

# Emerging Targeted Delivery Strategies of Nanosystems for Ischemic Stroke Treatment

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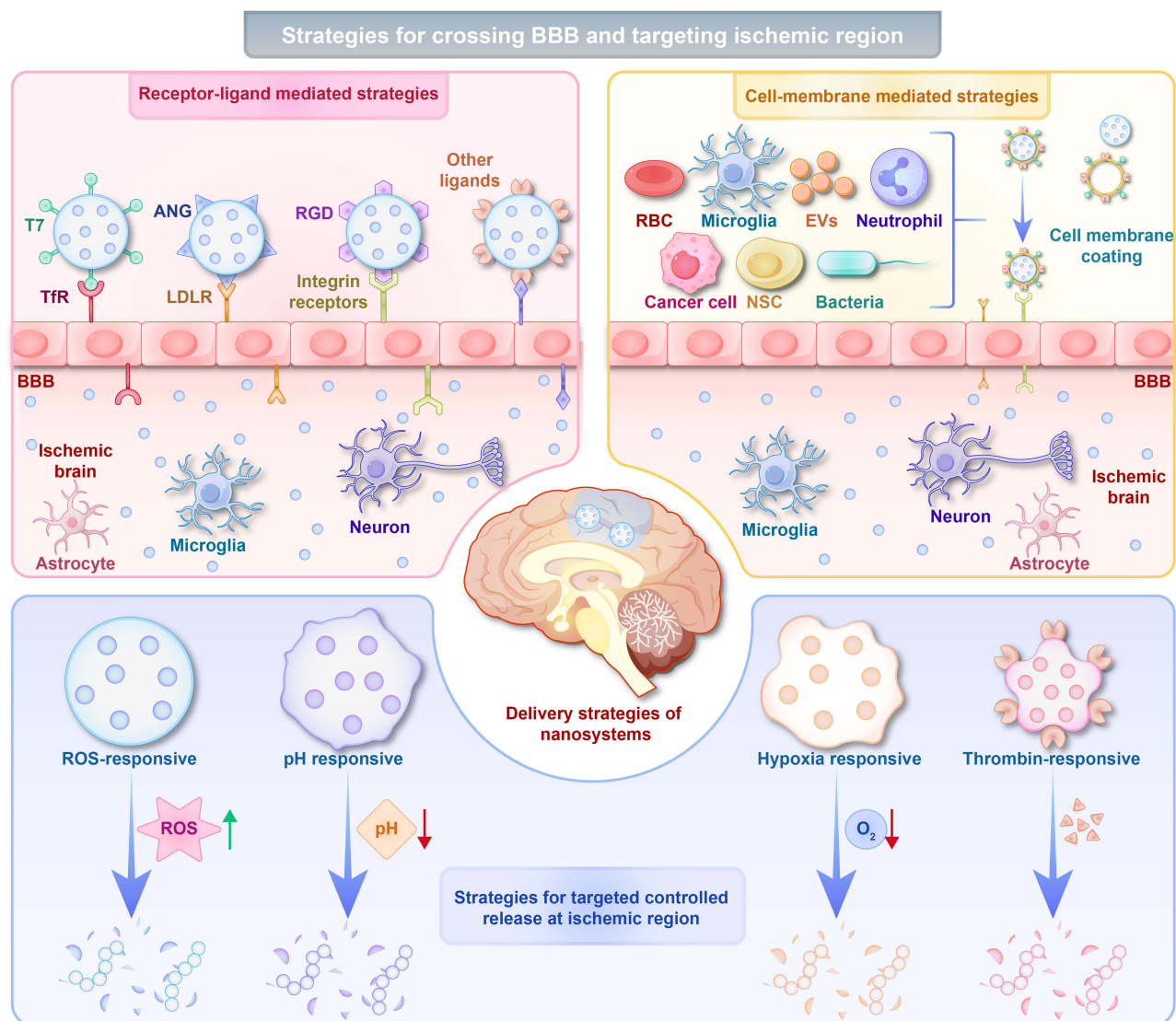
**Abstract:** Ischemic stroke is a leading cause of death and severe disability worldwide. Current treatments mainly focus on reperfusion and neuroprotection. However, due to limitations like a narrow treatment window, single therapeutic targets, and side effects, drug therapy effectiveness is often unsatisfactory. Additionally, the blood-brain barrier (BBB) and the pathophysiological changes following ischemia pose challenges in stroke treatment. Recent developments in nanomaterials have enabled the design of multifunctional drug delivery nanosystems to advance stroke therapeutic approaches. These novel treatments significantly overcome the shortcomings of current therapies and improve efficacy. This review comprehensively summarizes innovative strategies for drug delivery nanosystems, which include crossing the BBB to target the ischemic region and controllable release in a responsive manner. Smart nanosystems, due to the modification of specific ligands and/or cell membranes, can cross the BBB to target the ischemic region for precise treatment, achieving controlled release and specific accumulation of drugs through intelligent molecular switching. Combining different strategies to build drug delivery nanosystems allows for positioning and targeted accumulation of drugs in the ischemic region, thereby extending the therapeutic time window and synergistically improving the neuroprotective effects. Such combinations provide a powerful strategy for developing novel ischemic stroke treatments and establishing targeting delivery nanosystems. Furthermore, the review summarizes the challenges encountered by multifunctional drug delivery nanosystems including clinical translation, drug loading capacity, and safety concerns, and potential solutions.

**Keywords:** ischemic stroke, nanosystems, targeting therapy, blood-brain barrier, controllable release

## Introduction

Ischemic stroke (IS) is the leading cause of death and severe disability worldwide.<sup>1</sup> The mainstay of IS treatment is early revascularization and neuroprotection. Although many promising drugs have shown significant therapeutic effects in animal models, the benefit of clinical functional outcomes remains unsatisfactory. Thus, most of these drugs have not yet been successfully applied in clinics.<sup>2-4</sup> The main therapeutic limitations include the inability to cross the blood-brain barrier (BBB), low lesion targeting capability and bioavailability, and evident systemic side effects.<sup>5</sup> The BBB is a natural biological barrier that restricts drug transport between blood circulation and the central nervous system. Although BBB destruction occurs, to some extent, in the ischemic area after IS, cumulative doses of the drug in the infarct core and penumbra remain insufficient to reach a therapeutic effect. Moreover, traditional therapeutic strategies of neuroprotection drugs are mostly focused on one or two aspects of the complex pathophysiological mechanisms during IS progression, including calcium dysregulation, oxidative stress, neuro-inflammation, cellular apoptosis, and BBB impairment, which fail to exert beneficial efficacy comprehensively. Consequently, overcoming the BBB, targeting the

## Graphical Abstract



ischemic lesion, and exploring multifunctional drugs with diverse and synergistic efficacy are priorities for the new-generation IS treatment.

Nanomedicine, which combines nanotechnology and medicine, is an emerging field in the advancement of drug delivery systems. Drug delivery nanosystems has become an effective solution with considerable prospects for application due to recent advancements in nanotechnology.<sup>6</sup> Constructed nanosystems can cross the BBB to target the ischemic region for precise treatment through strategies such as ligand modification and the use of biomimetic cell membranes. Moreover, the nanosystem functions as a platform for drug delivery and release, achieving the specific accumulation and controlled release of drugs in the ischemic penumbra through intelligent molecular switching. Intelligent molecular switching are stimulus-responsive components integrated into nanomaterials, including pH-sensitive polymers, enzyme-sensitive peptides, thioketal linkers, and thrombolytic peptides, among others. These elements regulate drug release specifically within certain pathological microenvironments, such as those characterized by acidity, elevated ROS, or high levels of thrombolytic enzymes, thereby facilitating controlled drug delivery. The targeted strategies of drug delivery

nanosystems can improve bioavailability, prolong circulation time and reduce toxic side effects on normal tissues.<sup>7</sup> Synergistic drug treatment can be achieved by building multifunctional nanosystems to induce multi-mechanism therapeutic effects and alleviate the complex cascade of pathological responses after IS.

Therefore, this review summarizes the strategies underlying the targeted drug delivery of nanosystems in IS, including overcoming the BBB, targeting ischemic lesions, and responsively releasing drugs. These insights aim to usher in new cutting-edge ideas for drug delivery nanosystems and facilitate the advancement and application of nanosystems in IS.

## Pathophysiology of Ischemic Stroke

IS progression involves necrosis of the ischemic core, which is defined as the area where cerebral vessels occlude, and deterioration of the penumbra in the later phase. The blood from collateral vessels cannot compensate for the occlusion in time, resulting in oxygen and glucose depletion at the ischemic site, which leads to irreversible downstream neuronal death.<sup>8</sup> The penumbra represents a peri-infarcted area with limited affected neurons, thus potentially reversible injury following early reperfusion.<sup>9</sup> However, cerebral ischemia-reperfusion injury (CIRI) is a pathological response to further neuronal damage and increased dysfunction after reconstruction of the cerebral blood supply. The cellular and molecular mechanisms underlying the development of IS include excitotoxicity, calcium dysregulation, oxidative stress, neuro-inflammation, apoptosis, and BBB impairment, as detailed below.<sup>10</sup>

## Excitotoxicity and Calcium Dysregulation

Glutamate functions as a potent and rapid neurotransmitter. However, prolonged exposure to high levels of glutamate can lead to neurotoxic effects in the ischemic area through the excessive response of glutamate receptors, which facilitates the entry of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and water into the cells.<sup>11</sup> The accumulation of water and ions leads to cellular swelling and death. The calcium influx also triggers catabolic processes through  $\text{Ca}^{2+}$ -dependent proteases, lipases, and nucleases, serving as an extra trigger for neuron death.<sup>12,13</sup> The release of stored  $\text{Ca}^{2+}$  from the mitochondria further accelerates a cascade of catastrophic events that leads to acute cell death.<sup>14</sup>

## Oxidative Stress

The brain is more susceptible to oxidative stress due to its massive oxygen -consumption and high lipid content.<sup>15</sup> In the penumbra, following reperfusion and restoration of oxygen supply, there is a significant increase in the levels of reactive oxygen species (ROS), including superoxide radical anions, hydroxyl radicals, singlet oxygen, nitric oxide, and peroxynitrite, which disrupt the balance between oxidants and antioxidants. The interaction between the cellular components of neurons and oxidants results in protein oxidation and nitridation, lipid peroxidation, and DNA damage, exacerbating the damage to ischemic brain tissue.<sup>4,16</sup> Furthermore, hydroxyl radicals compromise mitochondrial membrane integrity and impair the electron transport chain during oxidative phosphorylation. Excessive ROS generation triggers mitochondrial damage and leads to cellular apoptosis.<sup>17</sup>

## Neuro-Inflammation

In the acute phase of IS, the endothelium of cerebral blood vessels promptly upregulates adhesion molecules, such as selectins, facilitating the adherence of leukocytes and platelets after reperfusion. BBB impairment, meningeal infiltration, and choroid plexus entry are examples of the diverse pathways through which immune cells, especially neutrophils and monocytes, access the brain during ischemic events.<sup>18–20</sup> Microglia function as major immune cells in the maintenance of CNS stability.<sup>21</sup> After the onset of cerebral ischemia, damaged brain cells release danger-associated molecular patterns that can activate microglia, transform microglia into distinct phenotypes, and engage in their organized division of labor.<sup>22</sup> The classical activation (M1 type) of microglia is characterized by the secretion of pro-inflammatory mediators, which lead to the release of more chemokines and cytokines, promoting the influx of leukocytes, and exacerbating brain damage. The alternative activation (M2 type) of microglia primarily functions to repair damage to the brain tissue.<sup>23,24</sup> The balance between the two types of activated microglia is vital for regulating the neuroinflammatory process in IS.<sup>25,26</sup>

## Apoptosis

Various cell death pathways are associated with the pathophysiological processes of IS, such as intrinsic and extrinsic apoptosis, necroptosis, autophagy, ferroptosis, parthanatos, phagoptosis, and pyroptosis. Apoptosis, the predominant form of programmed cell death, can be activated through intrinsic or extrinsic pathways. Following the occurrence of IS, the intrinsic pathway of apoptosis is initiated, involving mitochondrial dysfunction, the release of cytochrome C, and apoptosis-inducing factor, or both, leading to caspase-dependent or independent cell death. Rapid translocation of apoptosis-inducing factors released by mitochondria to the nucleus mediates large-scale deoxyribo nucleic acid (DNA) damage and cell death in a caspase-independent manner. Furthermore, the nuclear pathway of neuronal cell death is activated in response to DNA damage by either phosphorylation and activation of p53 or the translocation of nuclear phosphoproteins from mitochondria.<sup>27</sup>

