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MN_3O_4@NANOERYTHROCYTE-T7 PEPTIDE: A BIOMIMETIC NANOPLATFORM FOR TARGETED ISCHEMIC STROKE THERAPY

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ABSTRACT

Ischemic stroke is a major cause of mortality and long-term neurological disability worldwide, primarily resulting from blockage of cerebral blood vessels and subsequent ischemia-reperfusion injury. Excessive production of reactive oxygen species (ROS), inflammation, neuronal apoptosis and blood-brain barrier (BBB) disruption significantly contribute to neurological damage following stroke. Conventional therapies mainly focus on restoring blood flow but provide limited protection against oxidative stress and reperfusion-associated injury. Therefore, the development of multifunctional neuroprotective nanomedicines has emerged as a promising therapeutic strategy. Nanosponges are porous, biocompatible nanoparticles capable of encapsulating both hydrophilic and lipophilic substances, offering advantages such as enhanced stability, targeted delivery and improved therapeutic efficacy. Among these, Mn_3O_4@nanoerythrocyte-T7peptide (MNET) represents an innovative biomimetic nano sponge system designed for targeted ischemic stroke therapy. This formulation consists of manganese oxide (Mn_3O_4) nanoparticles enclosed within nano erythrocyte membranes and functionalized with T7 peptide to enhance BBB penetration and brain targeting. The Mn_3O_4 nanoparticles exhibit potent ROS-scavenging nanoenzyme activity, thereby reducing oxidative damage in ischemic brain tissue. The nano erythrocyte membrane improves biocompatibility, prolongs circulation time and supports adaptive oxygen regulation during ischemia brain regions. Experimental studies have demonstrated that MNET significantly reduces infarct volume, decreases neuronal apoptosis and improves neurological recovery. Overall, Mn_3O_4@nanoerythrocyte-T7 offers a promising biomimetic nanotherapeutic approach for ischemic stroke management by simultaneously addressing oxidative stress, hypoxia and reperfusion injury, thereby presenting potential advantages over conventional stroke therapies.

KEYWORDS: Biomimetic nanomedicine, Neuroprotection, Ischemia-reperfusion injury, Nano neurology, Brain targeting.

INTRODUCTION

Ischemic stroke is one of the leading causes of death and long-term neurological disability worldwide. It occurs when a blood vessel supplying the brain becomes blocked, resulting in reduced oxygen and nutrient delivery to brain tissue. The subsequent ischemia and reperfusion processes generate excessive reactive oxygen species (ROS), inflammation, neuronal apoptosis, and blood-brain barrier (BBB) damage, which significantly worsen neurological injury.

Conventional therapies such as thrombolysis and mechanical thrombectomy primarily restore blood flow but do not adequately protect neurons from oxidative stress and reperfusion injury. Therefore, the development of multifunctional neuroprotective nanomedicines has become an important research focus in modern stroke therapy.

Nanosponges are three-dimensional, solid, porous, biocompatible nanoparticles that can encapsulate both hydrophilic (water-soluble) and lipophilic (fat-soluble)

substances. Roughly the size of a virus (typically under 1 micrometre), these tiny structures feature an intricate mesh-like network of microscopic cavities capable of carrying or absorbing diverse molecules. The nanosponge drug delivery system possesses various applications in various ailments such as cancer, autoimmune diseases, theranostic applications, enhanced bioavailability, stability, etc.

Mn_3O_4@nanoerythrocyte-T7, also called **MNET**, is an experimental biomimetic nanomedicine {Nanosponges} designed for treatment of acute ischemic stroke and ischemia-reperfusion injury.

Mn_3O_4@Nanoerythrocyte-T7 (MNET) is a bioinspired nanosponge system developed to treat ischemic stroke through targeted antioxidant and oxygen-regulating mechanisms. The formulation consists of manganese oxide (Mn_3O_4) nanoparticles encapsulated within nano erythrocyte membranes and modified with T7 peptide for enhanced brain targeting and BBB penetration.

