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How can targeted protein functionalisation improve transport of RNA therapeutics across the BBB for treating neurodegenerative diseases?

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ABSTRACT

Neurodegenerative diseases remain among the leading causes of disability worldwide, yet effective disease-modifying therapies capable of slowing or halting disease progression remain largely unavailable. RNA therapeutics have emerged as a promising disease-modifying modality through their ability to selectively target the molecular mechanisms underlying neurodegeneration; however, their clinical application is severely constrained by the blood–brain barrier (BBB), which restricts efficient delivery to the central nervous system. Consequently, considerable effort has been directed towards developing delivery platforms capable of overcoming this barrier. Among these, protein functionalisation of nanoparticles has emerged as a particularly promising approach by enabling receptor-mediated or adsorptive transport across the BBB. This review critically evaluates current protein functionalisation strategies for BBB-targeted RNA delivery, comparing endogenous receptor-targeting ligands, engineered targeting peptides, receptor-independent approaches, and covalent versus non-covalent conjugation methods using a mechanistic framework encompassing targeting, intracellular trafficking, transcytosis, and therapeutic delivery. Comparative analysis indicates that lactoferrin currently represents the strongest evidence-supported targeting ligand owing to its comprehensive mechanistic validation and consistently superior quantitative delivery, whereas rabies virus glycoprotein-derived peptides demonstrate the greatest targeting specificity and long-term potential through neuron-selective delivery. Covalent conjugation emerged as the preferred functionalisation strategy by providing superior control over ligand stability, orientation, and surface architecture, enabling more predictable BBB transport than non-covalent approaches. However, clinical translation remains limited by heterogeneous preclinical methodologies, incomplete understanding of intracellular trafficking, and manufacturing challenges. Establishing standardised evaluation frameworks, advancing mechanistic characterisation, and developing rationally engineered multifunctional nanocarriers will be essential for enabling the clinical translation of BBB-targeted RNA therapeutics.

INTRODUCTION

Neurodegenerative diseases, particularly Alzheimer's disease (AD) and Parkinson's disease (PD) constitute a growing global health burden with substantial medical, social, and economic implications.[1] In 2019, Parkinson's disease affected over 8.5 million individuals worldwide and contributed to approximately 329,000 deaths and 5.8 million disability-adjusted life years (DALYs), a measure that combines years of life lost due to premature death and years lived with disability. [2,3] Similarly, Alzheimer's disease, the leading cause of dementia, currently accounts for an estimated 37 million cases worldwide and has become the seventh leading cause of death globally. [4] Crucially, beyond mortality, these disorders display progressive neurodegeneration, requiring decades-long increases in care burden. Consequently, patients often require prolonged multidisciplinary care, placing substantial financial and caregiving demands on families and healthcare systems. With increasing life expectancy and ageing populations across many regions, the prevalence of Parkinson's and dementia is expected to rise substantially in the coming decades, further amplifying its societal and economic impact.[4]

Unfortunately the molecular mechanisms which underlie these disorders is heavily disputed, with theories suggesting a complex interplay of ageing, genetic susceptibility, and environmental factors.[5] Critically, the pathological processes underlying neurodegenerative diseases often begin years before the onset of overt clinical symptoms, allowing substantial and irreversible neuronal damage to accumulate before diagnosis. Consequently, current treatments remain largely symptomatic and are unable to halt disease progression. For example, current standard-of-care Alzheimer's treatments, including acetylcholinesterase inhibitors such as galantamine, donepezil, and rivastigmine, can provide symptomatic improvements in cognition and daily functioning by enhancing cholinergic neurotransmission. However, they do not halt the underlying neurodegenerative processes driving disease progression. In contrast, Parkinson's disease therapies primarily focus on dopamine replacement or dopaminergic stimulation to alleviate motor symptoms, but they do not prevent the ongoing loss of dopaminergic neurons that underlies disease progression.[5] Consequently, there is a major unmet need for disease-modifying therapies that target the underlying molecular drivers of these disorders.[5]

Contrastingly, conditions such as Huntington's disease, C9orf72-linked frontotemporal dementia (FTD), Familial Alzheimer's disease, and parkin-associated parkinsonism are linked to identifiable genetic or molecular abnormalities that contribute to disease pathogenesis. [5] The presence of defined molecular drivers makes these disorders attractive candidates for therapeutic approaches that intervene upstream of neuronal dysfunction by modulating pathogenic gene expression. Here, RNA-based therapeutics, particularly small interfering RNAs (siRNAs) and microRNAs (miRNAs), have emerged as a promising strategy because they enable precise, sequence-specific regulation of gene expression. [5] Through selective silencing of mutant genes, reduction of toxic protein production, and modulation of dysregulated cellular pathways, these approaches have the potential to target neurodegenerative disease molecular mechanisms rather than solely manage clinical symptoms. The therapeutic potential of RNA-based approaches in this context is supported by advances in other disease areas, where antisense oligonucleotide (ASO), siRNA, and miRNA therapies have successfully progressed through clinical development. Notable examples

include the siRNA therapeutic ALN-TTR02 for transthyretin-mediated amyloidosis and the miRNA-targeting drug miravirsen for hepatitis C virus infection. [6] These studies demonstrate that RNA-based therapeutics can achieve clinically meaningful gene modulation in humans, providing proof-of-concept for the application of gene-silencing strategies to neurodegenerative diseases with well-defined molecular targets.

However, the widespread application of RNA-based therapeutics in neurodegenerative disease therapeutics faces significant obstacles. Principally, RNA molecules are intrinsically unstable. This results in susceptibility to rapid nuclease-mediated degradation, and limited membrane permeability due to size and charge, severely restricting effective systemic delivery [7]. In addition, central nervous system (CNS) therapeutics must pass the Blood Brain Barrier (BBB), a highly selective and tightly regulated endothelial interface. The BBB maintains central nervous system homeostasis by restricting the entry of peripheral immune cells, pathogens, and circulating macromolecules, thereby preserving the specialised microenvironment required for normal neuronal function. The BBB prevents the passage of most macromolecules larger than approximately 400–500 Da, including proteins, antibodies, and RNA therapeutics such as siRNAs and antisense oligonucleotides. [7] Consequently, only small lipophilic molecules (e.g., O_2 and CO_2) diffuse freely. This presents a major obstacle for RNA therapeutics in neurodegenerative diseases, which require an effective delivery system to reach the brain. Larger or charged macromolecules are excluded unless transported via highly specific carrier- or receptor-mediated mechanisms, such as those required for glucose, amino acids, or insulin [8]

Preserving BBB integrity is essential because disruption of this highly selective barrier permits uncontrolled entry of circulating toxins, pathogens and inflammatory mediators into the CNS, potentially exacerbating neuronal injury and disease progression.[9] Hence, any viable RNA delivery platform must simultaneously achieve three interdependent objectives: protection of the RNA cargo from degradation, maintenance of sufficient circulation time within the bloodstream to allow delivery to target tissues, and efficient, targeted transport across the BBB. Achieving all three in combination has proven to be a persistent and unresolved challenge. Here, nanoparticle-based delivery systems, such as liposomes, polymeric nanoparticles, and inorganic nanoparticles, have emerged as a leading strategy to overcome these constraints because they protect therapeutic cargo, prolong systemic circulation, and enable targeted delivery while improving stability in biological environments. [10,11] Recent evidence has demonstrated significant potential from validation in peripheral drug delivery applications.[6,12] Encouragingly, these nanoparticles are also being actively investigated for targeted brain delivery, as with the addition of specific surface functionalisation, they may enable transport across the blood–brain barrier (BBB) without disrupting its structure. [9]

Although nanoparticles can be functionalized with numerous classes of targeting ligands, including antibodies, aptamers, peptides, and small molecules, protein-based functionalization strategies have emerged as particularly promising because they can directly exploit endogenous receptor systems responsible for molecular transport across the BBB. Protein and protein derived ligands such as transferrin[13,14], lactoferrin[15], apolipoprotein E[14,16], insulin[14], and engineered peptides such as rabies virus

glycoprotein (RVG)[17] have been used to engage receptor-mediated transcytosis. These are incorporated into nanocarriers using functionalisation methods including covalent conjugation[18,19], non-covalent adsorption (protein corona / surface adsorption approaches)[18–20] . However, both the choice of protein ligand and the functionalisation strategy used to attach it can significantly influence BBB recognition, receptor-mediated uptake, and the overall transport efficiency of RNA-loaded nanoparticles.[21]

Accordingly, this narrative review examines how both protein functionalisation strategies and attachment methodologies influence RNA transport across the BBB, stability during systemic circulation, and functional efficacy following delivery. The review focuses on identifying the key constraints limiting clinical translation and highlighting future directions for protein functionalisation and attachment research.

The literature search was conducted primarily through Google Scholar, using combinations of terms relating to the blood–brain barrier (BBB), RNA therapeutics, nanoparticle delivery, protein functionalisation, and neurodegenerative disease, supplemented by ligand-specific searches for transferrin, lactoferrin, ApoE, insulin, RVG, LMWP, and CBSA. The evidence base was centred on peer-reviewed primary research published during the preceding decade, while earlier studies were retained where they established foundational mechanisms or provided baseline data against which subsequent advances could be evaluated. Relevant review articles and citation networks were also examined to identify seminal studies and trace the development of individual targeting strategies. Particular weight was given to studies offering quantitative evidence or mechanistic insight into receptor recognition, cellular trafficking, transcytosis, brain accumulation, and therapeutic efficacy. Studies lacking a substantive connection to BBB transport or CNS delivery were not considered. Where the literature yielded multiple investigations of the same targeting approach, emphasis was placed on studies offering greater mechanistic resolution, robust quantitative characterisation, or evidence extending beyond BBB crossing to functional therapeutic outcomes. This selection framework provided a consistent basis for evaluating how protein functionalisation influences the progression from receptor engagement to effective CNS delivery.