## BBB Impairment

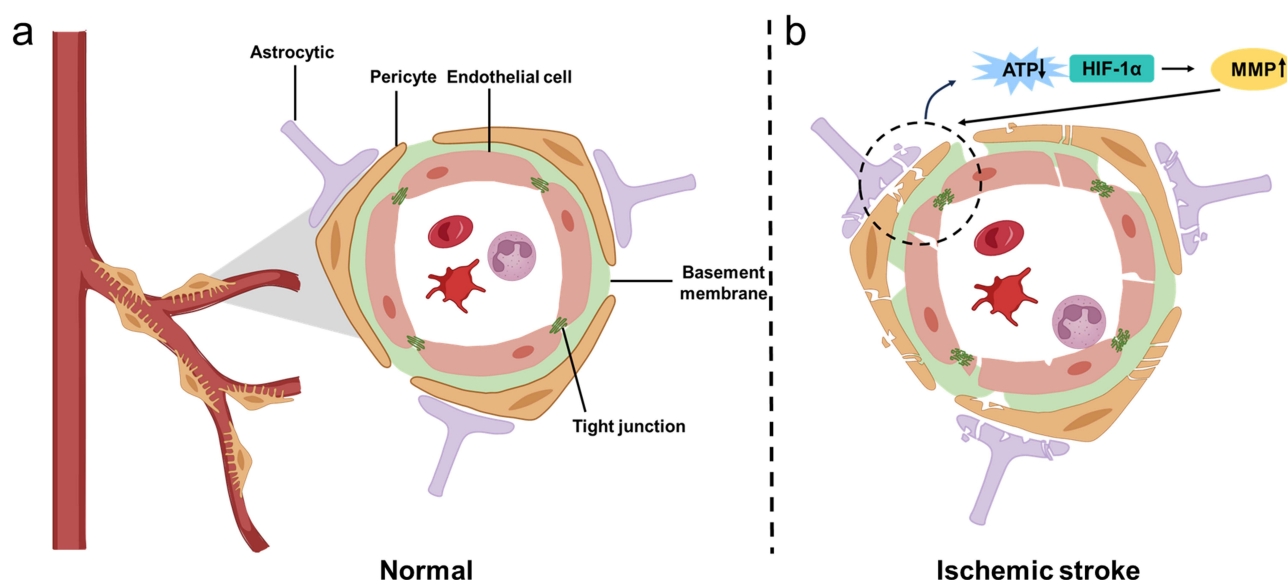
The BBB is a highly selective and regulated structure that limits the exchange of substances between blood circulation and the CNS. It is composed of the cerebral microvascular endothelium, surrounded by astrocytes, pericytes, neuronal terminals, the basement membrane, and the extracellular matrix. Collectively, these elements constitute a neurovascular unit that is essential for the health and stability of the CNS.<sup>28,29</sup> The causes of BBB deficits after IS are mainly the progressive impairment of the transcellular barrier mechanism followed by paracellular barrier mechanisms. Studies have shown that BBB function is impaired as early as 6 hours after IS. Although the tight junction was found to exhibit severe structural deficits two days later, the number of endothelial caveolae and the rate of transcytosis increased as early as 6 hours after IS.<sup>18</sup> The enzymatic breakdown of the basement membrane and tight junction and the loss of endothelial cells, pericytes, and astrocytes can be observed during a 24–48-hour period after IS onset. Therefore, the biphasic increase in BBB permeability exists after IS, which expands the therapeutic window for neuroprotective treatment and enhances drug delivery. After the breakdown of the BBB, leukocytes infiltrate and adhere to the walls of endothelial cells.<sup>30,31</sup> Excessive ROS accumulation and impairment of endogenous antioxidant mechanisms lead to the peroxidation of DNA, proteins, and lipids, resulting in tissue damage and breakdown of the BBB.<sup>32,33</sup> Matrix metalloproteinase (MMP) levels are significantly elevated following the activation of microglia and astrocytes. The loss of adenosine triphosphate and oxygen and other induced cytokines, such as hypoxia-inducible factor-1 $\alpha$ , triggers the activation of the MMP family, particularly MMP9 and MMP2. In conjunction with other chemokines, cytokines, and vascular endothelial growth factor released by activated microglia and astrocytes, the tight junction between the cells and basal lamina undergoes degradation, leading to compromised BBB integrity. This degradation increases BBB permeability, facilitates the infiltration of blood-derived immune cells, and leads to vasogenic edema, hemorrhagic transformation, and an elevated mortality rate (Figure 1).<sup>31,34</sup>

## Current Clinical Treatments for IS and Limitations

### Reperfusion Treatments for IS

Reperfusion therapy is the primary treatment for IS, which mainly includes endovascular intervention and thrombolysis. Endovascular therapy has emerged as a highly effective intervention for IS caused by large vessel occlusion, including mechanical thrombectomy, thrombus aspiration, angioplasty and stenting placement.<sup>35</sup> The meta-analysis of large-scale clinical trials on endovascular treatment of IS demonstrated that recanalization was achieved in 71% of patients following thrombus extraction. However, only 46% of patients experienced a favorable prognosis, and futile recanalization was observed in 50% of the cases. The causes of futile recanalization include tissue damage, cerebral edema, no-reflow phenomenon, reperfusion injury, procedural features, and variations in postprocedural management.<sup>36</sup> In addition, the application of endovascular therapy remains constrained by the requirement for advanced stroke center infrastructure, specialized neurointerventional teams, and substantial healthcare expenditures. These limitations highlight the urgent need for adjuvant neuroprotective strategies to improve therapeutic benefits.

So far, the only drug approved by the US Food and Drug Administration is recombinant tissue-type plasminogen activator (rt-PA), a fibrin-specific thrombolytic agent. The clot-dissolving mechanism of rt-PA involves activating



**Figure 1** The structure of the BBB (a) and the BBB impairment after ischemic stroke (b). BBB is composed of the cerebral microvascular endothelium, surrounded by astrocytes, pericytes, neuronal terminals, the basement membrane, and the extracellular matrix. The enzymatic breakdown of the basement membrane and tight junction and the loss of endothelial cells, pericytes, and astrocytes can be observed during a 24–48-hour period after IS onset. The loss of adenosine triphosphate and oxygen and other induced cytokines, such as hypoxia-inducible factor-1 $\alpha$ , triggers the activation of the MMP family, particularly MMP9 and MMP2.

**Abbreviations:** BBB, blood-brain barrier; MMP, matrix metalloproteinase; HIF-1 $\alpha$ : hypoxia-inducible factor-1 $\alpha$ .

endogenous plasminogen into plasmin, which rapidly dissolves the fibrinogen surrounding the thrombus.<sup>37</sup> However, due to the narrow therapeutic window (<4.5 h), only a small proportion of patients are eligible for rt-PA treatment, and hemorrhagic transformation and CIRI limit their efficacy. The extremely short half-life of rt-PA is approximately 4–6 minutes, mainly due to the presence of fast-acting endogenous inhibitors, such as fibrinogen activator inhibitor-1, in the circulation.<sup>38</sup> Therefore, continuous intravenous infusion is necessary to achieve effective thrombolysis, which poses a risk of bleeding complications. Despite strict adherence to these indications, there is a 2–7% risk of fatal intracranial hemorrhage.<sup>39</sup> Additionally, there is a risk that the dissolved embolic components from thrombolysis may spread to distal small arteries, potentially leading to fluctuating symptoms and worsening functional prognosis.<sup>40</sup>

## Drug Treatment

Neuroprotection is achieved by inhibiting the pathogenic cascade to from preserving, recovering, or regenerating the nervous system.<sup>41</sup> Various neuroprotectants are currently being investigated in preclinical research. Neuroprotective agents that target different pathophysiological processes can be divided into four categories: glutamate antagonists, calcium-channel blockers, free radical scavengers, and immunosuppressants.<sup>42</sup> Although these promising candidates have shown significant therapeutic effects in animal models, they have yet to be successfully translated into clinical use.<sup>4,43</sup> Efforts to develop neuroprotective agents such as edaravone and butylphthalide have also faced challenges related to the limited bioavailability and potential side effects of these drugs in clinical trials.<sup>44</sup> The limited permeability of drugs to the brain may decrease the efficacy of neuroprotectants due to the protective function of the BBB. Moreover, the need for precise site-specific delivery of the drugs within a narrow treatment window, their side effects, and their short half-life after administration all present challenges for the clinical use of neuroprotective agents.<sup>4,5</sup> To circumvent the BBB limitations of conventional drug delivery, intranasal administration has emerged as a promising strategy for bypassing the BBB.<sup>45,46</sup> This approach utilizes olfactory and trigeminal neural pathways to facilitate rapid (within 15 minutes) transport of substances from the nasal cavity to the brain, achieving cerebral drug concentrations that are 3 to 5 times higher than those attained via intravenous administration. This method enhances drug absorption efficiency, minimizes extensive systemic exposure, and reduces hepatic and renal toxicity. At present, the intranasal route for the treatment of IS facilitates the delivery of small molecules, proteins, gene vectors, stem cells, and drug-loaded vesicles.<sup>47,48</sup> Nevertheless, challenges such as limited drug retention time due to rapid nasal clearance, which impacts drug absorption, uneven drug



distribution resulting from individual variability, and drug degradation by local enzymes in the nasal cavity, present significant obstacles. Consequently, the current therapeutic approach to intranasal drug delivery remains primarily in the basic research phase, with clinical translation facing substantial bottlenecks.<sup>49</sup> Therefore, overcoming the limitations of current stroke therapeutics in crossing the BBB and targeting the ischemic region are key challenges that need to be addressed in the pharmacological treatment of stroke. How can therapeutic drugs be intelligently released only to the ischemic region to improve targeting and biological responsiveness? The drug delivery nanosystems designed using various strategies offer a promising avenue, and the current strategies for the use of nanosystems are described in detail below.

## Targeted Delivery Strategies of Nanosystems

The function of drug delivery nanosystems for IS is site-specific targeting and releasing, including crossing the BBB to target the focal lesion and releasing drugs in a target-responsive manner. Detailed information on drug-delivery nanosystems is listed in Table 1.

First, the ability to cross the BBB and reach the focal zone primarily relies on the presence of ligands on the nanocarriers that specifically bind to receptors mainly on the brain microvascular endothelial cells (BMECs) highly expressed at the focal site, including transferrin receptor (TfR), low-density lipoprotein receptor (LDLR), integrin receptors, and so forth. In addition to these receptors that are highly expressed on BMECs, the nanosystems can also target ischemic neurons, microglia, and mitochondria. Another strategy is to deliver the drug to the ischemic site by using biomimetic cell membranes or cellular components as nanocarriers, including neutrophil membranes, erythrocytes, macrophages, microglia, cancer cells, neural stem cells, and extracellular vehicles (EVs). Natural cell membranes and cellular components can also be modified with specific peptides to increase their targeting properties. In addition, external ultrasound or magnetic fields can be used to assist the nanocarriers in crossing the BBB and targeting the ischemic region.

Second, target-responsive drug release is a promising method for overcoming dose limitations, reducing systemic toxicity, and enhancing drug concentration at the lesion site. This strategy involves designing intelligent and controllable “switches” that respond to specific microenvironmental changes in the ischemic region. Specific chemical groups or other protein structures are attached to the nanocarriers to respond to the changes in the ischemic region, including changes in pH, ROS, thrombin aggregation, hypoxia, and inflammatory changes. The strategy of using nanosystems for thrombolytic therapy when the stroke has thromboembolic causes is to target the thrombus. Similarly, receptors expressed on thrombus-forming erythrocytes, platelets, and fibronectin are targeted via specific ligands, and thrombi are targeted via cell membranes and cellular components. Recently, multi-targeted, cascade-delivered nanosystems can both target thrombi and reach the ischemic site to achieve multiple functions, including thrombolysis and neuroprotection.

## Strategies for Crossing BBB and Targeting Ischemic Region

Crossing the BBB and targeting the ischemic region are the primary hurdles for drug delivery nanosystems. Although some degree of BBB disruption occurs in the ischemic region after IS, the accumulated drug in the infarct core and penumbra remains insufficient to have therapeutic effects. Researchers have simulated the natural biological crossing of the BBB and selected various receptor–ligand pairs, cell membrane coatings, extracellular or cell membrane-derived vesicles, exosomes, peptides, and proteins to cross the BBB for drug delivery successfully. Regulating the permeability of the BBB also constitutes an effective strategy to enhance transport by crossing BBB. Furthermore, the current nanosystems not only cross the BBB, but also precisely target the infarcted brain region to exert multifunctional neuroprotective effects, such as anti-apoptosis, anti-inflammation, and anti-oxidative stress.