\The Mn_3O_4 nanoparticles function as nanoenzymes with strong ROS-scavenging activity, helping reduce oxidative damage in ischemic brain tissue. The nano erythrocyte membrane, derived from red blood cells, improves biocompatibility, prolongs, blood circulation, and provides self-adaptive oxygen regulation during ischemia and reperfusion. Meanwhile, the T7 peptide facilitates targeted delivery across the BBB to ischemic regions of the brain.

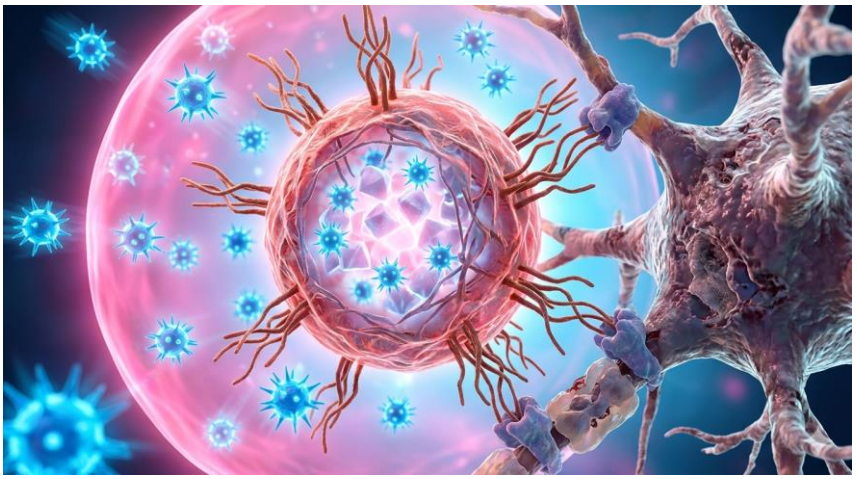

Fig. 1: A 3D medical illustration of a biomimetic nanoerythrocyte for ischemic stroke.

Through the combined effects of antioxidant activity, oxygen homeostasis regulation, immune evasion, and targeted brain delivery. Mn_3O_4@nanoerythrocyte-T7 has shown promising neuroprotective effects in experimental ischemic stroke models, including reduced infract volume, decreased neuronal apoptosis, and improved neurological recovery.

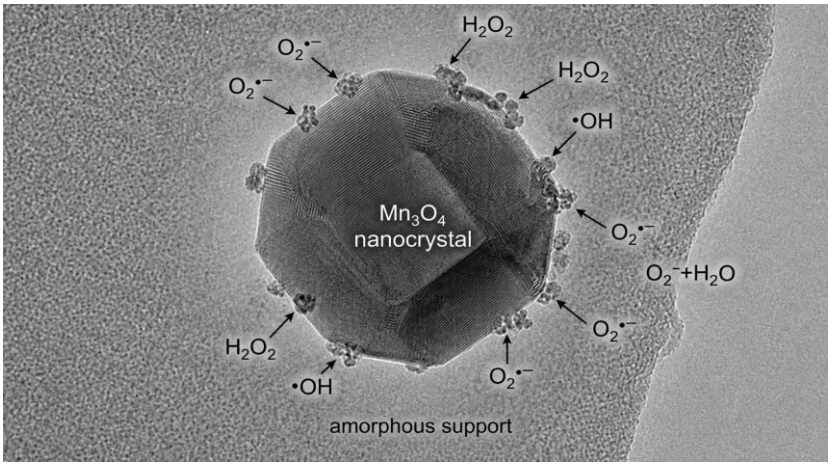

Fig. 2: Visual representation of multi-enzyme like catalytic activities on the facets of an individual Mn_3O_4 nanocrystal support structure.

This biomimetic nanoplatform represents an emerging therapeutic strategy in nano neurology and targeted stroke treatment, offering potential advantages over conventional therapies by simultaneously addressing oxidative stress, hypoxia, and reperfusion injury.