PROTEIN-BASED FUNCTIONALISATION FOR BBB TRAFFICKING

General mechanism

To overcome the challenge of the BBB which restricts delivery into the CNS, protein functionalisation strategies have been developed to enable active nanoparticle targeting and transit across the BBB through receptor-mediated transcytosis (RMT), a physiological transport mechanism involving ligand–receptor binding on brain capillary endothelial cells (BCECs), receptor-mediated endocytosis, and subsequent vesicular trafficking across the endothelial layer. The efficiency of protein-mediated BBB transport is governed by several interrelated biological and physicochemical factors that dictate nanoparticle uptake behaviour in three key processes: targeting, intracellular trafficking, and CNS release.

First, receptor expression and accessibility on the luminal surface of brain capillary endothelial cells strongly influence nanoparticle targeting, as receptors with high abundance provide a greater number of binding sites for circulating ligands. Endogenous ligand competition further affects transport efficiency.[22–26]

Following receptor engagement, intracellular trafficking behaviour becomes a critical determinant of delivery success. Here, protein–receptor affinity must also be carefully balanced; while sufficient affinity is required to promote endothelial binding and internalisation, excessively strong interactions may result in prolonged intracellular retention or lysosomal trafficking rather than productive transcytosis and release into the brain parenchyma.[22,23] However, transport efficiency is also influenced by cargo size and physicochemical properties, as excessively large or complex constructs may undergo less efficient receptor-mediated transcytosis and exhibit reduced release into brain tissue following endothelial uptake.[22,23]

Following successful transcytosis, nanoparticles must be efficiently released from the abluminal surface of brain capillary endothelial cells into the brain parenchyma, where the therapeutic cargo becomes available for uptake by target neural cells.[22,23] Effective CNS delivery therefore depends not only on crossing the BBB but also on successful dissociation from endothelial transport pathways, diffusion through the extracellular environment, cellular uptake by neurons or glial cells, and, for RNA therapeutics, efficient intracellular release into the cytoplasm where gene silencing can occur. Consequently, productive BBB transport represents only the first step in achieving functional therapeutic efficacy within the CNS.[22,23]

Meanwhile, In AMT systems, surface charge density, nanoparticle size, and colloidal stability become particularly important determinants of BBB penetration. Collectively, these observations demonstrate that successful BBB transport depends not only on ligand selection but also on receptor biology, intracellular trafficking pathways, cargo characteristics, and nanoparticle physicochemical properties.

Transferrin

The transferrin receptor (TfR) is the most extensively investigated receptor-mediated target for blood–brain barrier (BBB) drug delivery owing to its high expression on brain capillary endothelial cells, its physiological role in iron transport into the central nervous system, and decades of research exploiting this pathway for therapeutic delivery. [22,27] The human BBB possesses a saturable TfR transport system, with isolated human brain capillaries demonstrating specific transferrin binding, supporting its suitability as a receptor-mediated transport pathway for therapeutic delivery.[28] As a result, transferrin-functionalised nanoparticles and TfR-targeting antibodies have been widely explored for the delivery of proteins, nucleic acids, and nanomedicines across the BBB.[22] For example, transferrin-conjugated melittin-loaded iron oxide nanoparticles reduced hippocampal amyloid plaque density by approximately 60% in a 5XFAD Alzheimer's disease mouse model, demonstrating successful therapeutic delivery across the BBB.[29] Compared with passive diffusion, which permits negligible accumulation of most biologics and

nanomedicines within the brain, TfR-mediated transport represents one of the most effective receptor-mediated approaches currently available for enhancing BBB penetration.[22] Consistent with this, transferrin-conjugated nanoparticles were detected within the mouse brain within 10 minutes of intravenous administration and remained detectable after 24 hours before being largely cleared by 48 hours, indicating rapid BBB transport and transient brain retention.[29]

TfR offers several advantages as a targeting receptor, including high endothelial expression and established receptor-mediated transcytosis pathways, which collectively promote efficient nanoparticle binding and endothelial internalisation.[22,27] However, targeting performance is influenced by receptor occupancy and competition with endogenous transferrin, which continuously circulates at relatively high concentrations and may reduce available binding sites for therapeutic carriers.[22] Ligand affinity also plays a critical role; while sufficient receptor affinity is required to promote endothelial uptake, excessively strong TfR binding may negatively affect downstream transport.[22]

Following receptor-mediated endocytosis, successful BBB transport depends on the intracellular fate of the receptor-cargo complex. A major limitation of TfR-targeted systems is endosomal sequestration, whereby receptor–cargo complexes become retained within endosomal or lysosomal compartments rather than being transported across the endothelial cell.[22] Reducing anti-TfR antibody affinity enhances brain exposure by promoting receptor recycling and productive transcytosis rather than intracellular retention, illustrating that moderate receptor affinity can improve overall BBB transport despite weaker receptor binding.[22]

Importantly, increased endothelial uptake should not be interpreted as successful BBB transcytosis, as nanoparticles may accumulate within brain endothelial cells without undergoing productive abluminal transport into the brain parenchyma.[22] Evidence suggests that high-affinity TfR-binding constructs are particularly susceptible to this phenomenon such as OX26-derived antibodies, which showed substantial accumulation within brain endothelial cells but comparatively limited delivery into the brain parenchyma due to intracellular sequestration following receptor-mediated endocytosis.[22] Consequently, optimisation of ligand–receptor interactions represents a key determinant of successful endothelial transit.

Nevertheless, targeted nanocarriers have demonstrated measurable functional benefits *in vitro*. Transferrin-functionalised liposomes carrying vitamin B12 delayed amyloid-β fibrillation by extending the aggregation lag phase approximately 29-fold, reducing the fibril elongation rate by nearly 80%, and decreasing fibril-associated fluorescence by approximately 31% compared with unloaded transferrin liposomes, demonstrating enhanced anti-amyloidogenic activity following transferrin-mediated delivery.[30] However, these findings were obtained using in vitro aggregation assays rather than BBB transport studies, meaning that functional anti-amyloid activity does not directly confirm enhanced brain delivery. Limited abluminal release following transcytosis, receptor saturation, and intracellular trafficking losses can reduce the fraction of administered cargo that ultimately reaches neural tissue.[22,27] Therefore, while TfR remains one of the most extensively validated BBB-targeting strategies, the absolute fraction of administered cargo reaching the brain remains very small, and despite decades of intensive investigation,

clinical translation has remained limited, indicating that further optimisation of intracellular trafficking, transcytosis and post-transcytotic release is still required.[22]

Lactoferrin:

While transferrin receptor (TfR)-targeted systems benefit from high receptor expression at the BBB, their performance is often constrained by competition with endogenous transferrin and the risk of receptor saturation, both of which can reduce effective nanoparticle binding and subsequent transport. Lactoferrin (Lf) has therefore emerged as an attractive alternative owing to the comparatively low endogenous occupancy of lactoferrin receptors, resulting in greater receptor accessibility at the brain endothelium and reduced competitive inhibition.[31] This advantage is supported by receptor binding data demonstrating a high-affinity lactoferrin receptor population (Kd ≈ 6.8 nM), while endogenous plasma lactoferrin concentrations are only ~5 nM, suggesting that a substantial proportion of receptors remain available for therapeutic targeting under physiological conditions.[31]

Relative to transferrin-functionalised systems, Lf-functionalised nanocarriers demonstrated superior in vivo brain targeting, exhibiting approximately 2.2-fold greater brain uptake than an equivalent transferrin nanocarrier, supporting the hypothesis that reduced receptor competition can translate into improved BBB delivery.[31] Consistent with this enhanced targeting capability, Lf modification produced approximately 4.6-fold greater brain-targeting efficiency and 4.9-fold higher transgene expression than unmodified formulations, demonstrating that receptor accessibility can translate into substantially improved functional delivery.[32] However, despite these advantages, targeting performance remains dependent on receptor expression, which may vary under pathological conditions and potentially affect reproducibility across disease states.[31,33,34]

From an intracellular trafficking perspective, Lf-functionalised nanoparticles appear to exploit a broader range of uptake mechanisms than TfR-functionalised nanocarriers, incorporating receptor-mediated, adsorptive-mediated, and macropinocytic pathways.[32] Here, following clathrin- and caveolae-mediated internalisation, Lf-functionalised nanoparticles rapidly enter acidic endolysosomal compartments within 5 minutes and demonstrated partial escape by 30 minutes, reducing intracellular retention and facilitating more efficient endothelial trafficking.[32] As a result, a greater proportion of Lf-functionalised nanoparticles successfully complete transcytosis and reach the brain parenchyma relative to TfR systems that remain susceptible to extensive endosomal trapping.[32] Consistent with this mechanism, Lf-modified vectors and nanoparticles exhibited significantly greater BBB-crossing efficiency than unmodified counterparts, while transport was markedly reduced in the presence of excess free lactoferrin, further confirming receptor-mediated transcytosis as a major contributor to endothelial transit.[32]

The transit advantages associated with Lf targeting translate into improved delivery outcomes. Compared with transferrin-functionalised systems, Lf-functionalised carriers consistently demonstrated superior CNS delivery, including approximately 2.3-fold greater brain gene expression than transferrin-targeted formulations following intravenous administration.[31] Consistent with this principle, increased CNS accumulation following Lf functionalisation has been associated with improved therapeutic efficacy in

multiple neurodegenerative disease models. Repeated administration of Lf-modified nanoparticles encoding GDNF produced sustained neuroprotective effects in rotenone-induced Parkinsonian models, while Lf-functionalised polymersomes carrying S14G-humanin reduced amyloid-β-associated neurotoxicity and cognitive impairment.[33,34] These findings suggest that, relative to transferrin-mediated approaches, Lf functionalisation may achieve more efficient conversion of endothelial uptake into therapeutically meaningful delivery within the brain. Transferrin -targeted nanoparticles often show good receptor binding and endothelial uptake, but a significant fraction may become trapped in endothelial cells or be recycled back into the bloodstream, limiting drug delivery to the brain. Lactoferrin-targeted nanoparticles appear to result in greater amounts of therapeutic cargo reaching neurons, leading to measurable improvements such as neuroprotection and cognitive recovery.