### Receptor-Ligand Mediated Strategies

Transporter receptors on the surface of the BBB are responsible for the intracranial transport of biomolecules. Receptor-mediated transcytosis (RMT) is one of the most widely used and effective strategies to help biomolecular drugs cross the BBB. The receptors contain TfR, LDLR, integrin receptors and other ligands decorated on the surface of nanoparticles, which not only bind to BMECs to achieve BBB crossing, and even to target lesion site to fulfill specific brain cells

**Table I** Targeted Delivery Strategies of Nanosystems

Strategies	Drug Delivery Nanosystem	Drugs Loaded	Ligand on Nanoparticles	Receptors	Cell-Membrane Coating	Responsive-ness	Function	Year	Ref.
I	PLGA-CTX/LEX NPs	BBB modulator lexiscan; Nogo-66 receptor antagonist peptide NEPI-40	Chlorotoxin (CTX)	MMP-2	NA	NA	Enhanced BBB permeability, BBB crossing and target ischemic microenvironment	2016	[50]
I+2+3	SHp-RBC-NP	A neuroprotective agent, NR2B9C	SHp	Apoptotic neuronal cells	Red blood cell	ROS responsive	Immune-evading capabilities, prolonged blood retention, crossing BBB, enhancing the active targeting of the ischemic area and controllable release	2018	[51]
I	Cerium oxide nanoparticle (E-A/P-CeO <sub>2</sub> )	Edaravone	Angiopep-2 (ANG)	LRP	NA	NA	BBB crossing, ROS-scavenging, and neuroprotection	2018	[52]
I+3	AMD3100-conjugated, size-shrinkable nanoparticles (ASNPs)	Glyburide	CXCR4 antagonist: AMD3100	CXCR4: a chemokine receptor	NA	Thrombin-responsive/ROS responsive	Penetrating the ischemic brain with high efficiency, shrink ability and excellent antioxidant bioactivity	2018 2022	[53,54]
I+3	A-BAM NPs	Betulinic amine (BAM)			NA	pH responsive	Crossing the damaged BBB to target ischemic regions, controllable release and antioxidant effect	2022	[55]
I+2+3	Brain-derived neurotrophic factor transfected neural stem cells	Brain-derived neurotrophic factor transfection	VLA-4	VCAM-1	Neural stem cells	ROS responsive	Targeting to injured brain regions and controllable release	2019	[56]
I+2	Arg-Gly-Asp -extracellular vesicles (RGD-EV)	Hypoxia-inducible factor 1- $\alpha$ mutant form plasmid DNA	RGD peptide (ACDCRGDCFC)	Integrin $\alpha\beta 3$	EV derived from neural progenitor cell	NA	Crossing BBB, targeting to ischemic region and functioning as a safe nonviral gene vector	2019	[57]
I+2	Nanovesicle-based delivery system	Resolvin D2 (RvD2)	The expression of integrin $\beta 2$ , Mac-1, and LFA-1 on the neutrophil membrane	ICAM-1, was upregulated on inflamed brain microvascular endothelial cells	Neutrophil	NA	Crossing BBB, target ischemic region and prevent neuroinflammation	2019	[58]
I+2	Neutrophil-like cell-membrane-coated mesoporous Prussian blue nanozyme (MPBzyme@NCM)	Mesoporous Prussian blue nanozyme				NA	Crossing BBB, target ischemic region, microglia polarization, decreased apoptosis of neurons, up-regulation of neurogenesis	2021	[59]
I+2	A biomimetic nanosystem (Leo@NM-Lipo) composed of neutrophil membrane-fused nanoliposomal leonurine (Leo)	Leonurine (Leo)				NA	Crossing BBB with high delivery efficiency to exert anti-oxidant, anti-inflammatory, and anti-apoptotic effects	2023	[60]

(Continued)

Table I (Continued).

Strategies	Drug Delivery Nanosystem	Drugs Loaded	Ligand on Nanoparticles	Receptors	Cell-Membrane Coating	Responsive-ness	Function	Year	Ref.
I+2+3	Succinobucol-loaded pH-sensitive polymeric nanovehicle with a 4T1 cell membrane (MPP/SCB)	Succinobucol (SCB)	Syndecan-1 (CD138) on 4T1 cancer cell membranes	The platelet endothelial cell adhesion molecule-1 (CD31) on vascular endothelial cells, platelets, and leukocytes	4T1 cancer cell	pH responsive	BBB penetration, controllable release, ROS elimination, inflammation inhibition and neuron protection	2021	[61]
I+2	Macrophage-disguised Fingolimod (FTY)-loaded MnO <sub>2</sub> nanoparticles (Ma@ (MnO <sub>2</sub> +FTY))	Fingolimod (FTY)	CD44 and CD11b on leukocytes	ICAM-1 and P-selectin on microvascular endothelial cells	Macrophage	NA	Crossing BBB, targeting to ischemic region, consuming excessive ROS, reducing oxidative stress and regulating proinflammatory microenvironment	2021	[62]
I+3	Liposome nanosystem	NF-κB inhibitor, caffeic acid phenethyl ester	Specific peptide ligands c (RGDyK)	Integrin αvβ3	NA	Reactive nitrogen species responsive	BBB crossing, NVU target Anti-nitration, anti-neuronal apoptosis, regulating microglial cell polarization and repairing the BBB	2022	[63]
I	SS31-HA-QT	Quercetin (QT)	Bendavia (SS-31) peptides	Cardiolipin at mitochondrial	NA	NA	BBB penetration, ischemic area-targeting mitochondrial targeting	2022	[64]
			Hyaluronic acid (HA)	CD44					
I+3	Fluorescein isothiocyanate (FITC)-labeled SiO <sub>2</sub> @PAA-MT /ACh nanospheres	Acetylcholine and Melatonin	Anti-complement component 5a (aC5a) aptamers	C5a	NA	pH responsive	Crossing the damaged BBB to target ischemic regions, controllable release, suppressing inflammatory response and eliminating ROS to alleviate oxidative stress	2023	[65]
I+2	OMV@PGZ	Pioglitazone (PGZ)	Lipopolysaccharide	Neutrophil Toll-like receptors	Bacteria-derived outer-membrane (OMV) hitchhike on neutrophil	NA	Crossing BBB with high delivery efficiency to protect against lipid peroxidation, prevent mitochondrial damage, and inhibit ferroptosis.	2023	[66]
I+2+3	Nanosystem	Ligustrazine	The peptide cinnamyl-F-(D)L-F-(D)L-F (CFLFLF)	FPR	Neutrophil	ROS responsive	Crossing BBB and improve drug enrichment in the ischemic brain	2023	[67]
I+2+3	NSC-Lipo	Metformin	VLA-4	VCAM-1	The EV derived from stem cell	Bioresponsive	Multifunction specific recognition and binding capability to inflamed BBB	2023	[68]
I+3	Multifunctional nanoparticle	Puerarin (PU)	Bendavia (SS-31) peptides	Cardiolipin at mitochondrial	NA	ROS-responsive	Mitochondrial-targeted	2023	[46]



1+3	CPLSeP/IDBN Micelles	idebenone	CREKA peptides	Fibrin	NA	ROS-responsive	Targeting thrombus, crossing BBB and ROS-responsive drug release	2023	[69]
	Self-assembled liposomal nanocarriers (C-Lipo/CA)	Cl-amidine, an inhibitor of peptidylarginine deiminase 4 (PAD4)			NA		Targeting thrombus and ischemic injured brain areas, controllable drug release	2023	[70]
1+2	Nanovehicle composed of polyfluorocarbon and Pluronic P123	Drug-free	LFA-1 on the surface of M2 microglia	ICAM-1	M2 microglia	NA	Crossing BBB, targeting to ischemic region, inhibition of MMP-9 secretion and protection of BBB	2023	[71]
1+3	Triple-targeting nanoplatform	dl-3-n-butylphthalide	T7	TfR	NA	ROS responsive	Enhanced BBB permeability, BBB crossing and neuron-targeting	2023	[72]
			SHp	Apoptotic neuronal cells					
1+2+3	SHp-NM@Edv/RCD (SNM-NPs)	Edaravone	SHp	Apoptotic neuronal cells	Neutrophil	ROS responsive	BBB crossing, target the inflammatory site, target injured neurons, ROS scavenging.	2024	[73]
1+3	Peptide-templated MnO <sub>2</sub> nanozyme (PNzyme/MnO <sub>2</sub> )	Edaravone	T7 sequence (HAIYPRH)	TfR	NA	Thrombin responsive	Fibrin-binding, thrombolysis, thrombin cleavage, BBB crossing, and apoptotic neuron targeting	2024	[74]
			Stroke homing peptide (CLEVSRKNC)	Apoptotic neuronal cells					
1+2+3	Cu <sub>4</sub> .6O@zein-Se-DHA @PLM@rt-PA	rt-PA	Docosahexaenoic acid (DHA)	DHA receptor	Platelet	ROS-responsive	Thrombolysis, BBB crossing, and neuroprotection	2024	[75]
3	Calixarene-grafted self-assembled peptide hydrogel	Fingolimod	NA	NA	NA	Hypoxia-responsive	Functioning as a smart hypoxia-responsive drug depot	2024	[76]
1	TPP@(CeO <sub>2</sub> +ROF)	PDE4 inhibitor, roflumilast	Mitochondrial targeting	NA	NA	NA	Mitochondrial targeting, neuroprotection, ROS scavenging	2024	[77]
2+3	Polyprodrug nanoparticle	Fingolimod hydrochloride	NA	NA	Neutrophil membrane	ROS-responsive	BBB crossing, ROS scavenging and neuroprotection	2024	[78]
2	MM@AK137-FN/EDV	Edaravone	NA	NA	Macrophage membrane		BBB crossing, ROS scavenging, neuroprotection, anti-inflammation	2024	[79]

**Notes:** 1 represents “receptor-ligand mediated strategies”. 2 represents “cell-membrane mediated strategies”. 3 represents “strategies for targeted controlled release at ischemic region”.

**Abbreviations:** BBB, blood brain barrier; EV, extracellular vesicle; ICAM-1, Intercellular adhesion molecule-1; FPR, formyl peptide receptor; LFA-1, lymphocyte function-associated antigen 1; LRP, LDLR-related protein receptor; Mac-1, macrophage-1 antigen; ROS, reactive oxygen species; SHp, stroke-homing peptides; TfR, transferrin receptor.

targeting ability, taking the example of ischemic neurons and activated microglia. This so-called “molecular Trojan horse technology” provide potential strategies for achieving trans-BBB and targeted drug delivery.<sup>80</sup>

### TfR-Medicated Targeting

Transferrin is responsible for transporting iron across the BBB by binding to TfR expressed on BMECs. Previous studies have shown that TfR is significantly overexpressed in the vascular endothelium 1 day and 2 days after in middle cerebral artery occlusion (MCAO) rat model to mimic middle cerebral artery occlusion.<sup>81</sup> Raza et al developed transferrin-coupled pH-responsive nanomicelles to encapsulate NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome inhibitor. The nanomicelles were synthesized by using poly (2-methyl-2-oxazoline) (a hydrophilic and pH-responsive polymer), poly (l-lactic-co-glycolic acid) (PLGA) and holo-transferrin. These nanomicelles specifically bind to TfR and cross the BBB. The nanomicelles were found to significantly increase the overall survival of MCAO rats and act as a therapeutic agent for CIRI injury by inhibiting the activation of NLRP3 inflammasome.<sup>82</sup> In addition, a specific ligand peptide of TfR, namely HAIYPRH (T7) mediates the transport of the nanocarriers across the BBB. Gu et al and Fan et al found that nanomaterials with T7 loading were translocated between BMECs in contrast to non-targeted liposomes.<sup>74,83</sup> Furthermore, Li et al used a mild biomimetic mineralization technique to build a transferrin-based manganese dioxide nanozyme ( $\text{MnO}_2@\text{Tf}$ , MT) loaded with neuroprotective agent edaravone ( $\text{Eda-MnO}_2@\text{Tf}$ , EMT). The decoration of transferrin enabled EMT to be targeted to the ischemic BMECs. Then, the EMT not only crossed the BBB and aggregated at the ischemic lesion with good biocompatibility and biosafety.  $\text{MnO}_2$  nanozyme and edaravone endowed the EMT with superoxide dismutase and catalase-like antioxidant activity to scavenge overwhelmed ROS, ameliorate ROS-mediated damage and reduce the levels of inflammatory factors. Additionally, EMT released  $\text{Mn}^{2+}$  ions in the weak acid environment of the ischemic lesion area, which can be used for dynamic tracking of the treatment process through magnetic resonance imaging.<sup>84</sup>