Composition

I) Mn_3O_4 nanoparticles (the core)- Mn (II, III) shows action of potent nanoenzymes. It exhibits superoxide dismutase (SOD) & catalase activities. Superoxide dismutase allows them to scavenge toxic free radicals (ROS) generated during an ischemic stroke.

II) Nano erythrocyte (the cloak)- Mn_3O_4 Nanoparticles are encapsulated within natural red blood cell (RBC). These coating particles provides a "stealth effect" to prevents the nanoparticles to extend the circulation time in the blood stream.

III) T7-peptide (the key)- Outer source of the nano vesicles is functionalized with T7 peptides. T7 peptides functions as a specific ligand that targets the Transferrin Receptor (TFR), highly expressed on the brain capillary endothelial cells. Its actively facilitates drug particles to cross the blood brain barrier.

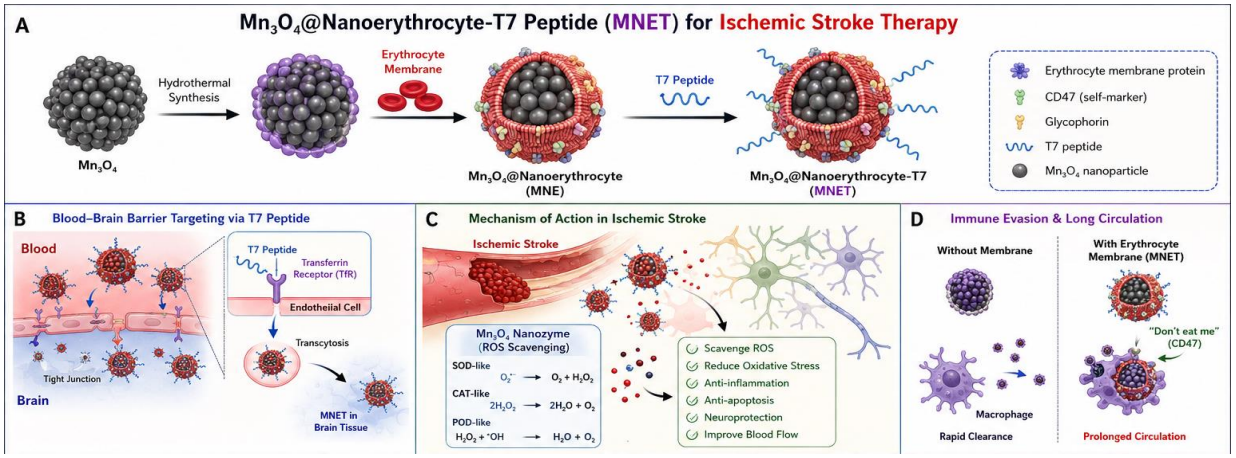

Fig. 3: Schematic illustration of the synthesis, blood brain barrier targeting, therapeutic mechanism and immune-evasive properties of Mn_3O_4@nanoerythrocyte-T7 peptide (MNET) nanozymes for ischemic stroke therapy.

Biphasic therapeutic window (mechanism)

Ischemic stroke damage occurs in two distinct phases. This formulation is uniquely evaluated for its ability to treat both:

Phase	Pathophysiology	@nanoerythrocyte-T7 Action
1. Ischemia (pre-thrombolysis)	Blood clot blocks flow; brain cells starve of oxygen; local hypoxia triggers cell death.	**Oxygen-Regulating Rescue:** The nanozyme structure helps stabilize and modulate local oxygen tension, mitigating early-stage hypoxic damage.
2. Reperfusion (post-thrombolysis)	Clot dissolve; blood rushes back, bringing a surge of oxygen that creates a massive "ROS storm."	**ROS Scavenging**: The core acts as a multi-enzyme mimic, rapidly neutralizing superoxide radicals and hydrogen peroxide to stop secondary tissue necrosis.

METHOD OF FORMULATION

(I) The formulation is synthesized through a sequential, **three-step biomimetic process**

Step 1: synthesis of nanoparticles

Tri Manganese tetroxide (Mn_3O_4) nanoparticles are typically synthesized using either a hydrothermal method or a precipitation technique.