Despite these advantages, translational challenges remain. Notably, receptor-mediated uptake remained partially suppressible by excess free lactoferrin, indicating that transport capacity remains dependent on receptor availability and may therefore be susceptible to pathological alterations in receptor expression.[32] The contribution of adsorptive uptake mechanisms may reduce transport specificity and increase the risk of off-target biodistribution, while scalable ligand conjugation remains difficult owing to challenges associated with controlling ligand density, colloidal stability, and batch-to-batch consistency.[31,33,34] Nevertheless, when evaluated across the key metrics of targeting, transit, and delivery, current evidence suggests that Lf functionalisation may overcome several of the principal limitations associated with TfR-targeted systems, particularly those related to receptor competition and endosomal sequestration.[31–33]

ApoE

While transferrin (TfR)- and lactoferrin (Lf)-based strategies primarily exploit endogenous transport receptors to enhance BBB penetration, ApoE-functionalised nanoparticles target low-density lipoprotein receptor (LDLR) family pathways that are directly implicated in both brain lipid homeostasis and Alzheimer's disease pathogenesis. Unlike TfR-mediated delivery, which depends upon competition with circulating transferrin, ApoE-functionalised nanoparticles exploit physiological LDLR family pathways that normally mediate lipoprotein transport.[25,35]

Compared with TfR-targeted systems, which may experience competition from endogenous transferrin and receptor saturation[22] and Lf-functionalised systems, which benefit from lower endogenous receptor occupancy[31], ApoE-functionalised nanoparticles exploit endogenous lipoprotein transport mechanisms involved in cholesterol redistribution and lipid trafficking within the central nervous system. Experimental studies demonstrate that ApoE functionalisation significantly enhances nanoparticle uptake by BBB endothelial cells, with ApoE-functionalised solid lipid nanoparticles exhibiting approximately 1.8–1.9-fold greater uptake in hCMEC/D3 endothelial monolayers than non-functionalised controls.[36] These findings suggest that ApoE-mediated targeting can effectively promote receptor engagement and endothelial uptake while maintaining favourable biocompatibility, as limited pro-inflammatory immune activation was observed following administration.[37] ApoE141–150-functionalised nanoliposomes increased endothelial uptake by approximately 60% compared with non-functionalised nanoliposomes

while preserving amyloid-β binding [38]. mApoE-functionalised solid lipid nanoparticles increased BBB permeability and accumulated within the brain parenchyma within 30 minutes following administration.[39]

Following receptor-mediated endocytosis, ApoE-functionalised nanoparticles appear to undergo efficient receptor-mediated transcellular transport predominantly through clathrin-mediated pathways. In contrast to TfR-targeted systems, where endosomal sequestration represents a major barrier to productive transcytosis[22], current evidence suggests that ApoE-functionalised nanoparticles undergo efficient receptor-mediated transcellular transport, predominantly through clathrin-mediated pathways.[36] However, unlike Lf-functionalised nanoparticles, whose intracellular trafficking and endosomal escape have been directly characterised[32], ApoE trafficking remains comparatively poorly understood, making its overall transcytotic efficiency less certain. mApoE-functionalised liposomes reached brain concentrations of up to 0.6 μg phosphatidic acid per gram of brain tissue following pulmonary administration, demonstrating that the formulation successfully crossed both the pulmonary epithelium and the BBB in vivo.[40] Similarly, ApoE-derived peptide-functionalised PLGA (poly(lactic-co-glycolic acid) nanoparticles successfully crossed the BBB following intravenous administration and accumulated within the cerebral cortex in vivo.[37]

The transport advantages associated with ApoE targeting translate into improved therapeutic delivery. Importantly, enhanced BBB transport translated into measurable therapeutic benefit in vivo, as repeated administration of mApoE-functionalised liposomes reduced total brain amyloid-β burden by approximately 60% in an Alzheimer's disease mouse model.[40] Consistent with this therapeutic efficacy, liposomes bi-functionalised with phosphatidic acid and a modified ApoE-derived peptide increased BBB permeability approximately five-fold compared with phosphatidic acid-functionalised liposomes, demonstrating a substantial improvement in transendothelial transport efficiency. Furthermore, these liposomes inhibited amyloid-β aggregation by approximately 70% after 72 hours and reduced pre-formed aggregates by approximately 60% after 120 hours, indicating that ApoE functionalisation may provide therapeutic benefits extending beyond BBB transport alone.[41] Surface plasmon resonance additionally demonstrated high-affinity amyloid-β binding (KD ≈ 0.6 μM), indicating that ApoE bi-functionalisation preserves strong interaction with pathological amyloid species while simultaneously improving BBB transport.[41]However, direct quantitative comparisons of brain accumulation remain less extensive than those reported for Lf-functionalised systems, where superior BBB permeability and brain uptake relative to transferrin-targeted carriers have been demonstrated experimentally.[33]

However, this strategy introduces additional formulation complexity, as receptor recognition depends on maintaining appropriate ApoE presentation on the nanoparticle surface, potentially affecting reproducibility between formulations[42]. Consequently, ApoE differs from TfR and Lf in that its therapeutic relevance extends beyond BBB transport alone and may additionally influence disease-associated biological processes.[25,35] Nevertheless, ApoE possesses a distinct advantage not shared by either TfR or Lf targeting approaches: the receptor pathway itself is closely linked to Alzheimer's disease biology. ApoE isoforms influence amyloid-β aggregation and clearance, neuroinflammation, lipid metabolism, synaptic maintenance, mitochondrial function, and neuronal repair.[25,35] In particular, impaired

amyloid-β clearance across the BBB is considered a major contributor to sporadic Alzheimer's disease, suggesting that ApoE-mediated transport pathways may provide both enhanced CNS delivery and interaction with disease-relevant mechanisms.[25,35]

Collectively, ApoE functionalisation occupies a unique position among protein-based BBB targeting strategies by combining receptor-mediated delivery with direct relevance to Alzheimer's disease pathology. Although ApoE-functionalised nanocarriers consistently enhance BBB transport, endothelial uptake and amyloid-β modulation, current evidence does not yet demonstrate the degree of transport optimisation reported for Lf-functionalised systems. Furthermore, ApoE-targeting approaches remain heterogeneous, employing direct protein conjugation, ApoE-derived peptides and indirect surfactant-mediated ApoE adsorption, limiting standardisation and complicating comparisons between nanoparticle platforms.[42] Most studies also remain preclinical, with few direct quantitative comparisons against established targeting ligands, making it difficult to determine whether ApoE provides superior transport efficiency or represents an alternative receptor-targeting strategy.[42,43] Consequently, while ApoE offers distinct therapeutic advantages through its direct involvement in Alzheimer's disease biology, current evidence does not demonstrate superior BBB transport efficiency relative to established TfR- and particularly Lf-functionalised systems.[25,35,42,43]

Insulin

Taking advantage of a similar metabolic signaling cascade to ApoE, insulin receptor (IR)-targeted systems utilise a receptor that plays a fundamental role in metabolic signalling and glucose homeostasis.IR-targeted functionalisation has demonstrated the capacity to enhance BBB uptake through receptor-mediated internalisation pathways. Engineered insulin-fusion proteins undergo receptor-mediated macropinocytosis, while cysteine-modified insulin B-chain variants improve cargo transport, demonstrating that ligand engineering can optimize BBB uptake.[26] Mathematical modelling indicates that BBB transport depends on receptor affinity, binding kinetics, receptor recycling and intracellular trafficking, demonstrating that transport kinetics are more important than affinity alone.[44] Importantly, the translational potential of insulin receptor targeting is supported by the clinical development of BBB “molecular Trojan horse” fusion proteins, including the human insulin receptor antibody–iduronidase construct valanafusp alfa, which has progressed into clinical trials for lysosomal storage disorders, demonstrating that IR-mediated transcytosis can be exploited for therapeutic delivery to the brain.[45] The principal challenge for IR-targeted delivery is therefore not receptor competition but receptor dysfunction. Because the insulin receptor performs essential physiological functions, excessive receptor engagement may interfere with endogenous insulin signalling and increase the risk of off-target metabolic effects.[46] Unlike transferrin receptors, which are almost completely occupied by endogenous transferrin, circulating insulin concentrations (~0.3 nM) remain substantially below the receptor dissociation constant (~2.2 nM). Consequently, endogenous ligand competition is predicted to be considerably lower for IR-targeted systems than for TfR-targeted approaches.[44]