### LDLR-Medicated Targeting

LDLR is expressed on BMECs and other peripheral tissues and is responsible for transporting lipoproteins such as cholesterol, tocopherol, and apolipoproteins.<sup>85</sup> LDLR-related protein receptors 1 and 2, which are structurally similar to LDLR, are also expressed in BMECs. Various ligands, including L-carnosine, angiopep-2 (ANG), lactoferrin, and melanotransferrin, can bind to these receptors and mediate nanocarriers across the BBB via RMT.<sup>52</sup> Therefore, Shi et al ingeniously developed an edaravone-loaded ceria nanoparticle for crossing the BBB and combating IS. The engineered nanoparticles performed a core-shell structure with monodisperse ultrasmall ceria as the core and organic ANG/polyethylene glycol (PEG) loaded by edaravone as the shell. Firstly, PEG was able to significantly improve biocompatibility and monodispersity and prolong the half-life in the blood. Second, ANG was the targeted ligand on cerium oxide nanoparticles that binds specifically to the LDLR of the BBB via receptor-mediated transcytosis and makes nanoparticle high accumulation in intracerebral lesions. Meanwhile, the core of the ceria nanoparticles and the absorbed edaravone synergistically eliminate ROS, which mitigates oxidative stress and reduces infarct size.<sup>52</sup> In addition, Liu et al also created a nanoprobe decorated by ANG peptides, which penetrated BBB and were activated by a highly specific biomarker of neuroinflammation through up-conversion with near-infrared emission. The ANG peptides can bind to the LDLR overexpressed on the BBB and then deliver the nanoprobe into the brain tissue to allow non-invasive neuroinflammation assessment.<sup>86</sup>

### Integrin Receptor-Medicated Targeting

Integrin  $\alpha_v\beta_3$ , an important member of the integrin family, is significantly upregulated on stressed vascular endothelial cells during cerebral ischemia.<sup>87,88</sup> One of the identified ligands for integrin is the RGD integrin-binding peptide.<sup>89</sup> Arg-Gly-Asp (RGDyC) is selective for  $\alpha_v\beta_3$  integrin peptides and also binds to integrin receptors on the surface of leukocytes (neutrophils and monocytes).<sup>90</sup> Therefore, drug delivery systems enriched with the  $\alpha_v\beta_3$ -specific ligand are one of the potential ways to target lesions.

Xin et al constructed c (RGDyK)-modified reactive nitrogen species stimuli-responsive liposomes loaded with NF- $\kappa$ B inhibitor, caffeic acid phenethyl ester (R-lipo-CAPE) to remodel the neurovascular unit and reduce the progression of

CIRI. The lipid-containing o-phenylenediamine structure was first synthesized and self-assembled with phospholipids, cholesterol, c (RGDyK)-modified phospholipids, and CAPE in proportion to create a targeted R-Lipo-CAPE in response to reactive nitrogen species stimulation. The c (RGDyK) peptide promoted the targeted accumulation of R-Lipo-CAPE to cross the BBB and reach the ischemic lesion. Subsequently, the o-phenylenediamine lipid hydrolyzed in response to RNS activation accumulated in the lesion focus, causing alterations in the lipid bilayer's curvature and regional instability, which ultimately led to the intelligent release of CAPE. The results suggested that R-lipo-CAPE remodels neurovascular unit and exhibits good protection against IS by inhibiting NF- $\kappa$ B activation in neurons, glial cells and endothelial cells, and exerting anti-nitrosative stress, anti-neuronal apoptosis, modulation of microglia polarization and repair of vascular endothelial cells.<sup>63</sup> Similarly, Deng et al encapsulated the plasmid DNA containing the mutant form of the hypoxia-inducible factor 1- $\alpha$  (HIF-1 $\alpha$ -AA) in a hyperbranched cationic amylopectin derivative (DMAPA-Amyp) nanocarrier, which was decorated by RGD peptides to increase aggregation in BMECs at the infarct region and penumbra, which significantly contributed to the recovery of neurological function.<sup>57</sup>

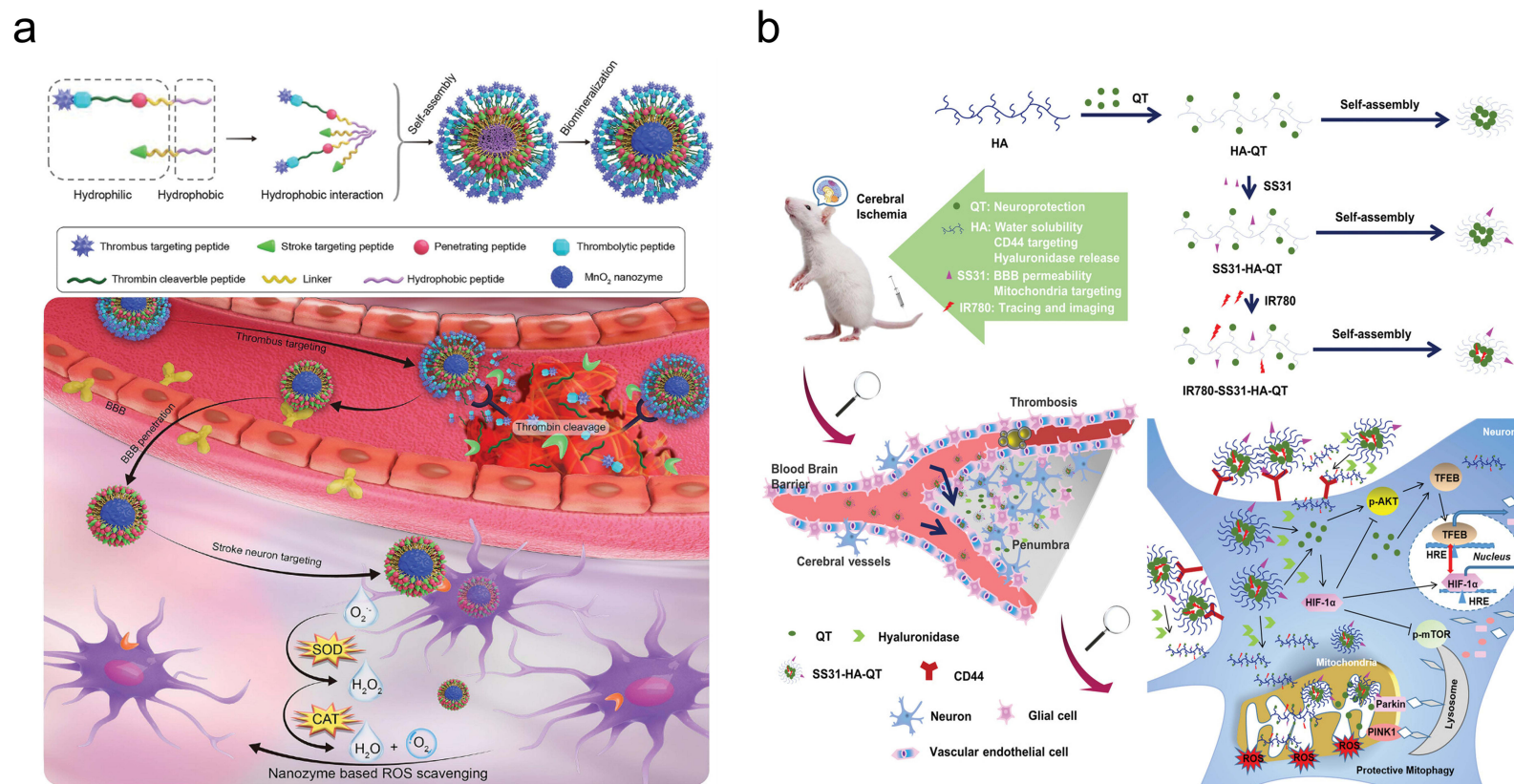
## Other Ligands-Medicated Targeting

### Stroke-Homing Peptides

The stroke-homing peptides (SHp) are peptides obtained by in vivo screening of phage libraries with a high affinity for glutamate receptors on the cell surface during neuronal apoptosis.<sup>51,91</sup> Modifying targeted peptide on the surface of nanoparticles is a feasible and promising strategy. Self-assembled nanoparticles with peptides have abundant metal-binding sites, making them effective biomineralization scaffolds. These nanoparticles are more advantageous in terms of biocompatibility and targeting than inorganic nanomaterials. Therefore, Fan et al designed a novel peptide-templated manganese dioxide nanozyme (PNzyme/MnO<sub>2</sub>), which combined the ability of nanozymes to scavenge ROS with the thrombolytic activity of functional peptides (Figure 2a).<sup>74</sup> There were two functional peptide chains included to construct peptide templates for the thrombin response. The functional peptide chain 1 contained the following structural motifs: a fibrin binding sequence, a thrombolytic sequence (CREKA), a sequence recognized and cleaved by thrombin, a transferrin receptor binding sequence, and a hydrophobic metal ion binding peptide. The functional peptide chain 2 consisted of a sequence that targeted ischemic neuronal tissues and a hydrophobic sequence that bound metal ions. A mixture of the two peptide chains self-assembled into nanoparticles and was biomineralized with MnO<sub>2</sub> initiated by KMnO<sub>4</sub> to form PNzyme/MnO<sub>2</sub>. Based on self-assembling polypeptides with different functional motifs, the PNzyme/MnO<sub>2</sub> performed step-wise targeting thrombus, BBB and ischemic lesion. First, the PNzyme/MnO<sub>2</sub> was particularly localized at the thrombus site via the fibrin-binding motif CREKA, which is then cleaved by thrombin to release the thrombolytic peptide, GRPAK to exert thrombolytic effects. Then, the rest of PNzyme/MnO<sub>2</sub> bound to the TfR on endothelial cells via the T7 sequence HAIYPRH. Finally, the stroke homing sequence CLEVSRKNC targeted to the apoptotic neurons in the IS tissue to exert antioxidant effect. Moreover, the PNzyme/MnO<sub>2</sub> prolonged the blood-circulation time and exhibited effective thrombolytic action, and reduced the ischemic damages and neuroinflammation in brain tissues. Similarly, Wang et al and Zhang et al used SHp-modified nanoparticles that could selectively target the site of cerebral ischemia and co-localize with neurons undergoing apoptosis in ischemic brain tissue.<sup>72,73</sup> In addition, the targeted delivery of nanoparticles to the stroke site can be achieved by attaching other stroke-targeting peptides (eg, T6 or T7 peptides) to the surface of liposomes, cyclodextrins, or nanozymes.<sup>83</sup>

### Mitochondria-Targeting Peptide Bendavia

The mitochondria-targeting peptide Bendavia (SS-31) (a member of the Szeto-Schiller peptide family) is a short peptide that freely crosses cell membranes and selectively binds to cardiolipin, independent of the mitochondrial transmembrane potential, which is capable of increasing the mitochondrial accumulation 1000 to 5000-fold.<sup>64,92</sup> Wang et al instructed the SS-31 peptide-modified multifunctional nanoparticles with ROS-responsiveness and mitochondrial-targeted (SPNPs). The SPNPs encapsulated in a thermo-sensitive gel were delivered to ischemic penumbra via nose-to-brain pathway. Apparently, the SPNPs were shown using MitoTracker Red staining to achieve mitochondrial targeting with the help of SS-31 rapid accumulation in the ischemic penumbra. The thioketal cross linked skeleton of SPNPs were fractured in response to the ROS in the oxidative microenvironment, which exerted ROS-scavenging activity and achieved controlled



**Figure 2** The different ligands-mediated targeting for crossing BBB and targeting ischemic region. **(a)** Schematic illustration of the biomimetic synthesis of a novel peptide-templated manganese dioxide nanozyme (PNzyme/MnO<sub>2</sub>) and its application as a smart multifunctional therapeutic for the treatment of ischemic stroke. Reprinted from Wang Z, Zhao Y, Hou Y, et al. A thrombin-activated peptide-templated nanozyme for remedying ischemic stroke via thrombolytic and neuroprotective actions. *Adv Mater.* 2024;36(10):e2210144. © 2023 Wiley-VCH GmbH.<sup>74</sup> **(b)** Triple targeting SS31-HA-QT and its fluorescent indicator IR-780-SS31-HA-QT exerts neuroprotective effect against cerebral ischemia. Reprinted from Cen J, Zhang R, Zhao T, et al. A water-soluble quercetin conjugate with triple targeting exerts neuron-protective effect on cerebral ischemia by mitophagy activation. *Adv Healthcare Mater.* 2022;11(22):e2200817. © 2022 Wiley-VCH GmbH.<sup>64</sup>

release of loaded Puerarin. Triggered by abundant ROS at mitochondria, ROS-responsive skeleton in SPNPs and SS-31 could eliminate ROS to reduce oxidative stress and cascade inflammatory response. The encapsulated Puerarin release can repair mitochondrial function by maintaining adenosine triphosphate metabolism and membrane function, thereby inhibiting cytochrome C-mediated apoptosis.<sup>46</sup> Additionally, Guo et al constructed a novel delivery system through decorating SS-31 peptide to a water-soluble conjugate hyaluronic acid -Quercetin, which was capable of penetrating the BBB and indiscriminately targeting mitochondria. The delivery system could be targeting to the mitochondria of damaged neurons and exerted a neuroprotective effect by activating mitochondrial autophagy (Figure 2b).<sup>64,77</sup>

