclusions. First, TfR-mediated transcytosis of these T7-functionalised polymersomes likely occurs through a PACSIN2-independent pathway. While direct evidence of the precise endosomal route remains to be established, the most plausible alternative is a clathrin-dependent

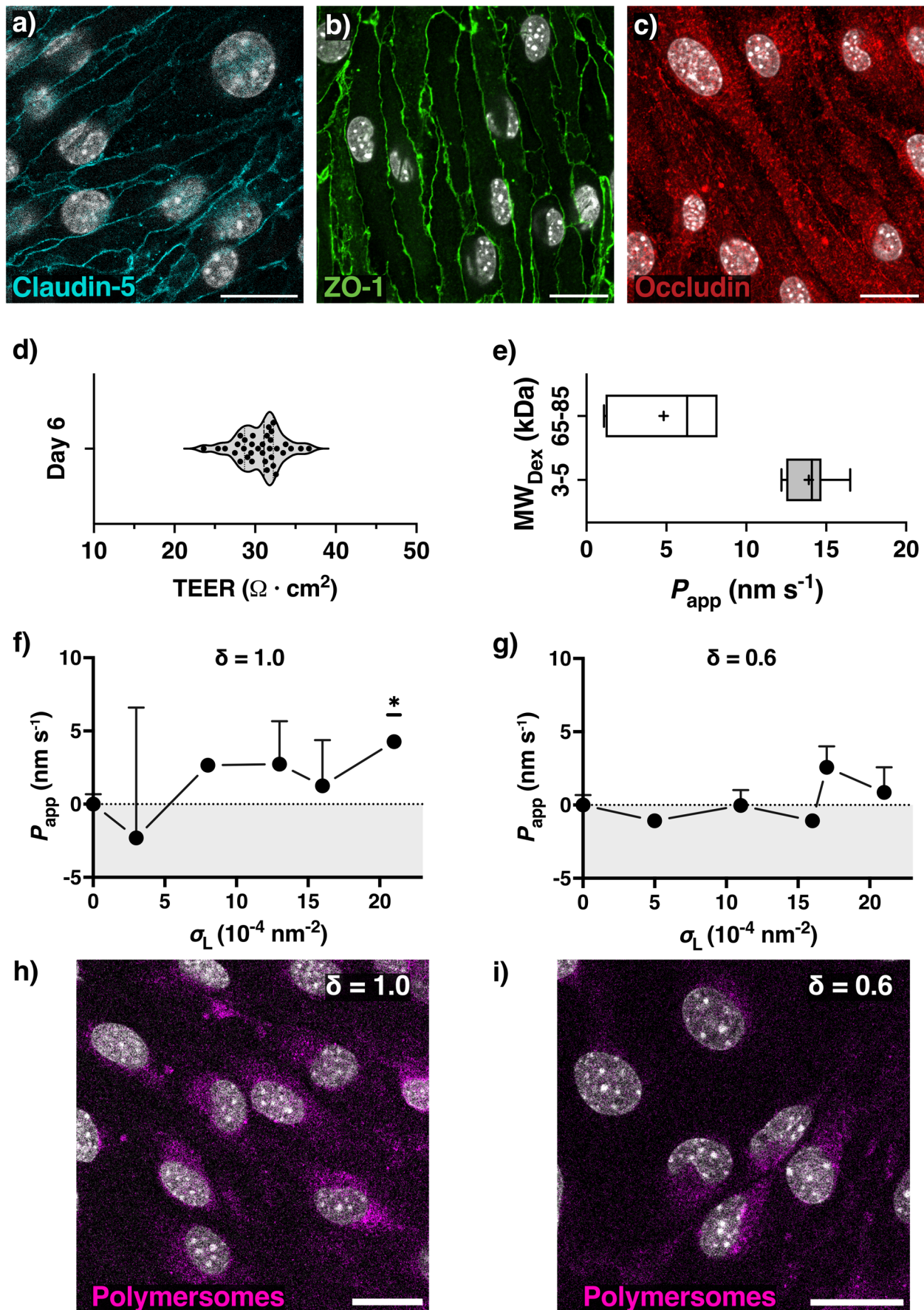


Fig. 3 In vitro BBB transcytosis of T7-conjugated polymersome. **a–c** Confocal microscopy images demonstrating expression and localisation of tight junction proteins in confluent bEnd3 monolayers: (**a**) Claudin-5 (cyan), (**b**) ZO-1 (green), and (**c**) Occludin (red). Nuclei were counterstained with Hoechst (grey). Scale bars: 20 μm . **d** Quantitative assessment of barrier integrity showing TEER values at 6 days in vitro. **e** Apparent permeability coefficients (P_{app}) for FITC-dextran (3–5 kDa) and TRITC-dextran (65–85 kDa). **f, g** P_{app} for T7-functionalised polymersomes with $\delta=1.0$ (**f**) and $\delta=0.6$ (**g**). The non-specific TfR transport was removed by subtracting the P_{app} values of pristine polymersomes ($n=3$). * $p<0.05$, Kruskal-Wallis test did not show significant overall differences, but exploratory pairwise comparisons using uncorrected Dunn's test suggested a potential difference between pristine polymersomes and T7-polymersomes ($\sigma_L=21$, $\delta=1.0$). **h, i** Confocal microscopy images of T7-functionalised PEG-*b*-PDPA polymersomes (magenta) with (**h**) $\sigma_L=21$, $\delta=1.0$ and (**i**) $\sigma_L=17$, $\delta=0.6$, demonstrating nanoparticle internalisation after 2 h incubation in the P_{app} assay. Scale bars: 20 μm