Early proof-of-concept studies demonstrated that insulin itself can function as an effective BBB-targeting ligand. Insulin-conjugated HSA nanoparticles transported the BBB-impermeable drug loperamide into the

brain following intravenous administration, an effect abolished by anti-insulin receptor antibodies, confirming receptor-mediated transport.[47] However, unlike Lf-functionalised nanoparticles, which produced approximately 2.3-fold greater exogenous gene expression in the brain than transferrin-functionalised nanoparticles following intravenous administration, comparable in vivo demonstrations of enhanced genetic cargo delivery remain limited for IR-targeted systems.[48] Unlike many insulin delivery systems that simply demonstrate BBB penetration, insulin-conjugated poly(N-vinyl pyrrolidone) nanogels preserved insulin bioactivity after covalent conjugation, maintaining receptor binding, activating AKT signalling, resisting protease degradation, and successfully crossing an in vitro BBB model, demonstrating that receptor engagement remained functionally intact after nanoparticle functionalisation.[49]

These nanogels maintained receptor binding, activated AKT signalling, and crossed an in vitro BBB model while preserving insulin bioactivity.[49] The available literature provides comparatively limited mechanistic insight into the intracellular trafficking fate of IR-targeted nanoparticles following endothelial uptake. Consistent with this uncertainty, the mechanism underlying enhanced transport has not been fully resolved, with papers suggesting that receptor-mediated transcytosis via endothelial insulin receptors requires further investigation.[50] However, these findings were demonstrated primarily using in vitro BBB models, with no quantitative in vivo pharmacokinetic, biodistribution, or brain accumulation data reported, limiting assessment of whether similar transport efficiencies can be achieved under physiological conditions.[49] The PNPHO nanocarrier also protected insulin from enzymatic degradation, preserving approximately 74% of insulin following aminopeptidase exposure and approximately 85% following insulin-degrading enzyme exposure, compared with substantial degradation of free insulin.[50] Beyond protecting insulin from proteolytic degradation, NG-insulin also reduced amyloid-β-induced neuronal dysfunction following BBB transport, indicating that receptor-mediated delivery translated into measurable neuroprotective activity rather than simply improved endothelial uptake.[49]

The major limitation of IR-mediated delivery emerges at the level of effective therapeutic delivery in disease-relevant populations. IR-targeted delivery may be compromised by the disease process itself. Alzheimer's disease is associated with impaired insulin signalling, and cerebrovascular insulin receptor function progressively declines during disease progression. Because insulin regulates neuronal survival, synaptic plasticity, cerebral glucose metabolism and amyloid-β clearance, progressive disruption of insulin signalling in Alzheimer's disease may impair not only therapeutic response but also the receptor pathway exploited for BBB delivery.[51] Since IR-targeted delivery depends directly on receptor availability, this reduction is likely to proportionally decrease receptor-mediated uptake capacity, potentially limiting delivery precisely in advanced neurodegenerative disease where therapeutic demand is greatest.[52,53] Similarly, in Alzheimer's model studies, mice demonstrated reduced BBB insulin receptor activation associated with increased BACE1 activity and reduced vascular receptor expression, further limiting receptor-mediated transport capacity.[54] Consequently, although IR-targeted systems may successfully enhance delivery in healthy models, disease-associated receptor dysfunction may reduce therapeutic accumulation within the brain precisely in the patient populations most likely to require treatment. Although intranasal insulin improved cognition and reduced cerebral hypometabolism in

Alzheimer's disease, clinical translation remains limited by formulation stability, mucociliary clearance, enzymatic degradation and inconsistent brain deposition.[53,55] Unlike TfR-targeted delivery, for which receptor-mediated transport kinetics have been extensively quantified in vivo, comparable quantitative pharmacokinetic analyses of IR-targeted nanoparticles remain scarce, making optimisation of ligand density, receptor affinity and transport efficiency substantially more difficult.[44]

For RNA therapeutics, these findings suggest that insulin functionalisation could enable receptor-mediated delivery of RNA loaded nanoparticles across the BBB.[45] However, when evaluated against the key metrics of targeting, transit, and delivery, the principal limitations of IR-targeted systems differ from those of previously discussed ligands. IR-targeted approaches are primarily limited by disease-dependent receptor dysfunction and concerns regarding metabolic safety.[46,54] These factors may ultimately reduce the robustness and predictability of IR-mediated delivery in neurodegenerative disease settings. Nevertheless, the preservation of receptor signalling, protease resistance, and demonstrated neuroprotective activity following BBB transport suggest that insulin functionalisation remains a promising platform for delivering biologically active macromolecular therapeutics, including RNA cargoes, provided disease-associated receptor dysfunction and transport kinetics can be effectively addressed.[44,49]

Low Molecular Weight Protamine (LMWP)

In contrast to previously discussed systems, which depend on receptor-mediated uptake and transcytosis, low molecular weight protamine (LMWP) employs a cell-penetrating peptide (CPP)-based mechanism that promotes membrane translocation through physicochemical interactions rather than specific receptor engagement. Specifically, LMWP primarily exploits adsorptive-mediated transcytosis (AMT), a receptor-independent transport mechanism driven by electrostatic interactions between the positively charged nanoparticle surface and negatively charged endothelial membranes. Derived from enzymatically digested protamine, LMWP possesses a high arginine content that confers strong cationic charge and enables uptake through both lipid raft-mediated endocytosis and direct membrane translocation pathways.[56,57] LMWP also improves intracellular diffusion following covalent conjugation to albumin nanoparticles through the Cys34 thiol group, enhancing penetration into brain tissue after BBB transport.[58]

A key benefit of LMWP-mediated uptake is that it is largely independent of receptor expression and occupancy. This receptor-independent mechanism avoids endogenous ligand competition and variability in receptor availability. Nevertheless, overall BBB delivery remains inefficient, with <1% of ligand-functionalised nanoparticles successfully crossing the BBB, illustrating that LMWP does not eliminate the intrinsic transport barriers associated with nanoparticle delivery and that improvements remain incremental rather than absolute.[58] The absence of receptor specificity is also a limitation. Whereas ApoE, TfR, and Lf functionalisation direct nanoparticles towards defined BBB transport pathways,[22,27,31,32,36,37] LMWP relies primarily on electrostatic interactions with cellular membranes, increasing the risk of non-specific uptake and off-target biodistribution.[56,57,59] Consequently, enhanced

permeability does not confer disease-specific targeting, requiring optimisation of peptide density and nanoparticle formulation to minimise off-target accumulation.[58]

Receptor-targeted systems often encounter intracellular trafficking bottlenecks following endothelial uptake. In contrast, LMWP facilitates transport through direct membrane translocation and lipid raft-mediated uptake mechanisms, reducing dependence on receptor-controlled intracellular sorting pathways.[56,57] LMWP was developed to overcome the extremely slow diffusion of macromolecules within brain tissue, improving intracellular penetration following BBB transport.[58] Furthermore, evidence suggests that LMWP promotes transport through neuronal routes following intranasal administration, enabling access to the brain via olfactory and trigeminal pathways rather than relying exclusively on endothelial transcytosis across the BBB.[56]

The transport advantages associated with LMWP translate into measurable improvements in brain accumulation. Following intranasal administration, LMWP-functionalised nanoparticles increased brain exposure across brain regions approximately 2.0- to 2.8-fold greater than those achieved by non-functionalised nanoparticles.[56] For example, LMWP-functionalised albumin nanoparticles increased BBB permeability by approximately seven-fold compared with free temozolomide in the PAMPA-BBB model. Co-encapsulation of curcumin and temozolomide further increased permeability to $>10 \times 10^{-6}$ cm/s compared with 0.21×10^{-6} cm/s and 0.75×10^{-6} cm/s for the free drugs, respectively.[58] In a haloperidol-induced Parkinson's disease mouse model, intranasally administered LMWP/lactoferrin co-functionalised PLGA nanoparticles achieved maximum brain accumulation within 2 h and increased brain endothelial uptake 6.53-fold, improving motor function, reducing oxidative stress, and preserving hippocampal neuronal architecture.[60,61] Importantly, these improvements were achieved without compromising short-term biocompatibility.[61] However, unlike ApoE-targeted or Lf-functionalised nanoparticles that have demonstrated improved CNS biodistribution through receptor-mediated transport[33,34] , the delivery advantages of LMWP derive primarily from enhanced membrane permeability rather than biological targeting specificity.