### Chlorotoxin

Chlorotoxin (CTX) is a 36-amino acid peptide closely related to the cerebral ischemic microenvironment, for which the upregulated MMP-2 has a high specificity and affinity.<sup>93</sup> CTX had been shown to transiently enhance the BBB permeability and drug delivery to the brain.<sup>94,95</sup> As we all know, conventional targeted drug delivery is achieved using ligands with a high affinity for the ischemic microenvironment, which allows a small fraction of the nanoparticles to enter the ischemic region of the brain. Departing from the aforementioned targeting mechanisms, Zhou et al innovatively used an autocatalytic targeting approach to release the BBB modulator-lexiscan, which in turn transiently enhances BBB permeability, allowing more nanoparticles to enter the ischemic region.<sup>50</sup> Through this secondary autocatalytic mechanism, the delivery process created a positive feedback loop that promoted drug accumulation in the ischemic region. Based on this concept, Zhou et al synthesized the novel nanoparticles using PLGA anchored by CTX and encapsulated as the BBB modulator. The nanoparticles were not only efficiently and precisely accumulated in the ischemic brain, but also the targeting efficiency autocatalytically became superior with following administration. The results showed that the aggregation of nanoparticles on the infarct side was 12 times higher than it on the healthy side.

### Docosahex Aenoic Acids

Approximately 50% of the polyunsaturated fatty acids (PUFA) on cell membranes in the CNS are docosahex aenoic acids (DHA).<sup>96</sup> DHA can pass through the BBB owing to its ability to absorb plasma-unesterified PUFA.<sup>97,98</sup> Wang et al encapsulated Cu4.6O nanoparticles in the diselenide bond-conjugated zein and DHA, which was coated by plate membrane and rt-PA was further conjugated on the plate membrane. The platelet membrane represents a highly promising endogenous carrier for the targeted delivery to thrombotic sites. The structural domains of platelet surface membrane proteins, such as CD62p and CD41, facilitate the evasion of immune clearance and enhance the targeting efficacy towards blood clots. Through the synthesis methods, the nanoparticle was finally constructed, which possessed multiple functions of ROS responsiveness, targeted thrombolysis and neuroprotection. The nanoparticles evaded phagocytosis by macrophages via plate membrane, crossed the BBB based on the DHA receptor-mediated endocytosis of BMECs, and Cu4.6O and DHA would be released in ROS-rich ischemic microenvironment. Therefore, the nanoparticles exerted strong ROS scavenging and neuroprotection abilities and had good therapeutic effects on embolic MCAO rats.<sup>75</sup> Besides, liposomes can selectively aggregate in the ischemic hemisphere and maintain long-term liposome colocalization in the neurovascular unit, thereby enhancing drug accumulation after stroke.<sup>30</sup> Consequently, Wang's group proposed a strategy to form covalent complexes and encapsulate Ginkgolide B liposomes with PUFA. The amount of Ginkgolide B liposomes-DHA targeted to the ischemic hemisphere was 2.2 times that of the free solution, which increased the accumulation of Ginkgolide B in the ischemic area.<sup>99</sup>

### Complement Component 5a

Inflammation-related molecules highly expressed in the ischemic region can be used in targeting strategies. Complement component 5a (C5a), an anaphylatoxin consisting of 74 amino acids, is highly expressed in the ischemic penumbra, and can thus be a potential target for drug delivery. C5a binds to its receptor on microglia and releases a variety of pro-inflammatory cytokines that further promote inflammatory responses.<sup>100,101</sup> Given their capability of precise chemical modification and great potential to target ischemia, anti-C5a aptamers specifically target C5a and block C5a binding to the C5a receptor to inhibit inflammatory responses. Therefore, based on the biocompatibility and abundance of binding sites of poly (acrylic acid), Peng et al combined the anti-C5a aptamer onto the poly (acrylic acid)-encapsulated and fluorescein isothiocyanate-labeled SiO<sub>2</sub> nanospheres, which could be regarded as scaffolds of anti-C5a aptamer and other



drugs.<sup>65</sup> The “navigation” - amino-modified anti-C5a aptamers were covalently cross linked with poly (acrylic acid) of nanospheres by pH-sensitive amide bonds. The fluorescein isothiocyanate label could track the real-time delivery institution in vivo. Effectively, the nanospheres targeted the ischemic penumbra, alleviated oxidative stress and polarized microglial phenotype to suppress inflammatory response in ischemic stroke.

### C-X-C Motif Chemokine Receptor 4

C-X-C motif chemokine receptor 4 (CXCR4) is a chemokine receptor that is highly expressed in ischemic tissues. AMD3100, a fully characterized CXCR4 antagonist, has previously been used as a ligand for targeted drug delivery in CXCR4-overexpressing diseases.<sup>102,103</sup> Thus, Sheth et al attached AMD3100 to the surface of antioxidant betulinic amine, which was preferentially abundant in the ischemic brain and could enhance medication release in acidic environments. This approach could be used as an effective vehicle for targeted delivery of therapeutic drugs to the ischemic brain in addition to treating ischemic stroke.<sup>55</sup> Furthermore, based on the fact that nanoparticles can expand or shrink their size by reacting to enzymes enriched in the stroke microenvironment, Zhou et al assessed the delivery efficiency of nanoparticles in different formulations to MCAO mice. Zhou's group constructed nanoparticles using block copolymers consisting of PEG, poly ( $\epsilon$ -caprolactone), and enzyme-cleavable peptides. Then the AMD3100 was conjugated to the surface of nanoparticles above, which performed the maximum delivery efficiency. After thrombin trigger, the average size of the nanoparticles gradually decreased from 218.2 nm to 79.3 nm over 24 h and accumulated 30 times more in the ischemic region than control nanoparticles without ligands.<sup>53</sup>

### Cell-Membrane Mediated Strategies

The emergence of cell membrane-based biomimetic carriers provides new avenues for BBB recognition and transport as well as for biomultifunctionality and high biocompatibility. In recent years, cell membrane-camouflaged nanocarriers derived from neutrophils, macrophages, microglia, erythrocytes, neural stem cells, and cancer cells have shown great potential as IS therapeutic drug delivery systems.<sup>78,104,105</sup> At the onset of ischemia, leukocytes in the peripheral circulation are activated and recruited to the ischemic brain region, where they communicate with endothelial cells via receptor-ligand interactions and transport, cross the BBB, and release payload drugs into the inflamed reconstituted endothelium. Encapsulating cell membranes from leukocytes have been shown to confer nanoparticles the ability to precisely target inflammatory lesions. Besides largely improving targeting capability, as the native component in the systematic circulation, cell membrane coating by as drug carriers also improves bio-capacity and reduce immunogenicity, and thus extend the circulation time of the system in vivo and eliminate potential peripheral side effects.<sup>106</sup>

### Neutrophils

As the fastest immune cells to respond to inflammation, neutrophils are the first leukocytes to be activated and recruited from the periphery to the ischemic brain region. Neutrophils can detect the concentrations of inflammatory chemokines through their cytokine receptors (including CXCR2, CXCR4, integrin CD11b, lymphocyte function-associated antigen 1 (LFA1), very late antigen 4 (VLA-4) and migrate to the site of injury.<sup>107,108</sup> Intercellular adhesion molecule (ICAM)-1 expression is upregulated in inflamed BMECs after IS.<sup>109</sup> Neutrophils also interact with endothelial cells through the expression of integrin  $\beta$ 2, macrophage-1 antigen, and LFA1 on neutrophil membranes.<sup>110,111</sup> Moreover, RMT is enhanced after IS, and neutrophils actively move towards endothelial junctions by rolling, adhering, and crawling, eventually migrating to the BBB.<sup>112,113</sup> N-acetyl Pro-Gly-Pro (PGP) is an endogenous tripeptide derived from extracellular matrix degradation, which can act as a ligand with high affinity to CXCR on neutrophils.<sup>114</sup> Based on the properties of neutrophils, the main ideas of researchers to design nanomaterials include nesting neutrophil membranes to the surface of synthetic substrates or drug delivery function by means of interaction with peripheral blood neutrophils.

On the one hand, nanoparticles can be encapsulated directly using natural neutrophil membranes. Therefore, Wang et al constructed a biomimetic nanosystem (Leo@NM-Lipo) consisting of nanoliposomal leonurine nested with neutrophil membrane. In vivo and in vitro experiments indicated that the cell membrane-to-nanocore weight ratio significantly altered the targeting and therapeutic efficacy of biomimetic nanosystems in addition to changing their physicochemical characteristics. Finally, Leo@NM-Lipo constructed with a low cell membrane protein-to-lipid ratio of 1:10 greatly increased the delivery efficiency of leonurine across the BBB and performed the multiple roles including



anti-oxidant damage, anti-inflammation and anti-apoptosis, which remodeled the inflammatory microenvironment, reduced infarct size and improved neurological recovery.<sup>60</sup> Additionally, the drug delivery system encapsulated by the neutrophil membranes exerted a 5.16-fold targeting effect on the inflammatory microenvironment.<sup>73</sup>

On the other hand, neutrophils can act as a “hitchhiker” in drug delivery.<sup>67,115</sup> Wang et al developed cross-linked dendrigraft poly-L-lysine nanoparticles decorated with PGP. The nanoparticles crossed the BBB and targeted to ischemic brain by PGP binding to neutrophils and neutrophil-mediated inflammatory migration, which demonstrated notable binding effectiveness to neutrophils and effective transport to recipient cells.<sup>116</sup> Zhou et al used a strategy based on bacterial-derived outer membrane vesicles (OMV) hitchhiking on neutrophils to enhance the intracranial delivery of pioglitazone for the treatment of IS. OMVs contain lipopolysaccharides, which are specifically recognized by neutrophils through the toll-like receptor-specific recognition, which promotes endocytosis of vesicles by neutrophils. OMV was extracted by differential centrifugation and pioglitazone was loaded into OMV by electroporation. Ultimately, OMV@PGZ was able to ride on neutrophils to penetrate the BBB into the infarcted brain. When neutrophils reach the ischemic region, excess ROS promoted neutrophil chromatin depolymerisation and formation of neutrophil extracellular traps. OMV@PGZ exhibited the neuroprotective effect through preventing lipid peroxidation, mitochondrial damage, ferroptosis, and the activation of nucleotide oligomerization-like receptor protein 3 inflammasomes while minimizing reperfusion injury (Figure 3a).<sup>66</sup>

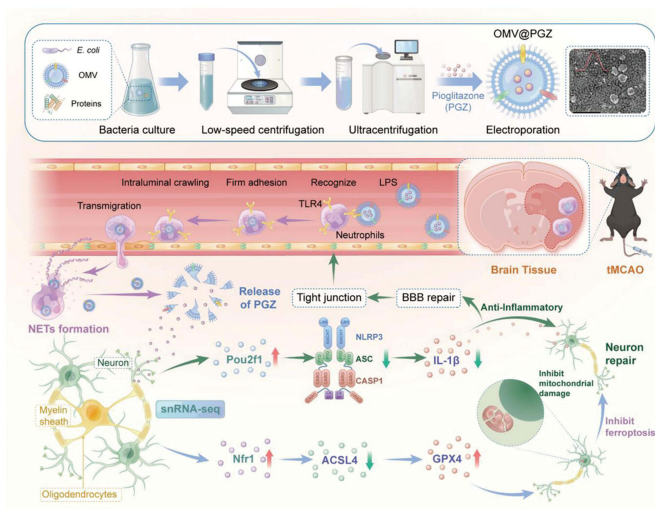
Furthermore, neutrophils are associated with proinflammation, and reducing neutrophil infiltration into ischemic areas is a potential approach to treating stroke. Inspired by this, Zhou et al designed rod-shaped piceatannol-encapsulated PLGA nanoparticles, which were prepared by solvent evaporation method to fabricate Pic@Spheres, followed by stretching the spheres to rod-shaped nanoparticles by film stretching method. Then, the nanoparticles with an aspect ratio of 5 were found to be more likely to be endocytosed by neutrophils. Therefore, the nanoparticles were filtered out to load with piceatannol, which blocked neutrophil adhesion to endothelial cells and inhibited neutrophil infiltration into the BBB. In addition, even if a small fraction of nanoparticles-carrying neutrophils entered the BBB, inflammatory cytokines were reduced in the ischemic brain. The nanoparticles released piceatannol in the ischemic region, inhibited spleen tyrosine kinase signalling from microglia, and alleviated neuroinflammation.<sup>119</sup>