vesicular transcytosis pathway involving sequential engagement of Rab5-, Rab7-, and Rab11-positive endosomes. This canonical TfR route operates via small vesicles rather than PACSIN2-dependent tubules. Second, these findings reinforce the paradigm that distinct receptor systems at the BBB engage distinct intracellular trafficking machinery for nanoparticle transcytosis, yet ultimately facilitate similar transport outcomes across the endothelial barrier. The mechanistic divergence between LRP1 and TfR trafficking pathways has important implications for the rational design of receptor-targeted nanocarriers, suggesting that optimal surface functionalisation strategies may need to be tailored to the specific transcytosis pathways associated with each target receptor. This work lays the foundation for investigating the specific intracellular sorting pathways underlying differential nanoparticle trafficking, as well as for in vivo validation of these design principles. These results significantly advance our understanding of how nanocarrier surface architecture influences BBB transport mechanisms, providing concrete design guidelines for future neurotherapeutic development.

Therapeutic implications: a modular platform for BBB modulation

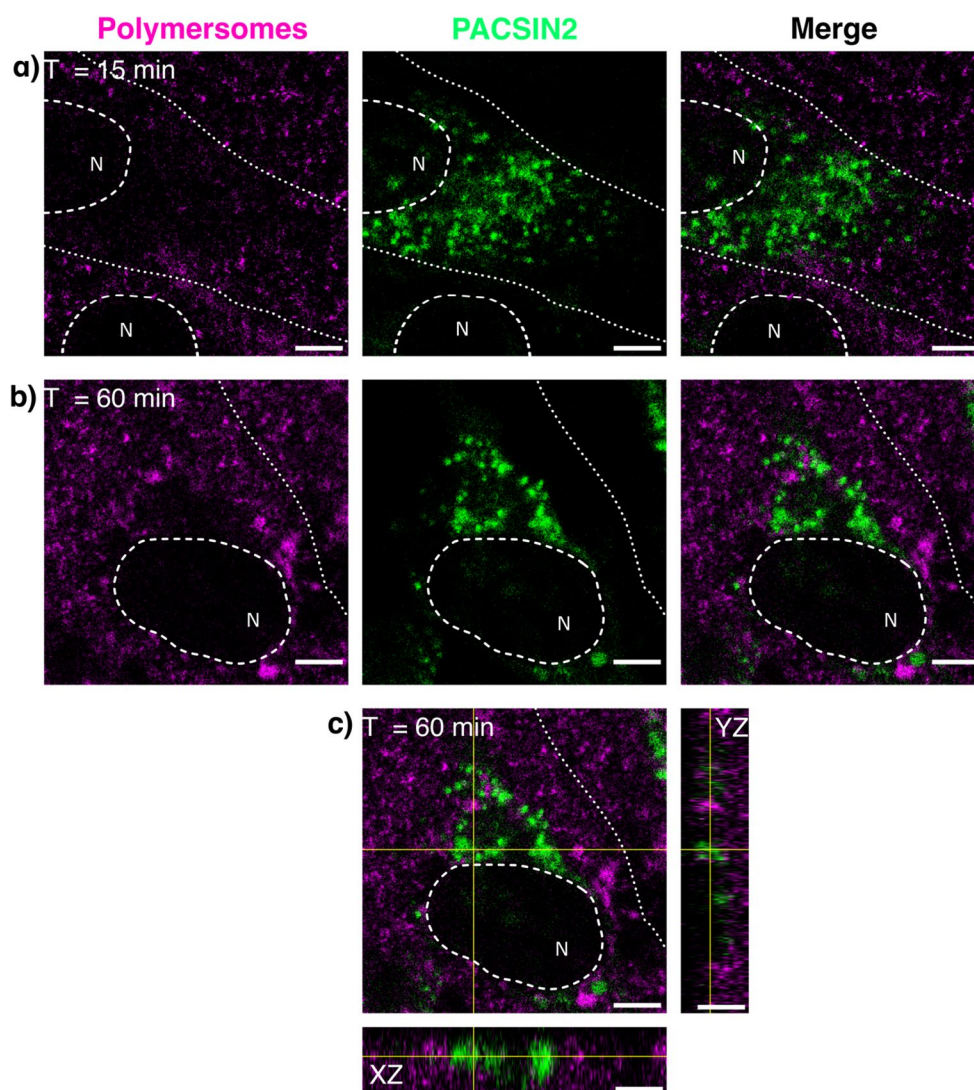
The findings presented in this work establish a framework for rationally designing nanotherapeutics with tailored interactions with the BBB by systematically controlling ligand presentation and density. Our results demonstrate that subtle modifications in nanocarrier architecture, particularly the spatial arrangement and stoichiometry of targeting ligands, can precisely dictate cellular fate, enabling two distinct therapeutic strategies: i) targeting brain endothelial cells for barrier modulation, and ii) transcytosis-enabled drug delivery to the brain parenchyma. The partially embedded T7 ligand placement ($\delta=0.6$ configuration) promotes prolonged endothelial engagement and lysosomal accumulation. While