Although LMWP substantially enhances nanoparticle transit and brain delivery, clinical translation remains limited by off-target biodistribution, optimisation of peptide density, manufacturing reproducibility, and an evidence base that remains almost exclusively preclinical, with no established clinical trials for CNS drug delivery. Consequently, its translational potential remains less well established than receptor-targeted platforms.[56,57,59–61]

Rabies Virus Glycoprotein (RVG)-Derived Peptides

Whereas transferrin (TfR), lactoferrin (Lf), ApoE, and insulin receptor (IR)-targeted systems exploit endogenous transport pathways, RVG-derived peptides represent an engineered targeting strategy designed to enhance CNS selectivity while minimising competition with naturally circulating ligands. By exploiting virus-inspired neuronal recognition mechanisms, RVG-functionalised nanocarriers achieve receptor-mediated uptake through interactions with neuronal receptors, particularly nicotinic acetylcholine receptors (nAChRs), and potentially γ-aminobutyric acid (GABA) receptors expressed on brain

endothelial cells and neurons. [17,62–64]

Unlike transferrin-, lactoferrin-, and LDL receptor-targeted systems, RVG29 provides dual functionality by simultaneously facilitating receptor-mediated BBB transcytosis through nAChRs expressed on brain endothelial cells while directly targeting neuronal nAChRs following BBB passage, thereby coupling BBB transport with neuron-specific delivery rather than relying solely on endothelial receptor recognition.[65]

Compared with TfR-targeted systems, which may be limited by endogenous transferrin competition and receptor saturation[22], or IR-targeted systems whose performance may decline in the presence of receptor dysfunction[46,54], RVG-derived peptides offer the advantage of reduced endogenous ligand competition and enhanced neuronal specificity.[66] This distinguishes RVG from ApoE and Lf functionalisation, which primarily target physiological transport pathways at the BBB.[31,32,36,37] Consequently, RVG-functionalised nanocarriers can achieve highly selective CNS targeting while reducing interference with endogenous transport processes. However, the receptor-binding affinity and trafficking dynamics governing RVG-mediated transport remain incompletely characterised, limiting the ability to predict and optimise delivery efficiency.[17,63,64]

Similar to other receptor-mediated strategies, RVG-functionalised nanoparticles rely on intracellular trafficking processes following endothelial uptake. Unlike Lf-functionalised or TfR-targeted systems, the intracellular fate of RVG-targeted nanocarriers remains less clearly defined. Nevertheless, multiple studies have demonstrated successful receptor-mediated transport of siRNA, miRNA, DNA, and protein cargoes across the BBB, indicating that a substantial proportion of internalised nanoparticles undergo productive transcytosis.[17,63,64]

RVG functionalisation consistently translates receptor engagement into therapeutically relevant CNS delivery. Consistent with these findings, RVG29-functionalised rifampicin-loaded nanoparticles produced approximately three-fold greater brain fluorescence intensity than non-targeted nanoparticles 48 h after intravenous administration, with detectable brain accumulation occurring within 30 min and persisting for at least 48 h, directly demonstrating enhanced BBB targeting and prolonged brain retention compared with non-functionalised carriers.[67] Similarly, RVG-modified nanocarriers successfully delivered BACE1 siRNA across the BBB, producing gene silencing of β-secretase 1 and attenuating Alzheimer's disease progression in preclinical models.[17,63] In APP/PS1 Alzheimer's disease mice, systemic RVG-mediated BACE1 siRNA delivery significantly reduced amyloid plaque burden, suppressed phosphorylated tau protein expression, restored cognitive performance to levels comparable with wild-type controls, and produced no significant adverse effects, demonstrating that enhanced BBB transport translated into meaningful disease modification rather than improved brain accumulation alone.[68]

Comparable therapeutic benefits have been reported in Parkinson's disease and Machado–Joseph disease models, where systemic administration reduced pathogenic protein expression and improved disease-associated outcomes.[17,63] For example, RVG-linked siRNA constructs have been developed to suppress α-synuclein expression by exploiting RVG-mediated neuronal targeting through nicotinic

acetylcholine receptors, providing proof-of-concept for RNA therapeutic delivery in Parkinson's disease models.[69] Unlike ApoE-functionalised systems, whose therapeutic value may partly derive from engagement with disease-relevant biological pathways,[25,35] the principal advantage of RVG lies in its ability to improve targeting specificity and neuronal delivery efficiency across multiple CNS disorders. This targeting capability also extends beyond conventional nanoparticles to engineered exosomes. RVG29-conjugated exosomes carrying BACE1 siRNA and berberine significantly improved cognitive function in Alzheimer's disease models, while miR-206-3p antagomir-loaded mesenchymal stem cell exosomes enhanced neurogenesis, reduced Aβ deposition and improved synaptic plasticity, demonstrating that RVG-mediated targeting can be successfully applied across multiple nanocarrier platforms and therapeutic cargos.[70]

Despite these advantages, several translational challenges remain. Peptide instability, endosomal entrapment, conjugation complexity, and difficulties in large-scale manufacturing may compromise delivery consistency and clinical scalability.[17,63,66] Despite improving several Parkinsonian behavioural measures, RVG-functionalised nanoparticles did not significantly improve all outcomes, with no significant differences observed in total travelled distance, movement speed, or time spent in the centre during the open-field test, indicating that therapeutic efficacy may be endpoint-dependent rather than universally beneficial.[71] Despite promising preclinical findings, RVG-derived therapeutics remain largely restricted to animal studies, with no approved CNS nanomedicines and only early-phase clinical evaluation of engineered exosomes. Major barriers include scalable GMP-compliant production, standardised cargo-loading methods, batch-to-batch reproducibility, and comprehensive long-term safety and immunogenicity assessment, all of which must be addressed before routine clinical application. Translation is further limited by peptide instability, manufacturing complexity, small preclinical cohorts, limited long-term toxicity and pharmacokinetic data, and the lack of robust human efficacy studies.[65,67,69,70]

Cationic Bovine Serum Albumin (CBSA)

Unlike transferrin (TfR), lactoferrin (Lf), ApoE, insulin receptor (IR), and RVG-derived targeting systems, which rely primarily on receptor-mediated recognition,[17,22,27,31,32,36,37,56,62–64] oneCBSA-functionalised nanoparticles exploit AMT. Consequently, CBSA largely avoids limitations associated with receptor competition, saturation, and disease-dependent receptor dysregulation that can affect endogenous transport pathways.[72,73]

Compared with receptor-targeted systems such as TfR, Lf, and ApoE, which derive specificity from ligand–receptor interactions,[22,31,36,37] CBSA promotes endothelial uptake through charge-mediated interactions with anionic endothelial microdomains. Competitive inhibition studies using excess free CBSA confirmed that transport occurs through AMT rather than disruption of BBB integrity or classical receptor-mediated transport mechanisms.[72,73] This receptor independence provides greater robustness against variations in receptor expression, including those observed in pathological conditions such as Alzheimer's disease.[46,54] However, the reduced reliance on specific receptors may also increase off-target interactions and uptake within non-CNS tissues.[74]

Whereas TfR-targeted nanoparticles are frequently limited by endosomal sequestration,[22] and RVG-functionalised systems continue to face uncertainty regarding intracellular trafficking efficiency,[17,63,64] CBSA-functionalised nanoparticles demonstrate efficient intracellular transport following endothelial uptake. In vitro BBB models showed a 7.76-fold increase in nanoparticle permeability relative to non-cationised albumin controls while maintaining unchanged ^14C-sucrose permeability, confirming enhanced transcellular transport without tight junction disruption.[72] Following internalisation, CBSA-functionalised nanoparticles exhibit partial endolysosomal escape and cytoplasmic redistribution, reducing intracellular retention and potentially improving delivery of nucleic acid therapeutics, for which endosomal entrapment remains a major barrier.[75]

The transport advantages of CBSA functionalisation translate into measurable improvements in brain accumulation. Optimised CBSA surface densities produced approximately 2.3-fold higher brain accumulation than non-targeted PEGylated nanoparticles in vivo.[73] Delivery benefits appear even greater in pathological settings; tumour-bearing models demonstrated approximately 17-fold higher brain tumour accumulation relative to controls, reflecting the combined effects of BBB transcytosis and enhanced interactions with tumour-associated anionic glycoproteins.[75] These delivery outcomes compare favourably with many receptor-mediated approaches, where increases in endothelial uptake do not necessarily translate into proportional increases in brain accumulation because of intracellular trafficking losses.[22,37] However, enhanced BBB delivery is accompanied by pharmacokinetic trade-offs. Increasing CBSA density improves BBB permeability but reduces systemic exposure, indicating accelerated clearance from circulation.[73]

Despite its strong transport performance, CBSA remains constrained by systemic delivery challenges.[73,74] Consequently, although CBSA avoids many receptor-associated limitations observed with TfR, Lf, ApoE, IR, and RVG targeting, its clinical utility depends on carefully balancing surface charge, ligand density, circulation time, and off-target biodistribution.