### Macrophages and Microglia

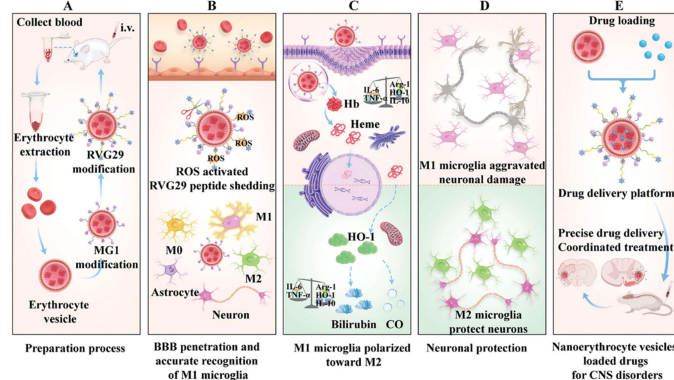
During CIRI, macrophages are recruited to and infiltrate the ischemic brain within hours.<sup>120</sup> ICAM-1 and P-selectin are overexpressed on the luminal side of stressed vascular endothelial cells,<sup>121</sup> and these molecules can interact with the CD44 and CD11b ligands on leukocytes recruited from the peripheral blood to promote infiltration into the lesion, whereas the recruitment is not affected by the degree of opening of the BBB.<sup>122,123</sup> Therefore, Jiang et al developed a macrophage-disguised honeycomb manganese dioxide (MnO<sub>2</sub>) nanosphere loaded with fingolimod (Ma@(MnO<sub>2</sub>+FTY)).<sup>62</sup> The macrophage enabled Ma@(MnO<sub>2</sub>+FTY) to have inflammation-directed chemotaxis and promoted the recruitment of CD11b and CD44 to the lesion via a volume-dependent caveolin-mediated endocytic pathway into the cells, thus crossing the BBB and accurately delivering drugs to the ischemic penumbra. The manganese dioxide nanospheres could consume excess hydrogen peroxide, convert it to oxygen demand, and break it down in acidic lysosomes to release fingolimod, which reduced oxidative stress, promoted phenotypic transition of microglia, and ultimately reversed the pro-inflammatory microenvironment and enhanced the survival of damaged neurons.<sup>79</sup>

Microglia are versatile macrophages found in the brain. Ischemia-activated anti-inflammatory M2 microglia are preferentially recruited to infarcted areas because lymphocyte LFA-1 on the surface of M2 microglia has a strong affinity for ICAM-1.<sup>59,124</sup> Previous studies using an in vitro BBB model have shown that the encapsulation of M2-microglia membranes significantly enhanced the BBB penetration of the nanovehicles, leading to an outstanding ability to target cerebral infarction regions.<sup>71</sup> Therefore, Sha et al designed a drug-free biomimetic nanovehicle, which was assembled from polyfluorocarbon and Pluronic P123 camouflaged with M2 microglia membranes. The perfluoro undecane-derived amphiphilic polyfluorocarbon was of exceeding ischemia-oxygenation capacity and the Pluronic P123 was uniquely characterized in downregulating MMP-9 and stabilized nanoparticles with excellent biocompatibility. The biomimetic nanovehicle targeted cerebral infarction regions by M2 microglia membranes, exerted neuroprotection effects and ameliorated neurological deficits by increasing oxygen to ischemic regions and inhibiting MMP-9 secretion.<sup>71</sup>

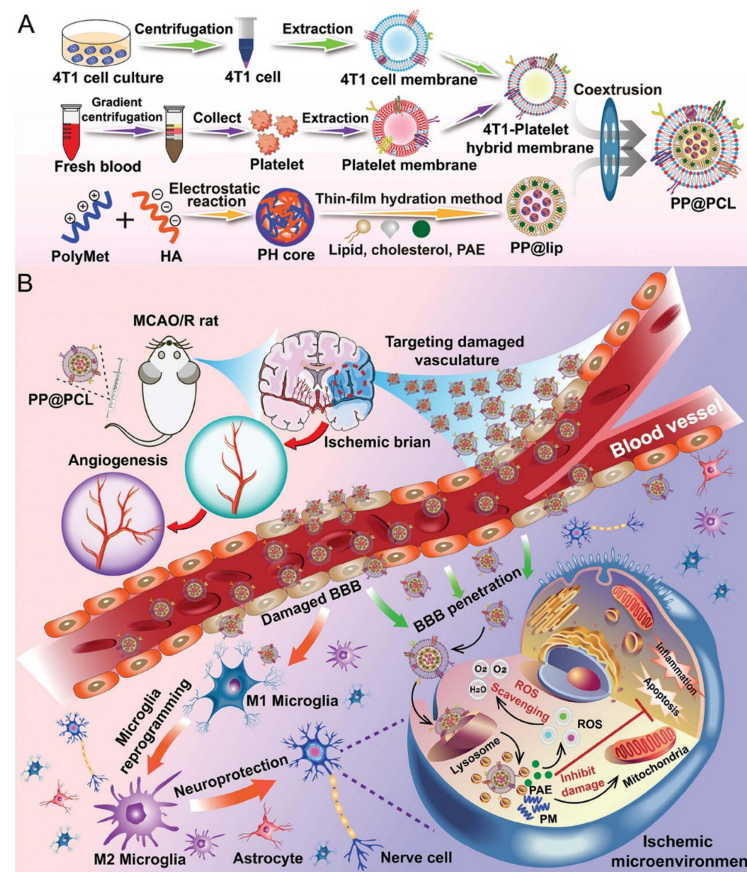
a



b



c



**Figure 3** The cell-membrane mediated strategies for crossing BBB and targeting ischemic region. **(a)** Schematic of ischemic stroke mediated by OMV@PGZ nanoparticles. OMV was extracted by differential centrifugation, and the PGZ was loaded into the OMV by electroporation. After the tail vein enters the blood circulation, OMV@PGZ is recognized and captured by the TLR receptor of neutrophils and brought to the stroke site. Then two pharmacological pathways occur, one is to inhibit the assembly of NLRP3 inflammasomes, and the other is to inhibit ferroptosis. Reprinted from Pan J, Wang Z, Huang X, et al. Bacteria-derived outer-membrane vesicles hitchhike neutrophils to enhance ischemic stroke therapy. *Adv Mater.* 2023;35(38):e2301779. © 2023 Wiley-VCH GmbH.<sup>66</sup> **(b)** Schematic of engineered nanoerythrocyte vesicles formation. Reprinted from Yin N, Zhao Y, Liu C, et al. Engineered nanoerythrocytes alleviate central nervous system inflammation by regulating the polarization of inflammatory microglia. *Adv Mater.* 2022;34(27):e2201322. © 2022 Wiley-VCH GmbH.<sup>117</sup> **(c)** Schematic illustration of the preparation of the nanojacket-cloaked drug-loaded liposome (PP@PCL) (c-A) and therapeutic mechanisms for precise ischemic stroke management. Reprinted from Tang L, Yin Y, Liu H, et al. Blood-brain barrier-penetrating and lesion-targeting nanoplateforms inspired by the pathophysiological features for synergistic ischemic stroke therapy. *Adv Mater.* 2024;36(21):e2312897. © 2024 Wiley-VCH GmbH.<sup>118</sup>

**Abbreviations:** NLRP3, NOD-like receptor thermal protein domain associated protein 3; OMV, outer membrane vesicles; PGZ, pioglitazone; TLR, toll-like receptor; HA, hyaluronic acid; MCAO/R, middle cerebral artery occlusion/reperfusion.

Additionally, Lin et al developed a magnetic field driven, mitochondria-targeted ceria nanosystem (MMTCe) anchored by BV2 cell membranes, which used an external magnetic field to achieve effective delivery of nanosystems in the brain to target damaged mitochondria and repair the ischemic microenvironment.<sup>125</sup>

### Red Blood Cells

Red blood cells (RBC)-based bionic systems have proliferated for drug delivery.<sup>126–128</sup> Critically, molecular signaling on the surface of the erythrocyte membrane, through hydrophilic glycans and negatively charged sialic acid residues, gives the nanoparticles advanced natural properties, such as excellent biocompatibility, better immune evasion, and longer blood circulation time. Typically, erythrocyte membranes are not targeted, but could theoretically increase the chance of passing the BBB by extending the half-life. Current erythrocyte membranes on IS therapy usually contain other targeting modifications at the same time to increase targeting. Xin et al constructed targeted “core-shell” nanoparticles (designated as SHp-RBC-NP), which is consisted of RBC membrane shell with stroke homing peptide (SHp) inserted, and a dextran polymer core modified with ROS-responsive boronic ester. The RBC membrane shell of SHp-RBC-NP reduced reticuloendothelial system clearance and prolong in vivo circulation time through the “don’t eat me” signaling molecule CD47 on the erythrocyte membranes used erythrocyte membrane.<sup>51</sup> Through the pharmacokinetic and pharmacodynamic studies in vivo, the SHp-RBC-NP group had a longer circulation time (more than 48 hours) than the control group. At 24 and 48 h, there was still about 17% and 12% of the SHp-RBC-NP remaining in the blood circulation, while the plain nanoparticles were found to be swiftly removed (could not be detected after 24 h). The successful coating of RBC membranes onto the nanoparticles was validated by the stealth capability of the RBC membrane, which is crucial for the smart bioengineered nanoparticles’ main in vivo mimicking properties.

Modifying nanoparticles by combining erythrocyte membranes with peptides can perform multiple functions of crossing the BBB and precisely targeting ischemic regions. Shi’s group fabricated engineered nanoerythrocytes by physical extrusion, which were doubly modified by MG1 peptide and RVG29 peptide for cascade targeting.<sup>117</sup> RVG29 peptide (YTIWMPENPRPGTPCDIFTNSRGKRASNGC) was conjugated to distearoyl phosphoethanolamine-selenium-selenium-poly (ethylene glycol) (3400)-maleimide (DSPE-Se-Se-PEG3400-Mal) with a ROS-responsive long arm through maleimide chemistry to enhance BBB targeting, which bond to cerebrovascular endothelial cells and improved delivery efficiency effectively. And the MG1 peptide (CHHSSSARC) was also coupled to DSPE-PEG2000-Mal (with a short arm) for precise M1 microglia recognition. After crossing the BBB, shedding of the outer RVG excited by ROS exposed MG1, which enabled precise recognition of microglia. Consequently, the inflammatory microglia absorbed the engineered nanoerythrocytes, which resulted in the release of exogenous heme. Hemoglobin initiated M2 polarization of microglia by inducing heme oxygenase-1, which in turn promotes the expression of endogenous anti-inflammatory cytokines and the production of two potent antioxidants, carbon monoxide and bilirubin, which significantly alleviated the inflammatory environment and had an excellent therapeutic role in the MCAO model. Promisingly, the engineered nanoerythrocytes provided a powerful tool for the precise delivery by encapsulating therapeutic drugs (Figure 3b).