- **Precipitation:** Manganese salts (e.g., manganese nitrate or manganese sulphate) are used as precursors and precipitated under basic conditions.
- **Hydrothermal Reduction:** Alternatively, potassium permanganate ($KMnO_4$) can be hydrothermally reduced in the presence of dimethylformamide (DMF) or water at to form octahedral nanoparticles, usually around 20 - 25 nm in size.

Step 2: isolation of RBC membranes

Red blood cell membranes are isolated from fresh blood using a hypotonic lysis method.

- Whole blood is centrifuged to collect the erythrocytes.
- The cells are subjected to **hypotonic lysis** using a buffer (e.g., phosphate buffer) to burst the cells.
- The haemoglobin is washed away through repeated centrifugation, leaving behind pure, translucent RBC membrane vesicles (RBC ghosts).

Step 3: encapsulation and T7 peptide modification-

The final nano sponge formulation is created by combining the components through **membrane extrusion**.

1. **Encapsulation (hyperosmotic method):** The nano particles and RBC vesicles are co-incubated, allowing the nanoparticles to be encapsulated inside the erythrocyte membrane.
2. **Surface modification:** The T7 peptide (a sequence known to enhance receptor-mediated transport across the blood-brain barrier) is chemically conjugated to the surface of the RBC vesicles.

3. **Extrusion:** The mixture is repeatedly extruded through polycarbonate membranes (commonly with a pore size of 200 nm) to yield uniform, stacked nanovesicles.

(II) This formulation is synthesized by **combining inorganic chemistry, cell biology and surface conjugation**. Below is a deep, step-by-step breakdown of the parameters, chemical reactions and specific protocols used in its preparation.

Step 1: **hydrothermal synthesis of nanoenzymes**

- **Chemistry:** Potassium permanganate ($KMnO_4$) is reduced using a mild reducing agent/solvent mixture to precipitate highly uniform, crystalline nanoparticles.
- **Protocol**

- Dissolve in a mixture of deionized water and Di-methylformamide (DMF). DMF acts as both the solvent and the controlled reducing agent.
- Seal the solution in a **Teflon-Lined stainless-steel autoclave.**
- Heat hydrothermally at to for 6 hours.
- Centrifuge the resulting brown precipitate, wash three times with ethanol and water, and dry. These produces monodisperse **octahedral nanoenzymes (~22nm).**

Step 2: isolation of RBC ghosts (hypotonic lysis)

- **Biology:** Erythrocyte membranes are isolated while maintaining their natural CD47 surface proteins (the "don't eat me" signal) to prevent immune clearance.
- **Protocol:**

- Collect fresh whole blood and centrifuge at **3000 rpm for 10 minutes** to isolate red blood cells (RBCs).
- Wash the isolated RBCs with cold Phosphate-Buffered Saline (PBS).
- Induce **hypotonic shock** by mixing the RBCs with a low-osmolality buffer (e.g., PBS) at for 2 hours.
- This causes the cells to swell and burst, releasing intracellular haemoglobin.
- Centrifuge at **14,000 rpm for 20 minutes** to pellet the pale, translucent RBC membrane ghosts, washing repeatedly until the supernatant is completely clear of haemoglobin.

Step 3: T7 peptide functionalization

- **The targeting mechanism:** The T7 peptide (sequence HAIYPRH) targets the **transferrin receptor (TFR)** overexpressed on blood-brain barrier (BBB) endothelial cells, allowing the nanosponge to cross into the brain.
- **Protocol:**

- Utilize **DSPE-PEG-MAL (**distearolylphosphatidyl-ethanolamine-polyethylene glycol-maleimide) as a linker.
- React the cysteine-terminated T7 peptide with DSPE-PEG-Mal via a thiol-maleimide coupling reaction in PBS (pH 7.4) for 4 hours to form **DSPE-PEG-T7.**
- Introduce DSPE-PEG-T7 into the isolated RBC ghost suspension.
- Incubate at **for 30 minutes**. The lipids of DSPE-PEG-T7 spontaneously inert themselves into the hydrophobic core of the RBC lipid bilayer via **lipid insertion**, exposing the T7 peptide on the exterior membrane surface.