METHODS OF PROTEIN FUNCTIONALISATION

Covalent conjugation

As successful RNA delivery to the brain depends not only on nanoparticle design but also on the stable presentation of BBB-targeting ligands, the method used to attach these ligands has become a major consideration in nanocarrier engineering. Attachment strategies influence ligand retention, receptor accessibility, circulation stability, and ultimately the efficiency of BBB transport. Here, protein functionalisation strategies can be categorised into covalent conjugation and non-covalent adsorption, with distinct functional capabilities associated with each approach. In addition to direct protein functionalisation strategies, surface modifications such as PEGylation are frequently incorporated to improve nanoparticle circulation, stability, and BBB targeting performance through enhanced pharmacokinetic behaviour and ligand presentation. Consequently, the integration of protein functionalisation and complementary surface engineering approaches has become central to the

development of effective BBB-targeted nanocarriers for neurotherapeutic RNA delivery. [18,19,76,77]

Covalent conjugation represents one of the most widely utilized strategies for nanoparticle surface functionalisation due to its strong ligand attachment, controlled surface architecture, and enhanced stability under physiological conditions.[18] Common covalent approaches include carbodiimide-mediated reactions such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) coupling. , maleimide–thiol chemistry, ligand exchange reactions, click chemistry, and gold–thiol interactions, all of which enable stable immobilisation of proteins, peptides, antibodies, or targeting ligands onto nanoparticle surfaces while reducing premature ligand dissociation during systemic circulation.[18,76–78] Covalent attachment provides greater control over ligand density, orientation, and long-term nanoparticle stability, factors that are critical for maintaining receptor accessibility and consistent blood–brain barrier (BBB) targeting efficiency.[18,19,76] Covalent surface engineering additionally modifies nanoparticle physicochemical behaviour, including colloidal stability, circulation half-life, bioavailability, tissue penetration, and resistance to degradation within biological fluids, thereby improving the pharmacological performance of RNA delivery systems intended for neurodegenerative disease treatment.[19,77]

Maleimide-mediated covalent conjugation has demonstrated particular effectiveness in BBB-targeted nanoparticle systems because it enables selective thiol-reactive binding under relatively mild conditions, thereby preserving maximum possible ligand functionality during synthesis. PEG–PLA nanoparticles functionalised with low molecular weight protamine (LMWP) through maleimide–thiol coupling showed significantly enhanced cellular accumulation through both lipid raft-mediated endocytosis and direct membrane translocation pathways without observable cytotoxicity.[56] Quantitatively, intranasal administration of LMWP-functionalised nanoparticles produced substantial increases in brain exposure, with AUC_{0-8} values in the cerebrum, cerebellum, olfactory tract, and olfactory bulb increasing by 2.03-, 2.55-, 2.68-, and 2.82-fold respectively compared with unconjugated nanoparticles.[56] The resulting nanoparticles retained a relatively uniform spherical morphology with particle sizes increasing only slightly after conjugation, highlighting modest impact to the nanoparticle structure. Importantly, the study attributed this enhanced delivery to improved transmucosal transport and peptide-mediated cellular internalisation, illustrating how stable covalent attachment can directly influence transport mechanisms relevant to CNS delivery.[56]

Similarly, covalent conjugation of cationic bovine serum albumin (CBSA) onto pegylated PEG–PLA nanoparticles using maleimide-functional PEG linkers significantly improved nanoparticle uptake by brain capillary endothelial cells relative to unconjugated controls.[79] The conjugation strategy was specifically designed so that maleimide groups protruded beyond the PEG corona, maximising ligand accessibility for absorptive-mediated transcytosis while simultaneously maintaining the stealth properties associated with PEGylation.[79] In vivo fluorescence imaging further demonstrated higher accumulation of CBSA-functionalised nanoparticles within the lateral ventricle, third ventricle, and periventricular brain regions following intravenous administration, while cytotoxicity remained comparable to unconjugated nanoparticles.[79] These findings suggest that covalent attachment not only improves targeting efficiency

but also permits rational spatial presentation of ligands on nanoparticle surfaces, an important consideration because receptor accessibility is highly dependent on steric orientation and surface architecture.[18,79]

Covalent functionalisation further improves nanoparticle retention and reduces instability associated with weakly adsorbed ligands, thereby maintaining targeting efficiency and preventing the premature loss of surface-bound ligands required for receptor recognition and BBB transport, which is particularly important in the bloodstream where competitive protein exchange may otherwise displace targeting molecules. [76,78] Additional studies demonstrated that increasing the density of reactive surface hydroxyl groups through Fenton chemistry, an oxidation process that generates highly reactive hydroxyl radicals through reactions between hydrogen peroxide and iron ions, substantially improved the extent of covalent functionalisation on diamond nanoparticles, enhancing nanoparticle polarity and solubility while enabling higher ligand densities on the nanoparticle surface.[80] High-density ligand presentation may be especially advantageous for BBB targeting because receptor-mediated transcytosis often depends on multivalent receptor interactions and sufficient ligand exposure at the endothelial interface.[24,81]

Despite these advantages, covalent conjugation introduces several limitations that may reduce translational feasibility. The chemical reactions used to covalently attach proteins or peptides to nanoparticle surfaces can alter protein conformation, disrupt receptor-binding domains and reduce biological activity despite improving attachment stability, particularly for targeting ligands whose tertiary structure is essential for receptor recognition at the BBB.[18,76] Furthermore, the multi-step synthesis, purification and surface activation procedures required for covalent functionalisation increase manufacturing complexity while reducing scalability and batch-to-batch reproducibility, making it challenging to maintain consistent ligand densities and nanoparticle characteristics required for predictable biological performance and regulatory approval.[18,76] Consequently, while covalent functionalisation remains one of the most effective approaches for achieving stable and targeted BBB transport, its overall therapeutic success depends on balancing ligand stability with preservation of biological functionality and scalable nanoparticle synthesis.

Non-covalent adsorption

In contrast to covalent conjugation, which relies on permanent chemical bonds to immobilise proteins on nanoparticle surfaces, non-covalent conjugation exploits reversible interactions including electrostatic attraction, hydrogen bonding, hydrophobic interactions, van der Waals forces, and π–π stacking to achieve surface functionalisation.[82] Compared with covalent attachment, non-covalent approaches require simpler fabrication procedures and avoid direct chemical modification of proteins, potentially preserving native protein structure and biological activity.[82] However, these advantages are achieved at the expense of the attachment stability and precise control over ligand orientation and surface presentation that characterise covalent functionalisation.[18,82] Despite this, non-covalent surface functionalisation has been shown to improve nanoparticle bioavailability, tissue penetration, retention, drug loading efficiency, and therapeutic efficacy, properties that are highly relevant for RNA delivery across the BBB.[19]

Whereas covalent conjugation relies on specific reactive functional groups to form stable chemical linkages that permanently immobilise proteins on nanoparticle surfaces, electrostatic adsorption relies on attractive charge interactions between oppositely charged proteins and nanoparticle surfaces. The extent of adsorption is therefore strongly influenced by factors such as surface charge density, protein charge distribution, pH, and ionic strength. Unlike covalent bonds, these electrostatic interactions are reversible and particularly susceptible to disruption under physiological or high-salt conditions, where charge screening can weaken binding forces and promote ligand dissociation, potentially reducing long-term ligand retention and functional stability.[24,82]

In contrast to electrostatic adsorption, hydrophobic interactions facilitate protein association through partitioning of hydrophobic protein domains onto lipophilic nanoparticle surfaces, enabling adsorption driven by hydrophobic compatibility rather than charge-based attraction.[24,82] Unlike covalent attachment, which immobilises proteins at defined surface locations, non-covalent interactions such as hydrophobic forces enable reversible protein association and encapsulation without chemical modification of either the nanoparticle or the protein, allowing dynamic assembly driven by interfacial affinity rather than fixed chemical bonding.[24]

A particularly important distinction from covalent functionalisation is the ability of non-covalently functionalised nanoparticles to dynamically acquire proteins from biological fluids through protein corona formation, whereby proteins spontaneously adsorb onto nanoparticle surfaces following biological exposure.[82] Whereas covalently attached ligands remain largely fixed after administration, proteins within the corona can undergo continuous adsorption, displacement, and exchange processes that modify the biological identity of the nanoparticle over time.[24,82] Because cells primarily interact with the adsorbed protein layer rather than the underlying nanoparticle surface, corona composition can significantly influence biodistribution, cellular uptake, bioavailability, and targeting efficiency.[82] For example, PLGA nanoparticles exposed to cerebrospinal fluid developed coronas enriched in transferrin and transthyretin, resulting in approximately two-fold greater association with neurons and glial cells than plasma-derived coronas.[82] Similarly, ionisable lipid nanoparticles enriched with ApoA-I- and ApoA-II-containing HDL proteins demonstrated enhanced mRNA delivery potency, highlighting how adsorption-mediated protein acquisition can improve nucleic acid delivery performance through biologically favourable corona formation; however, this mechanism also introduces variability and reduced control over nanoparticle identity due to dynamic protein exchange and the absence of stable covalent attachment.[82]

However, because non-covalent attachment relies on relatively weak intermolecular forces, adsorbed proteins remain more susceptible to dissociation, competitive exchange, and displacement than covalently immobilised ligands.[24,82] This contrasts with covalent functionalisation, where strong chemical bonding can prevent the displacement of targeting proteins by serum proteins following in vivo administration or purification procedures.[24] Consequently, while non-covalent functionalisation offers advantages in fabrication simplicity, preservation of protein structure, and dynamic biological interactions, covalent conjugation generally provides superior long-term attachment stability and retention of surface-bound targeting ligands under physiological conditions.[18,24,82]

In contrast to covalent conjugation, where ligand presentation can be controlled through surface engineering and site-selective coupling strategies, non-covalent adsorption is highly dependent on nanoparticle physicochemical properties including hydrophobicity, curvature, topography, and chemical composition.[83] Proteins exhibit distinct adsorption behaviours on graphite, polymeric materials, ceramic substrates, silica surfaces, and carbon nanotubes. For example, proteins adsorbed onto hydrophobic graphite undergo extensive surface spreading and irreversible adsorption, whereas adsorption onto hydrophilic poly(vinyl alcohol) is weaker and largely preserves native protein conformation. In contrast, adsorption on ceramic materials such as MgO and hydroxyapatite is primarily mediated by electrostatic interactions, while silica (SiO_2) surfaces promote different adsorption geometries due to their surface chemistry. Furthermore, even when surface chemistry is held constant, proteins adsorb differently to graphene and single-walled carbon nanotubes because nanoscale curvature and topography modify interaction strength, indicating that non-covalent functionalisation outcomes are strongly influenced by nanoparticle design.[83]