### Cancer Cells

Clinically, some malignant tumors (such as breast cancer cells) have been found to readily cross the BBB to form brain metastases.<sup>129,130</sup> After metastasis, syndecan-1 on the membrane of 4T1 tumor cells binds to platelet endothelial cell adhesion molecule-1 on BMECs, platelets, and leukocytes (monocytes and neutrophils) to achieve adhesion. Similarly, it was found that vascular cell adhesion molecule 1 was overexpressed on the cell membranes of 4T1 cancer cells, which showed high affinity for VLA-4 on leukocytes (eg, lymphocytes, monocytes, and eosinophils).<sup>131,132</sup> Therefore, Sha’s group developed the biomimetic nanovehicles by camouflaging a succinobucol-loaded pH-sensitive polymeric nanovehicle with a 4T1 cell membrane. After encapsulation of 4T1 cell membrane, the nanovehicles could be preferentially delivered to the ischemic hemisphere, which is 4.79 times higher than that of the normal hemisphere in the MCAO rat model. The 4T1 cell membrane not only made the nanovehicles penetrate the BBB to target inflammation sites in the ischemic parenchyma, but also had a prolonged circulation time through the signaling of CD47, which was highly expressed on the cell surface. Additionally, the nanovehicles were preferably delivered in a biomimetic manner to the stroke-affected sites, and were internalized by a wide variety of brain cells, and responded to the intracellular acidic



environment by releasing succinobucol, which had antioxidant and anti-inflammatory effects, thereby attenuating CIRI.<sup>61</sup> Furthermore, Wang et al originally developed a bio-derived nanojacket by fusing 4T1 tumor cell membrane with platelet membrane, which further clothes on the surface of paeonol and polymetformin-loaded liposome to obtain biomimetic nanoplatforms (PP@PCL) for ischemic stroke treatment.<sup>118</sup> The PP@PCL consisted of hydrophobic therapeutic agent PAE (encapsulated in the phospholipid bilayer of liposome) and hydrophilic and cationic polymetformin, which was fabricated by coextrusion according to well-established protocols. The innovative nanoplatforms exhibited the capability of penetrating the BBB, characteristic of CM, as well as the ischemic lesion-targeting ability inherent to PM, making it an exceptional delivery mechanism for overcoming the BBB to achieve precise drug transport. Significantly, the PP@PCL not only safeguarded damaged neuronal cells from additional harm by scavenging excessive ROS, mitigating mitochondrial dysfunction, and inhibiting neuroinflammation, but also restructured the ischemic microenvironment by polarizing microglial cell phenotypes, promoting angiogenesis, and reducing the secretion of pro-inflammatory cytokines, which remarkably alleviated ischemia-reperfusion injury and performed the neuroprotective influence (Figure 3c).

### Neural Stem Cells

Neural stem cells (NSCs) can spontaneously migrate to the vicinity of IS damage and communicate with damaged cells for precise targeting.<sup>56,133</sup> Thus, NSCs are regarded as carriers in CNS diseases. Endogenous NSCs actively home to damaged brain regions after IS, and then proliferate and differentiate into damaged cell phenotypes to self-repair and rebuild neural circuits. However, spontaneous recovery based on endogenous NSCs is insufficient for full functional recovery.<sup>134,135</sup> Previous studies have found that implanted NSCs secrete trophic factors, such as human insulin-like growth factor 1, vascular endothelial growth factor, epidermal growth factor and basic fibroblast growth factor to reduce the infarcted area.<sup>136</sup> Gao et al engineered gene transfection of NSCs overexpressing brain-derived neurotrophic factor (BDNF), and after intravenous injection in MCAO mice, the transfected NSCs (BDNF-NSCs) crossed the BBB and homed to the ischemic region just as efficiently as the original NSCs and produced BDNF more efficiently, leading to a significant increase in BDNF levels, which increased survival rate of the mice to a 60% and enabled faster and better neurological reconstruction.<sup>56</sup>

### Extracellular Vesicles

EVs are a heterogeneous collection of membrane-bound carriers with complex cargoes including proteins, lipids, and nucleic acids.<sup>137</sup> As natural intercellular shuttles, EVs have low immunogenicity and biodegradability, ability to encapsulate endogenous bioactive molecules, and capacity to cross the BBB. Previous studies have demonstrated that EVs can break through the intact BBB in vivo through transcytosis.<sup>138</sup> Stem cell-derived EVs have emerged as a new therapeutic agent for immunosuppression.<sup>139</sup> Gao et al used neural progenitor cells-derived EVs harboring the Arg-Gly-Asp (RGD) peptide (ACDCRGDCFC),<sup>57</sup> which is a ligand capable of binding to integrin  $\alpha_v\beta_3$  (integrin  $\alpha_v\beta_3$  is significantly expressed on endothelial cells in ischemic tissue, but not on blood vessels in normal tissue).<sup>140</sup> After intravenous injection, neural progenitor cell-derived EVs were found to be capable of targeting ischemic brain lesions and exhibiting intrinsic anti-inflammatory activity.<sup>141</sup> Stem cells are activated and recruited to the vicinity of the lesion after IS. Recruited stem cells can interact with damaged BMECs and cross the inflamed BBB through VLA-4, and thus enter the lesion site through the combination of VCAM-1/VLA-4.<sup>142</sup> Gao et al conceived stem cell-derived bio-responsive vesicles equipped with the functional ligand VLA-4 and packed with metformin, which could reverse the inflammatory response in BMECs, rapidly restore BBB integrity, and downregulate VCAM-1 expression.<sup>68</sup> With the alleviation of inflammation after therapeutic drug release, repair of BBB integrity, and downregulation of VCAM-1 expression, stem cell-derived vesicle migration and action are terminated.<sup>143</sup> Altered targeting capacity due to changes in the inflammatory environment confers stem cell bioreactivity, allowing for a smarter and more controllable drug delivery platform.

## Strategies for Increased Permeability of the BBB

Selective, controlled and reversible transient increase in BBB permeability is a potential delivery strategy of crossing BBB. Ultrasound and microbubble-mediated opening of the BBB represents a non-invasive and effective technique for targeted drug delivery to specific brain regions. The acoustic bubbles and targeted microbeads were proved to induce

reversible  $\text{Ca}^{2+}$  signaling and enhance paracellular transport in endothelial monolayer.<sup>144</sup> Some clinical trials are currently being conducted to demonstrate that ultrasound and microbubble-mediated techniques can facilitate localized, safe, temporary, and reversible opening of the blood-brain barrier in the human hippocampus (NCT03671889, NCT02253212). Davies et al explored Acoustic Cluster Therapy (ACT), which induced controlled volumetric oscillations of ACT bubbles at a lower frequency (500 kHz), thereby exerting biomechanical forces on capillary walls to enhance the regulation of BBB permeability.<sup>145</sup> Laser-induced stimulation of nanoparticles also modulates the BBB permeability.<sup>146,147</sup> Qin et al designed picosecond laser excitation of tight junction-targeted gold nanoparticles. Their study demonstrated that the interaction between gold nanoparticles and laser pulses can transiently open the BBB, with the permeability alterations being adjustable through laser intensity and entirely reversible. The findings indicated that targeting endothelial glycoproteins achieved a targeting efficiency over ten times greater than that of targeting tight junctions. Importantly, this strategy for modulating the BBB did not induce hemodynamic changes or cause significant disruption to the structure of the neurovascular unit.<sup>148</sup>

## Strategies for Targeted Controlled Release at Ischemic Region

To improve therapeutic efficacy and minimize complications, drug delivery systems have been designed to not only accumulated in the targeted area, but also trigger the release of the payload at the ischemic lesion site.<sup>149</sup> Modified moieties on nanomaterials can respond to the complex microenvironment at the ischemic site, for example to the pH conditions, ROS levels, presence of inflammatory factors.<sup>72</sup> Alternatively, hypoxia and thrombin responsiveness subtly employ ischemic site characteristics to implement a spatiotemporally targeted controlled release.

### ROS-Responsive Release

High ROS levels in ischemic regions can serve as intelligent and sensitive triggers for controlling drug release and can be applied to the development of site-specific drug delivery systems. Mitochondrial dysfunction in cerebral ischemia leads to oxidative stress and increased production of ROS, a class of oxygen-derived chemicals including hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide anions ( $\text{O}_2^{\cdot-}$ ), superoxide and hydroxyl radicals.<sup>150,151</sup> For increased ROS levels at the IS site, ROS-sensitive groups (aryl-boronate esters/acids, sulphides, thioethers, thioketal linkage, and proline) nanozymes can be engineered to construct ROS-responsive nanomaterials to deliver therapeutic drugs in a spatially and temporally controlled manner.<sup>152,153</sup>

Among these sites, thioketal linkers sensitive to  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  are ideal candidates for the construction of ROS-responsive nanoparticles.<sup>154</sup> It has been shown that sulfide groups can be converted to more polar hydrophilic groups (sulfoxide and sulfone) upon exposure to ROS, and often this increase in polarity/solubilization is used as a ROS-responsive release mechanism for encapsulated payloads for targeted drug release.<sup>155</sup> Drug-loaded nanopharmaceutical delivery systems prepared by Wang et al 's with the thioketal linker crosslink skeleton had been shown to break under the action of microenvironmental ROS, exerting ROS-eliminating activity and achieving the controlled release of loaded Puerarin, leading to oxidative stress-reducing therapeutic effects.<sup>46</sup> The nanoparticles were cross linked using the  $\text{H}_2\text{O}_2$ -sensitive cross linker, and the cumulative release of Puerarin from the system was approximately 48% in the presence of  $\text{H}_2\text{O}_2$ . In contrast, only approximately 18% of the Puerarin was released in the absence of  $\text{H}_2\text{O}_2$ . This suggested that the Puerarin release behavior was dependent on  $\text{H}_2\text{O}_2$  and that the accelerated release profile occurred in a ROS environment. In addition, Jiang et al<sup>69</sup> and Wang et al<sup>75</sup> doped diselenide bonds into nanomaterials, which converted the diselenide bonds within the materials to hydrophilic selenic acid under ROS stimulation to achieve synchronous ROS depletion and responsive drug release at the ischemic hemidiaphragm. The intelligent multifunctional delivery system developed by Xin et al, on the other hand, exploited the ability of thioketal functional group to be rapidly cleaved into non-toxic mercaptan and acetone products when exposed to ROS to release drugs in response to pathological ROS environment.<sup>70</sup>

Furthermore, borate, a smart ROS-responsive biomaterial, can be doped into nanoparticles via self-assembly.<sup>156,157</sup> Xin et al designed a ROS microenvironment using overexpressed ROS in IS lesions as a smart drug release switch, with phenylboronic ester modified dextran polymer vesicles carrying the neuroprotectant NR2B9C as the core, covered with a red blood cell membrane coat modified with the cerebral ischemia-homing peptide SHp. Ultraviolet-visible spectra

showed that 95% of the nanoparticles were hydrolyzed within 30 min in a medium containing 1 mM  $H_2O_2$ , suggesting that functional ROS triggered the sensitivity of nanoparticles made of phenylboronic ester modified dextran, promoting the rapid release of neuroprotective drugs in response to ROS to exert neuroprotective effects.<sup>51</sup> By using ROS-responsive charge-reversal poly[(2-acryloyl) ethyl (p-boronic acid benzyl) diethylammonium bromide] (B-PDEA), Gao et al could effectively condense the BDNF gene into polymeric B-PDEA nanoparticles, which could effectively protect DNA and be taken up by the cells. After internalization, B-PDEA oxidizes intracellular ROS into negatively charged polyacrylic acid, which rapidly releases the BDNF plasmid for efficient transcription and secretion of high levels of BDNF.<sup>56</sup> The ROS- responsiveness of B-PDEA not only led to the rapid release of plasmids with high levels of ROS from NSCs in the IS region, but also depleted some ROS, thereby alleviating ROS stress.<sup>158</sup>

Using cyclodextrin-sourced materials, Zhang et al<sup>72</sup> and Wang et al<sup>73</sup> constructed a ROS-responsive nanocarrier, which was triggered by pathological signals with a significantly enhanced function owing to a significant reduction in particle size, morphological alterations, and chemical switching of cellular surface uptake by brain endothelial cells. This ROS-responsive and transformable nanoplatform exhibited significantly higher brain drug accumulation in a mouse model of IS than non-responsive nanocarriers.

### pH-Responsive Release

Ischemic injury lowers pH by shifting the metabolism of the ischemic brain from aerobic to anaerobic glycolysis, resulting in the accumulation of lactate and protons. Through magnetic resonance spectroscopic imaging, the ischemic region of the brain was found to be acidic with a pH of 6.0 to 6.8. Acidosis persisted for more than 2 days, while the pH of the contralateral normal tissue remained neutral.<sup>55</sup> Considering the inherent pH gradient, brain- targeted nanoparticles with pH responsiveness may hold great promise for drug release in specific intracellular cavities. Typically, pH-responsive NPs are designed to deform (by swelling, dissolving, or collapsing) under the pH conditions of intracellular lysosomes, or have unstable linkers capable of cleavage under acidic pH conditions for efficient intracellular drug release.