Step 4: nanoenzyme encapsulation via extrusion

- **the assembly:** Mechanical forces are used to seal the inorganic nanoparticles within the functionalized RBC membranes.
- **Protocol**

- Mix the synthesized nanoparticles directly with the T7-functionalized RBC ghosts in a 1:1 mass ratio.
- Subject the mixture to physical extrusion using a handheld automated **liposome extruder**.
- Pass the mixture **11 to 21 times** through a porous **polycarbonate membrane (**pore size progressively decreased from 400 nm to 200 nm).
- The mechanical shear forces the open RBC membranes to self-assemble and wrap around the nanoparticles.
- Centrifuge at low speed to pellet any unencapsulated, heavy aggregates, leaving the completed **MNET nano sponges** (~150 - 180nm) suspended uniformly in the supernatant.

Administration

The specific method of administrating **Mn_3O_4@nanoerythrocyte-T7** (MNET) is through **intravenous (i.v.) injection**, typically via the tail vein in animal stroke models like the middle cerebral artery occlusion (MCAO) mouse model.

While some therapies require invasive direct brain injections to bypass the blood-brain barrier (BBB), MNET is specifically engineered to make standard systemic injection safe and effective.

♦ **step-by-step path of systemic administration**

1. **systemic injection**: The therapeutic nanosponges are introduced directly into the bloodstream.
2. **long-circulating stealth navigation**: Because the core of is wrapped in a natural **red blood cell (erythrocyte) membrane**, the body's immune system does not recognize it as a foreign threat. This prevents immediate clearance by the liver or spleen, keeping the nanozymes active in circulation longer.
3. **receptor-mediated BBB crossing**: As the particles travel through the brain's vascular network, the conjugated T7 peptides acts as key fragments. They specifically bind to transferrin receptors expressed on the brain capillary endothelial cells, safely pulling the nanosponges through the tight junctions of the BBB.
4. **ischemic core targeting:** Once inside the central nervous system, the particles automatically accumulate at the lesion site, or the infarcted area of

the stroke, where they initiate free radical scavenging and self-adapted oxygen regulation.

Advantages

1. **Biomimetic immune evasion**

- **Stealth delivery:** By encapsulating the Mn_3O_4 nanoparticles inside a natural red blood cell (RBC) membrane, the nanocarrier avoids rapid clearance by the body's immune system.
- **prolonged hair-life:** It significantly extends blood circulation time, allowing the therapeutic payload more opportunity to reach the target site in the brain.

2. **Active brain targeting**

- **T7 peptide modification;** The RBC membrane is surface conjugated with the T7 peptide a sequence known for its high affinity to transferrin receptors.
- **Enhanced BBB penetration:** This modification actively promotes the passage of the nanoparticle across the Blood-Brain Barrier (BBB) ensuring targeted accumulation directly in the ischemic region.

3. **Metabolic microenvironment remodelling**

- **O_2 balance regulation:** It restores cellular metabolic homeostasis by balancing oxygen supply and demand in the compromised ischemic penumbra.
- **Free radical scavenging:** The Mn_3O_4 nanoparticles exhibit the high enzyme-like activities (such as superoxide dismutase and catalase). They neutralize toxic Reactive Oxygen Species (ROS), protecting vulnerable neurons from oxidative stress-induced death.

4. **Neuroprotection and tissue recovery**

- **Limits damage:** By alleviating oxidative stress, reducing neuroinflammation and preventing neuronal apoptosis, the formulation limits infract volume and secondary brain injury.
- **improves long-term prognosis:** Studies indicate that restoring metabolic responsiveness leads to better recovery of behavioural and motor functions.

5. **Longer circulation time:** The Erythrocyte membrane camouflage helps the nanoparticles evade rapid clearance by the immune system, allowing them to remain in circulation longer and increasing the likelihood of reaching the brain.