Although non-covalent approaches generally exhibit lower attachment stability than covalent functionalisation, certain systems partially overcome this limitation through highly stable affinity interactions or biologically driven surface assembly. For example, avidin–biotin interactions, among the strongest naturally occurring non-covalent binding pairs, have been successfully exploited to immobilise ApoE onto human serum albumin (HSA) nanoparticles, producing well-defined surfactant-free formulations that mediated efficient receptor-dependent transport across the BBB. These ApoE-functionalised HSA nanoparticles delivered loperamide into the brain, produced superior antinociceptive effects in vivo compared with control formulations, and mechanistic studies identified low-density lipoprotein receptor-related protein 1 (LRP1) as the principal uptake receptor, confirming that the affinity-based immobilisation remained sufficiently stable during systemic circulation to preserve biological targeting activity.[24] Similarly, biologically driven non-covalent adsorption has been exploited through polysorbate 80 surface coating, which promotes the selective adsorption of endogenous ApoE from plasma onto polymeric nanoparticles. This adsorbed protein corona enabled LDL receptor-mediated uptake by brain endothelial cells, increasing PBCA nanoparticle internalisation by approximately 20-fold in cultured endothelial cells while facilitating delivery of therapeutics such as dalargin, nerve growth factor and doxorubicin across the BBB in multiple in vivo models.[24]

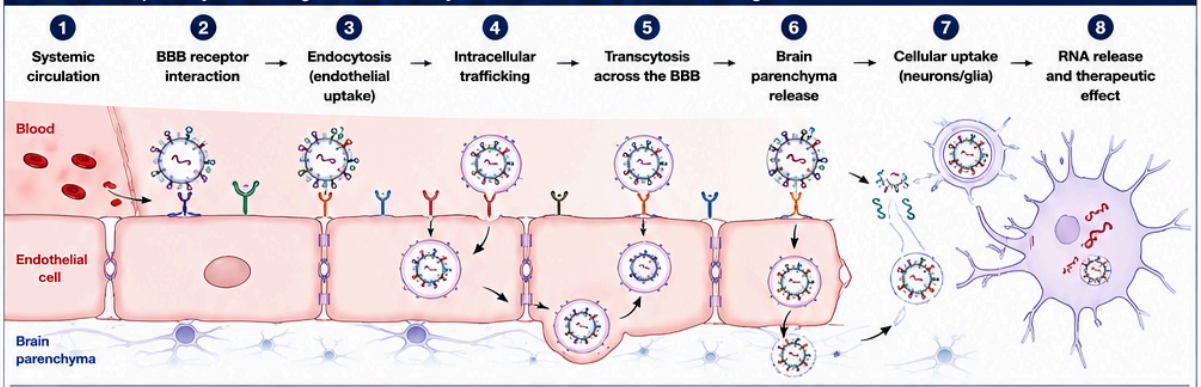
DISCUSSION/LIMITATIONS

This review demonstrates that successful blood–brain barrier (BBB) delivery of RNA therapeutics is determined by considerably more than efficient receptor binding. Rather, effective delivery depends upon the coordinated optimisation of multiple sequential biological processes, including selective endothelial targeting, productive intracellular trafficking, transcytosis across the BBB and functional therapeutic release within the brain parenchyma.

Comparison of the available evidence indicates that lactoferrin and RVG represent the two strongest targeting strategies currently available, consistently outperforming the remaining ligands across receptor accessibility, efficient BBB transcytosis and therapeutic efficacy. However, each achieves this through fundamentally different biological mechanisms. RVG provides the highest targeting specificity by coupling receptor-mediated BBB transcytosis with direct neuronal recognition through nicotinic acetylcholine receptors, thereby largely avoiding endogenous ligand competition while simultaneously increasing neuronal selectivity.[17,65,66] In contrast, lactoferrin derives its superiority from the breadth, consistency and mechanistic depth of the available evidence across targeting, BBB transit and therapeutic delivery, providing the most comprehensive quantitative validation among the currently investigated targeting ligands.[31–34] The comparative framework in Figure X synthesises the principal mechanisms, relative evidence base, and functionalisation considerations underlying the proposed hierarchy of BBB-targeting strategies.

Figure X. Comparative framework for protein functionalisation strategies in BBB-targeted RNA delivery

A. Mechanistic pathway of BBB-targeted RNA delivery and sites of action of different strategies



Strategies and their primary interaction/mechanism

- Lactoferrin (LfR-mediated)
- RVG (nAChR-mediated + neuronal targeting)
- ApoE (LDLR-mediated)
- Transferrin (TfR-mediated)
- Insulin Receptor (IR-mediated)
- CBSA (Adsorptive interaction)
- LMWP (Membrane translocation)

Key mechanistic strengths required for effective RNA delivery across the BBB

Selective targeting	Productive uptake	Favourable trafficking	Efficient transcytosis	Deep brain delivery	Cellular uptake & release	Therapeutic efficacy
High receptor accessibility and binding specificity	Efficient endocytosis without lysosomal degradation	Routing through recycling pathways and avoidance of lysosome	Sustained transport across the endothelial barrier	Release into brain parenchyma and wide distribution	Uptake by target cells and endosomal escape for RNA release	Functional RNA delivery leading to measurable therapeutic benefit

B. Relative strength of evidence across targeting strategies*

Stronger evidence base → Less comprehensive evidence

Relative position	Strategy (mechanism)	Key evidence strengths	Key limitations
1	Lactoferrin (LfR-mediated)	• Extensive mechanistic characterisation • Consistent BBB transcytosis • Robust quantitative brain delivery • Multiple therapeutic demonstrations	Endogenous ligand competition possible; context-dependent receptor expression.
2	RVG (nAChR-mediated + neuronal targeting)	• Strong mechanistic rationale • Demonstrated BBB transport • Neuronal targeting advantage • Therapeutic efficacy in several models	Short peptide stability, endosomal escape limitations and manufacturing scalability remain challenges.
3	ApoE (LDLR-mediated)	• Leverages endogenous pathway • Disease-relevant biology (AD) • Quantitative delivery reported • Less complete intracellular trafficking data	Intracellular trafficking and transcytosis mechanisms less defined.
4	CBSA (Adsorptive interaction)	• Receptor-independent uptake • BBB penetration demonstrated • Therapeutic benefit in models • Lower targeting specificity	Higher likelihood of off-target biodistribution and reduced cellular selectivity.
5	LMWP (Membrane translocation)	• Receptor- and saturation-independent • Efficient membrane translocation • BBB delivery demonstrated • Limited targeting specificity	Potential cytotoxicity at high doses; limited cell-type specificity.
6	Transferrin (TfR-mediated)	• Well-characterised biology • BBB transport demonstrated • Constrained by competition/saturation • Variable therapeutic outcomes	Endogenous competition and receptor recycling reduce transport efficiency.
7	Insulin (Receptor-mediated)	• BBB transport demonstrated • Limited by competition and receptor downregulation • Inconsistent therapeutic efficacy	Receptor downregulation in disease; lower transport efficiency.

*This hierarchy reflects the breadth, convergence and consistency of mechanistic, quantitative and therapeutic evidence available in the literature. It is derived from comparisons across independent preclinical studies conducted under heterogeneous conditions and should not be interpreted as a definitive ranking of intrinsic ligand efficacy.

C. Covalent vs. non-covalent protein functionalisation strategies

COVALENT CONJUGATION (benchmark approach)

- Stable attachment in circulation
- Precise control over ligand density and orientation
- Preserves ligand presentation and receptor accessibility
- Compatible with PEGylation and other surface engineering
- Consistent biological performance (higher uptake, brain accumulation)

Limitations: potential alteration of protein conformation and activity; more complex chemistry; scalability and batch-to-batch variability.

VS.

NON-COVALENT INTERACTION (complementary approach)

- Preserves native protein structure and biological activity
- Simpler and milder functionalisation
- Useful for biologically driven protein adsorption
- Examples: avidin–biotin immobilisation; polysorbate 80–mediated ApoE adsorption

Limitations: weaker attachment; ligand desorption in circulation; less control over density/orientation; greater lot-to-lot variability.

Functional implications for BBB-targeted RNA nanocarriers

Feature	Covalent conjugation	Non-covalent interaction
Ligand attachment	Strong, stable (e.g., amide, maleimide–thiol)	Reversible (electrostatic, hydrophobic, biotin–avidin, surfactant-mediated)
Control over surface architecture	High	Lower
Stability in circulation	High	Lower (risk of desorption)
Effect on targeting efficiency	Higher and more consistent	More variable
Compatibility with PEGylation	Excellent	Possible but less predictable
Impact on protein structure	Possible alteration	Preserved
Manufacturing complexity	Higher	Lower
Scalability & reproducibility	More challenging	Easier but less controlled
Overall outlook	Current benchmark for clinical translation	Useful for specialised applications

RNA nanocarrier · RNA cargo · LfR (Lactoferrin receptor) · nAChR (Nicotinic acetylcholine receptor) · LDLR (LDL receptor) · TfR (Transferrin receptor) · IR (Insulin receptor) · Adsorptive interaction (electrostatic) · Membrane translocation (peptide-mediated)

Consequently, lactoferrin represents the benchmark targeting strategy. However, this should not be interpreted as evidence of intrinsic biological superiority. RVG-derived peptides possess unique biological advantages that may ultimately provide greater control over BBB transport and cell-specific delivery than endogenous receptor-targeting approaches. Realising this potential will require more comprehensive characterisation of RVG-mediated intracellular trafficking, optimisation of peptide stability and endosomal escape, scalable GMP-compliant manufacturing, and robust clinical validation. Furthermore, combinatorial functionalisation strategies, for example coupling RVG with ligands that enhance BBB transcytosis or incorporating endosomal escape-promoting moieties, may further improve transport efficiency and intracellular cargo delivery. Consequently, although lactoferrin currently represents the present strongest evidence-supported strategy, RVG and other engineered protein functionalisations offer a high potential to ultimately establish the next generation of CNS-targeted nanomedicines as these mechanistic and translational challenges are overcome in the future.