Peng et al designed the “nano-courier”, which was based on the biocompatibility of poly acrylic acid and abundant binding sites. The ligand molecules were covalently cross linked to the receptor through pH-sensitive amide bonds, resulting in the controlled release of acetylcholine and melatonin triggered by the low pH in the ischemic region.<sup>65</sup> Similarly, Sha et al combined an amphiphilic pH-sensitive polymer of methoxypoly (ethylene glycol)-block-poly (2-diisopropyl methacrylate) onto a nano-loaded platform that released almost no drug at pH 7.4 but almost all at pH 4.7, further confirming that the pH-responsive approach was capable of the rapid release of loaded drugs.<sup>61</sup>

To design pH responsiveness, researchers had also considered the pH environment in which the neuroprotective drugs function. Sheth et al found that betulinic acid (BA) was one of the most potent antioxidants for the treatment of stroke, but that BA was released slowly at physiological pH. Encapsulated drug release is less than 30% in the first 24h, with complete release taking more than 6 days.<sup>159</sup> Therefore, given the narrow therapeutic time window of acute IS, BA was chemically modified to betulinic amine through the chemical conversion of the carboxyl group at the edge of the BA ring to an amino end group. This resulted in preferential release in the acidic ischemic brain tissues, with a release of 75% in 24 hours.<sup>55</sup>

### Thrombin-Responsive Release

Thrombin is a trypsin-like serine proteinase that plays a crucial role in the process of thrombosis, in which a large amount of thromboplastin is activated and induces the accumulation of thrombin at the thrombus site.<sup>160</sup> Thus, the thrombin-triggered release of rt-PA into the thrombus has great potential with a rapid response rate owing to its high sensitivity. Up-regulated thrombin triggers rt-PA release around the thrombus, converting fibrinogen to fibrinolytic enzymes, and inducing gradual loosening of the thrombus, which dissolves under the high shear forces of blood flow.<sup>161</sup> Fan et al devised a novel peptide-templated manganese dioxide nanozyme (PNzyme/ $MnO_2$ ), where thrombolysis is activated by thrombin. The thrombolytic peptide Gly-Arg-Pro- Ala-Lys (GRPAK) is released from the peptide after cleavage by thrombin at the thrombogenic site to initiate thrombolysis.<sup>74</sup>



Zhou et al suggested that, in addition to the surface affixation of targeted ligands, the delivery of nanoparticles in stroke therapy can be improved by designing nanoparticles that respond to the thrombin in hypoxic microenvironment and subsequently change their size. Variable-size nanoparticles were synthesized using block copolymers with peptides that can be cleaved by enzymes that are enriched in an ischemic microenvironment. This study confirmed that MMP-9 is an alternative protease for protease-triggered drug delivery in the treatment of stroke, and that thrombin and MMP-9 expression in the ischemic brain was significantly elevated 3–8 hours after ischemic injury. A block copolymer was used to synthesize swellable nanoparticles containing either thrombin-cleavable NH<sub>2</sub>-norleucine-TPRSFL-C-SH or NH<sub>2</sub>-LGRMGLPGK-C-SH (MMP-9 cleavable peptide), both of which responded within 5 min in the presence of protease. Comparing the drug delivery efficiency of the two, it was found that the nanomaterials containing thrombin-cleavable peptides were 2.2 times more efficient than those containing protease-cleavable peptides.<sup>53</sup> Similarly, Jiang et al conjugated polymers via the thrombin cleavable peptide NH<sub>2</sub>-norleucine TPRSFL-C-SH.<sup>162</sup> When used for nanoparticle construction, the resulting nanoparticles responded to thrombin, a protease that is highly enriched in the ischemic microenvironment, by shrinking in size to promote antioxidant release. This study further confirmed that designing nanoparticles to respond to the ischemic microenvironment by shrinking their size significantly enhances brain penetration and improves the efficiency of ligand-mediated targeting, although stimulus responsiveness may not provide higher efficiency than ligand-mediated approaches.<sup>54</sup>

### Hypoxia-Responsive Release

Engineered nanomedicine delivery systems based on the tissue microenvironmental response of lesions to achieve site-specific targeted delivery have received significant attention and represent a promising new strategy for targeted delivery. Therefore, the construction of an efficient targeted delivery system that incorporates the tissue microenvironmental characteristics of IS will provide a new way to enhance the efficiency of neuroprotective drug delivery and improve patient prognosis.

When assessing the microenvironment of infarcted tissue, it was found that a hypoxic environment coexists with the upregulation of cytochrome P450 reductase and inducible nitric oxide synthase enzymes, confirming the feasibility of the hypoxic response in IS.<sup>76</sup> The upregulated co-existence of reductases confirmed the feasibility of a hypoxic response in IS. The hypoxic features of IS provided an important breakthrough for further research on therapeutic strategies for IS. Gao et al prepared azocalixarene (CA), -grafted self-assembled peptide hydrogels, which were expected to serve as an intelligent hypoxia-responsive drug library for the on-demand release of anti-inflammatory drugs catalyzed by compliant reductases under hypoxic conditions, while maintaining their stability in the normal physiological state. CA had been utilized as a hypoxia-responsive controlled-release drug carrier. The complexation between CA and the drug was stable under normal conditions and hypoxia-responsive release was achieved by mimicking the reductase. Hypoxia-responsive hydrogels were prepared by phosphate buffer saline-induced  $\beta$ -fold formation following a click reaction between CA containing methacrylate groups and thiol-containing Q11 peptides.

## Clinical Application and Challenges of the Targeted Delivery Strategies of Nanosystems

Fewer clinical trials of nanosystems for IS have been conducted in phase I/II clinical trials, mainly focusing on exosomes from mesenchymal stem cells (NCT03384433/NCT06138210/NCT05326724), liposomes (NCT00507806) and metal nanoparticles (NCT06495671). In the small clinical sample size study (NCT02963376), intravenous dodecafluoropentane emulsion was safe with clinically improvements in NIHSS scores and mRS of IS patients (n=24, no statistically differences).<sup>163</sup> As an adjunctive therapy of reperfusion treatment, nanoparticle-mediated magnetically enhanced diffusion restored blood flow of blocked vessels by overcoming stagnant blood flow (NCT06495671). The magnetic nanoparticles may reduce the clot lysis time for reperfusion by 40% when compared to free rtPA at the same drug dosage.<sup>164</sup> Besides, nanosystems can be used as novel biomarkers to assess prognosis and guide the treatment of IS patients making clinical treatment more personalized and targeted (NCT05370105/ NCT06319742).

However, the clinical trials and translation of nanodrug delivery systems encounter significant challenges. Primarily, the complexity of safety assessments for nanosystems poses a major obstacle, as issues such as long-term toxicity and

potential bioaccumulation remain inadequately understood. Additionally, challenges such as low drug loading capacity contribute to the risks associated with clinical applications, thereby confining the research and development of nanomedicines to preclinical studies. Furthermore, technical bottlenecks in the large-scale production of drug delivery nanosystems hinder the achievement of efficient, stable, and cost-effective large-scale preparation, thereby restricting their widespread application.<sup>165</sup>

To address these challenges, researchers are advancing the development of intelligent responsive nanosystems designed to achieve precise drug release in response to microenvironmental changes within the body, thereby enhancing the performance potential of drug delivery nanosystems. Concurrently, during the construction of multifunctional nanosystems, researchers also focused on how to increase the drug loading capacity by innovative strategies and techniques.<sup>166</sup> Three principal methodological frameworks, alongside several innovative technologies, have been developed to achieve high drug-loading in nanoparticles: post-loading, co-loading, and pre-loading strategies. (1) The post-loading strategy typically utilizes carrier materials characterized by a high specific surface area or porous structure (eg, SiO<sub>2</sub>, carbon, metal-organic frameworks), which are subsequently combined with a drug solution. This allows for the drug to be loaded onto the nanoparticles through various mechanisms, including adsorption, electrostatic interactions, encapsulation, and hydrophobic forces. (2) The co-loading method involves coupling the drug to a polymer or macromolecule, where the drug is covalently attached to the carrier via cleavable bonds (eg, disulfide bonds, ester bonds), thereby enhancing the efficiency of drug loading.<sup>167</sup> (3) The pre-loading strategy entails the initial formation of drug nanoparticles, followed by the encapsulation of these nanoparticles with an additional material layer, resulting in nanoparticles with a drug core and a protective shell structure. To achieve synchronous and efficient delivery of both drugs, Chen et al encapsulated a prodrug into a nanocarrier, which enabled the drug loading capacity to reach as high as 99%, nearly achieving quantitative drug loading.<sup>168,169</sup>

Furthermore, the researchers have advanced novel technologies, such as modular design, microfluidics, 3D printing, and artificial intelligence-driven methodologies, which facilitate the precise construction and large-scale production of delivery nanosystems. By separating the functional modules (performing functions such as targeted, responsive release, imaging, etc.) from the drug-loaded modules, the drug loading capacity can be increased by avoiding the functional modules from crowding the drug space through plug-to-direct assembly.<sup>170</sup> Wan et al designed an innovative “dual chimeric nanoantibody-lipid-anchoring protein” scheme, which not only increased the preparation efficiency by more than three times, but also reduced the payload loss during preparation.<sup>171</sup> Hammond et al performed the engineering nanolayered particles for modular drug delivery, which allows for high drug loadings in nano-scale thin film coatings, thus enabling the delivery of quantities of drug. 3D printing opens a possibility for compartmentalization of modular drug delivery, which can spatially separate drug and functional excipient modules.<sup>172</sup> Zhao et al fabricated the microfluidic devices using polyethylene tubings by 3D and the nanoparticles were varied to synthesize by the flow ratio between the polymer solution and water. The microfluidic-assisted synthesis made the drug loading up to 50% and achieved continuous and homogeneous production of nano systems.<sup>173</sup> AI techniques can simulate different stages of biological processes and determine the proportions and optimal design parameters of the various components used to build delivery nanosystems. First, by simulating the behavior of nanosystems in complex biological environments (eg blood brain barrier), the penetration and distribution of nanosystems are enhanced to overcome biological barriers. Second, by predicting morphology, potentiation and targeted ligands, and optimizing the density and orientation of targeted ligands on the surface of the nanosystems, the drug loading rate and cellular uptake and transport of nanodrugs are increased. Based on the above-mentioned functions, AI-assisted drug delivery makes complex drug systems easy to build and enables faster, more efficient and more precise nanosystems manufacturing.<sup>174</sup> Besides, Santos et al proposed the DELIVER framework containing design, experimental, manufacturing, preclinical, clinical, regulatory and business considerations, which guide researchers to critically examine nanomedicine design and development at an early stage to mitigate risk and research time, as well as to accelerate clinical translation and maximize the impact of nanosystems.<sup>175</sup>

## Future and Prospects

Over the years, researchers have pursued improvements in the targeting and controllability of drugs in stroke therapy. Based on the unique characteristics of nanoparticles including increased drug solubility, reduced adverse drug reactions and enhanced

permeability and retention effect, smart drug delivery nanosystems can be constructed by diverse strategies to achieve targeting goal in IS. In this review, we provided a comprehensive summary of the targeting strategies of drug delivery nanosystems in stroke therapy to support further advances in the development of IS treatment. On the one hand, nanosystems can overcome the BBB and precisely target ischemic sites, mainly through receptor-ligand mediated strategies and cell-membrane mediated strategies, which improves drug accumulation at ischemic sites. In an innovative way, the researchers went beyond using ligands with high affinity for the ischemic site to achieve targeted delivery, and built on this by allowing the nanoparticles to locally release BBB modulators, which in turn transiently enhanced BBB permeability, allowing more nanoparticles to enter the ischemic region. Through this secondary autocatalytic mechanism, the delivery process generates a positive feedback loop that promotes further drug accumulation in the targeted region. On the other hand, specific changes in the microenvironment after ischemia have been used to equip nanosystems with targeted-release switches to improve delivery efficiency. Furthermore, nanosystems that combine thrombolysis and neuroprotectants, drug delivery systems that can target thrombolysis and deliver neuroprotectants to the ischemic brain in a sequential order, further compensate for the limitations of current treatments. Probably, combining approaches is needed to achieve a synergistic increase in the functionality of the nanosystems, and overlay the therapeutic efficacies of revascularization and neuroprotection. In the future, further preclinical and clinical studies are urgently warranted to evaluate the efficacy and safety of nanosystems for facilitating the transformation of scientific innovation to the clinical application.

## Abbreviations

BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; BMECs, brain microvascular endothelial cells; CNS, central nervous system; CIRI, cerebral ischemia-reperfusion injury; CTX, Chlorotoxin; CXCR, C-X-C motif chemokine receptor; DNA, deoxyribo nucleic acid; EVs, extracellular vehicles; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ICAM, intercellular adhesion molecule; IS, ischemic stroke; LDLR, low-density lipoprotein receptor; LFA1, lymphocyte function-associated antigen 1; MMP, matrix metalloproteinase; NLRP3, NOD-like receptor thermal protein domain associated protein 3; PLGA, poly (l-lactic-co-glycolic acid); PUFA, polyunsaturated fatty acids; RMT, receptor-mediated transcytosis; ROS, reactive oxygen species; TfR, transferrin receptor; VLA-4, very late antigen 4.

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## Disclosure

The authors report no conflicts of interest in this work.

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