6. **Biomimetic and potentially more biocompatible:** Because the carrier uses red blood cell membrane components, it may exhibit lower immunogenicity and better biocompatibility than many synthetic nanoparticles systems.

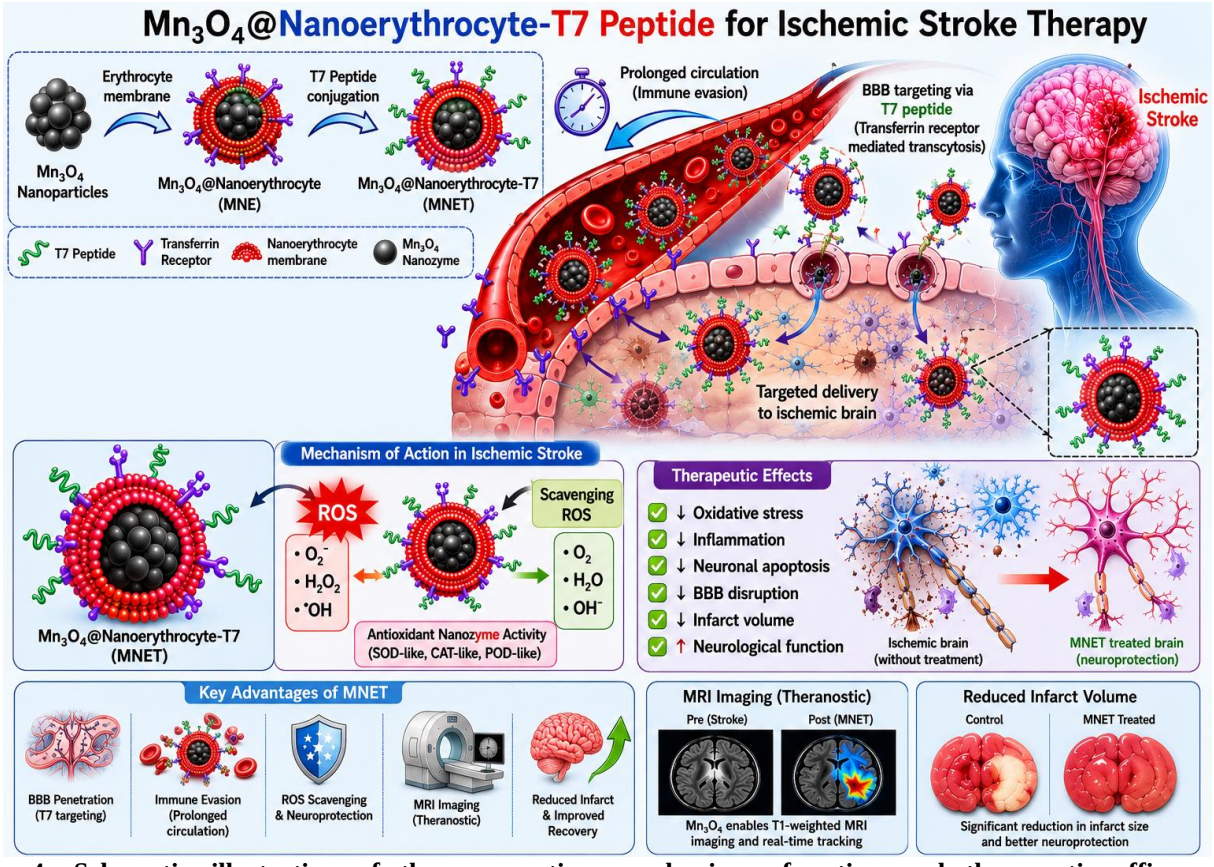

Fig. 4: Schematic illustration of the preparation, mechanism of action and therapeutic efficacy of Mn_3O_4@Nanoerythrocyte-T7 Peptide (MNET) for targeted ischemic stroke therapy.