The remaining ligands further reinforce that maximising endothelial uptake alone is insufficient for successful CNS delivery. Here, ApoE remains highly attractive because it exploits endogenous LDL receptor-mediated transport while simultaneously engaging biological pathways implicated in Alzheimer's disease, thereby coupling BBB transport with disease-specific therapeutic relevance.[36–38,40,41] Nevertheless, its intracellular trafficking remains considerably less characterised than that of lactoferrin, reducing confidence regarding productive transcytosis. Similarly, receptor-independent approaches such as CBSA and LMWP successfully circumvent receptor saturation and endogenous ligand competition through adsorptive-mediated transport and membrane translocation, respectively, but these advantages are partially offset by reduced targeting specificity and an increased likelihood of off-target biodistribution.[56,58,72,73,75] Conversely, transferrin and insulin receptor targeting occupy the lowest positions within the proposed hierarchy because their biological performance is intrinsically constrained by endogenous ligand competition, receptor saturation, receptor recycling and, in the case of insulin receptors, disease-associated receptor downregulation.[22,27,54] Collectively, these findings reinforce the notion that the most effective protein functionalisations are those capable of integrating receptor accessibility with productive intracellular trafficking and efficient therapeutic release, rather than simply maximising receptor affinity or endothelial uptake.

Beyond ligand selection, this review demonstrates that covalent conjugation currently represents the benchmark protein functionalisation strategy for BBB-targeted RNA nanocarriers because it provides the greatest control over nanoparticle surface architecture, preserving ligand stability, orientation and density while maintaining receptor accessibility and targeting efficiency throughout systemic circulation.[18,19,76–78] These advantages translate into more consistent biological performance, with studies demonstrating enhanced cellular internalisation, endothelial uptake and brain accumulation through optimised ligand presentation without compromising complementary surface engineering strategies such as PEGylation.[56,79] Consequently, the principal advantage of covalent conjugation lies not simply in stronger ligand attachment, but in its ability to rationally engineer nanoparticle surfaces for predictable receptor-mediated BBB transport. Nevertheless, its superiority is not absolute, as chemical conjugation may alter protein

conformation, reduce biological activity, and increase manufacturing complexity, scalability challenges and batch-to-batch variability.[18,76] However, unlike the inherent instability associated with reversible non-covalent interactions, these limitations principally reflect engineering and manufacturing constraints rather than fundamental biological limitations. Continued optimization of ligand orientation, surface architecture and complementary surface engineering strategies therefore offers greater potential to overcome these challenges while preserving the stability advantages of covalent attachment. Although non-covalent approaches remain valuable where preservation of native protein structure or biologically driven protein adsorption is advantageous, particularly in specialised systems such as avidin–biotin immobilisation and polysorbate 80-mediated ApoE adsorption,[24] the available evidence suggests that future advances in BBB-targeted RNA delivery are more likely to arise through refinement of covalent functionalisation strategies than through replacement by non-covalent alternatives. As summarised in Figure X, covalent conjugation provides greater control over surface architecture and ligand presentation than non-covalent functionalisation.

Unfortunately, despite considerable advances in BBB-targeted RNA delivery research, successful clinical translation remains limited by the absence of a standardised framework for nanoparticle evaluation. Relatively few studies directly compare multiple ligands on identical nanoparticle platforms, meaning that the proposed hierarchy necessarily integrates evidence generated under heterogeneous experimental conditions rather than controlled head-to-head evaluation. Differences in nanoparticle composition, ligand density, RNA cargos, administration route, biological model and outcome measures may therefore contribute to observed differences in delivery performance independently of the targeting ligand itself. Consequently, greater brain accumulation or therapeutic efficacy reported for one ligand cannot necessarily be interpreted as evidence of superiority over another evaluated under different conditions. This makes direct comparison between targeting strategies difficult. The proposed hierarchy should instead be understood as a synthesis of the breadth, convergence and consistency of mechanistic, quantitative and therapeutic evidence across the literature, rather than a definitive ranking of intrinsic ligand efficacy. Establishing standardised preclinical evaluation criteria, including consistent nanoparticle characterisation, quantitative measures of BBB transport, intracellular trafficking, cargo delivery and therapeutic efficacy, would enable more robust comparison between functionalisation strategies and facilitate translation into clinical development. Importantly, these experimental limitations reflect a broader translational challenge highlighted throughout this review. Although RNA therapeutics have demonstrated remarkable efficacy in preclinical models, many neurodegenerative disease clinical trials have failed to reproduce these outcomes because sufficient, reproducible delivery to the human brain remains difficult to achieve.

Clinical translation of BBB-targeted RNA nanocarriers remains constrained by a set of interdependent pharmacological, biological and regulatory barriers extending beyond manufacturing, intracellular trafficking and standardisation. Pharmacokinetic behaviour observed in preclinical models may not predict human exposure, as protein corona formation, systemic clearance, reticuloendothelial sequestration and altered ligand accessibility can substantially modify nanoparticle biodistribution and targeting in vivo. Consequently, altered nanoparticle disposition may reduce the fraction of systemically

administered particles that reaches the BBB in an intact, receptor-accessible form, thereby diminishing productive receptor-mediated transcytosis and lowering the concentration of intact RNA cargo available to the target cells within the CNS. These uncertainties are compounded by differences between animal and human BBB physiology, including receptor expression, endothelial trafficking pathways and neurovascular architecture, limiting the predictive validity of preclinical models for human CNS delivery. Long-term safety and immunogenicity also remain critical considerations, particularly for neurodegenerative diseases requiring repeated administration, where cumulative tissue exposure, complement activation or adaptive immune responses may alter efficacy or produce delayed toxicity. From a regulatory perspective, the structural complexity of these systems complicates the definition of critical quality attributes and demonstration of batch-to-batch comparability, since variation in particle size, morphology, RNA loading, surface chemistry or ligand density may alter biological behaviour. Such physicochemical variability can therefore translate directly into variability in pharmacokinetics, biodistribution and therapeutic potency, making reproducible product performance and the establishment of a consistent clinical dose–response relationship substantially more difficult. Consequently, clinical implementation requires demonstration not merely of BBB penetration, but of reproducible human-relevant pharmacokinetics, biodistribution, therapeutic efficacy and long-term safety, supported by scalable manufacturing and regulatory frameworks capable of controlling the nanocarrier–RNA system as an integrated therapeutic entity.

Future research should establish this framework through prospective head-to-head studies evaluating multiple ligands using identical nanoparticle platforms, RNA cargos, dosing regimens and disease models. Greater emphasis should also be placed on elucidating intracellular trafficking and endosomal escape mechanisms, particularly for highly promising ligands such as RVG. Finally, translation should increasingly focus on clinically scalable nanoparticle manufacturing, reproducible functionalisation strategies and clinical trials capable of determining whether the substantial therapeutic benefits observed preclinically can be replicated in patients.

Beyond improving individual targeting ligands, future advances are likely to arise from multivalent nanoparticle design, where ligand density and spatial organisation are engineered to actively regulate receptor trafficking rather than simply increase receptor binding. Recent evidence demonstrates that avidity-optimised multivalent LRP1-targeted polymersomes can bias receptor trafficking towards productive transcytosis, producing approximately 41–45% reductions in brain amyloid-β within hours, an eight-fold increase in plasma amyloid-β, restoration of BBB transport pathways, and cognitive performance comparable to wild-type animals for up to six months.[84] Importantly, these findings also show that excessive ligand density is not beneficial, as high-valency nanoparticles promoted less favourable receptor trafficking, highlighting that optimal ligand loading is critical for maximising therapeutic efficacy while avoiding receptor degradation.[84,85] Collectively, these studies suggest that future BBB-targeted RNA therapeutics may increasingly exploit rationally engineered multivalent nanocarriers capable of simultaneously enhancing transport efficiency and modulating endothelial biology, representing a promising direction for the next generation of precision nanomedicine.

CONCLUSION

Overall, this review demonstrates that successful BBB delivery of RNA therapeutics depends upon integration of targeting specificity, productive intracellular trafficking, efficient transcytosis and functional intracellular release rather than optimisation of any individual component alone. Among currently available protein ligands, lactoferrin provides the strongest overall evidence across targeting, BBB transit and therapeutic delivery because superior quantitative delivery is supported by the most comprehensive mechanistic validation, while RVG offers the highest targeting specificity and greatest potential for neuron-selective delivery. Covalent functionalisation remains the most reliable strategy for preserving ligand stability during systemic delivery, although selected non-covalent approaches continue to provide highly effective alternatives when biological simplicity or endogenous protein adsorption is advantageous. Collectively, these findings establish a mechanistically informed framework for the rational design of next-generation BBB-targeted RNA nanomedicines while highlighting the need for standardised comparative evaluation frameworks, direct head-to-head ligand comparisons, greater mechanistic understanding of intracellular trafficking and endosomal escape, and the development of scalable combinatorial functionalisation strategies capable of maximising BBB transport and intracellular cargo delivery.

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