this design proves suboptimal for transcytosis, it may be strategically repurposed for modulating BBB function. For instance, nanoparticles engineered for endothelial retention could deliver therapeutics to restore key barrier properties, such as enhancing amyloid- β clearance in Alzheimer's disease by upregulating efflux transporters or degrading receptors like RAGE. They could also deliver anti-inflammatory agents to brain endothelial cells to mitigate neurovascular dysfunction in neuroinflammatory disorders, all while avoiding unintended parenchymal exposure. Conversely, the $\delta=1.0$ design achieves efficient transcytosis by leveraging dynamic receptor interactions that evade lysosomal trapping. This architecture is ideally suited for delivering neural therapeutics, such as enzyme replacements, gene therapies, or neuromodulators, that require direct access to brain parenchyma targets. The lack of PACSIN2 involvement in TfR-mediated transport further suggests that this pathway favours conventional vesicular trafficking over tubular transcytosis, potentially offering greater predictability for drug-delivery applications.

Conclusion

By exploiting multivalency as a tunable parameter, we have developed a BBB-targeting platform that transcends the traditional “cross or block” dichotomy. Our study demonstrates that T7-functionalised PEG-*b*-PDPA polymersomes achieve precise targeting of BECs through TfR-mediated interactions, with ligand arrangement and density acting as tunable parameters that dictate their intracellular fate and enable the achievement of specific therapeutic objectives (endocytic retention or transcytosis into the brain parenchyma), all while preserving barrier function. The ability to switch between intracellular retention and transcytosis-competent behaviours within the same nanoparticle system underscores the potential for precision medicine in neurological disorders. Future work should explore hybrid designs that combine both strategies. By decoupling targeting efficiency from intracellular trafficking outcomes, this approach moves beyond empirical optimisation toward mechanism-driven nanocarrier engineering for the brain.

Fig. 4 Live-cell imaging analysis of T7 ($\sigma_L=21$, $\delta=1.0$) PEG-*b*-PDPA polymersomes (magenta) and PACSIN2 (green) co-localisation in bEnd3 cells. **a** Initial polymersome binding to the cell surface was detectable within 15 min. **b** Robust internalisation occurred by 1 h post-incubation. **c** High-resolution imaging revealed nanoparticle-containing vesicles distinct from PACSIN2-positive signal, confirming the absence of co-localisation. N: nucleus. Dashed lines: nuclear boundary. Dotted lines: cell border. Scale bars: 5 μ m



Supplementary information The online version contains supplementary material available at <https://doi.org/10.1007/s13346-026-02163-0>.

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Author contributions CDFL and MB performed material preparation, data collection, and analysis. The first draft of the manuscript was written by CDFL, MB, VB, and VC, and all authors commented on previous versions. All authors read and approved the final manuscript.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate This study was performed using commercially available mouse cell lines, and no ethical approval or consent to participate is required.

Consent to publish This study did not involve human participants, human data, or human tissue. Therefore, consent for publication was not required.

Conflict of interests The authors declare no competing interests.

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