Disadvantages

1. **formulation and biological risks**

- **erythrocyte degradation:** Bioinspired formulations rely heavily on maintaining the structural integrity of natural red blood cell membranes. Stresses such as shear forces in the bloodstream or prolonged storage can cause premature haemolysis and leakage of the encapsulated Mn_3O_4 payload.
- **immunogenicity:** Exogenous administration of biomimetic vesicles-even those derived from autologous cell membranes-can trigger unpredictable immune responses or complement activation in some patient cohorts.

2. **Potential manganese-related toxicity:** The therapeutic component contains **manganese oxide (Mn_3O_4)** nanoparticles. Potential concerns include-

- Accumulation of manganese in tissues.
- Neurotoxicity at high or prolonged exposures.
- Possible liver and kidney burden during nanoparticle clearance.

3. **Manufacturing complexity:** These production makes-

- More expensive than conventional drugs.
- Potentially difficult to scale consistently for pharmaceutical manufacturing.

4. **Potential immune and inflammatory responses:** Although erythrocyte membrane is designed to reduce immune recognition-

- Modified membranes may still trigger immune responses.
- Repeated administration could potentially induce antibody formation.
- Interactions with the complement system require further study.

5. **Uncertain long-term safety**

- Chronic manganese accumulation.
- Long-term effects on brain tissue.
- Repeated-dose safety.
- Metabolism of degradation products.

Challenges

→ Mononuclear phagocyte system blockade.
→ Limited BBB permeability and accumulation at the lesion spots.
→ Phased O_2 regulation failure.
→ Extreme Biochemical Chaos.
→ Microglial and Immune Hyperactivation.
→ Erythrocyte Membrane Instability.
→ Long-Term Biosafety Concern of Mn.

PHARMACEUTICAL APPLICATIONS AND BIOMEDICAL APPLICATIONS

1. **Remodelling the ischemic microenvironment MNET**: Operates through self-adapted oxygen regulation and free radical scavenging. It alters stroke pathology in two distinct phases:

- **before thrombolysis:** Rapidly scavenges toxic **Reactive Oxygen Species (ROS)** and supplies timely oxygen to rescue dying neurocytes (brain cells) in hypoxic conditions.
- **after thrombolysis:** Suppresses dangerous "oxygen-boost" microenvironments and handles reperfusion injuries to avoid further brain tissue damage.

2. **Active targeted delivery**

- **T7 peptide**: The T7 peptide specifically targets the transferrin receptor, which allows the nano sponge to preferentially accumulate at the exact site of cerebral infarcts (lesions).
- **erythrocyte membrane**: Encapsulating in an RBC membrane provides a "stealth effect" that allows the nano sponge to evade immune clearance, drastically prolonging its circulation half-life in the bloodstream.

3. **Nano enzyme activity**

- The core acts as a highly efficient **nanozyme**. It mimics natural enzymes (like superoxide to control de dismutase) to control oxidative stress and safely manage cell oxidation levels.

4. **Neuroprotection and neuronal regeneration.**
5. **Biomimetic immune camouflage.**
6. **MRI-guided stroke diagnosis and monitoring.**
7. **biomimetic immune-evasive drug delivery.**

CONCLUSION

In conclusion, **Mn_3O_4@Nanoerythrocyte-T7(MNET)** represents a next-generation biomimetic nanosponge platform that goes beyond conventional stroke treatment by addressing the fundamental pathological mechanisms of ischemic brain injury. By integrating the potent ROS-scavenging activity of Mn_3O_4 nanoenzymes, the prolonged circulation and oxygen-regulating properties of erythrocyte membranes and the BBB-targeting capability of T7 peptide, this multifunctional nanoplatform offers a comprehensive strategy for neuroprotection. Unlike traditional therapies that primarily focus on restoring blood flow, MNET simultaneously combats oxidative stress, hypoxia, inflammation and reperfusion damage at the cellular level. Its ability to intelligently navigate biological barriers and deliver therapeutic effects directly to ischemic regions highlights the transformative potential of nanotechnology in neurology. As research continues to advance, Mn_3O_4@Nanoerythrocyte-T7 may pave the way for safer, more effective and precision-guided treatments, bringing new hope for reducing disability and improving recovery in patients affected by ischemic strokes.

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