Title:

NANOFERROBLOCK: DNA Origami Nanostructures Equipped with ssDNA Aptamers as DMT1 and ACSL4 Inhibitors for Combating Ferroptosis during Neuroinflammation

Keywords:

DNA origami nanostructures, Ferroptosis, Aptamers, Novel Therapy, Dual targeting, Neuroinflammation

Background and Rationale:

Indication:

Neuroinflammation, an inflammatory response within the central nervous system (CNS), is triggered by various stimuli. While initial inflammation post-CNS injury aids in recovery, chronic neuroinflammation, particularly mediated by microglia, can damage healthy neural tissue and contribute to neurodegenerative diseases (1). With increasing life expectancy, there is a concurrent rise in the prevalence of neuroinflammation-related diseases. Ferroptosis, an iron-dependent cell death process, is associated with neuroinflammation (2). Targeting microglial iron accumulation and the resulting ferroptosis presents a promising avenue for preventing and treating neurodegenerative diseases (3).

Background:

Microglia are the primary immune cells in the CNS and serve as the first line of defense in the CNS innate immune system. They play critical roles in neurogenesis, synaptic density, and plasticity, along with phagocytosing pathological aggregates and dead cells in damaged CNS (4). Microglia dynamically polarizes between pro-inflammatory, M1 and anti-inflammatory, M2 phenotypes to maintain CNS homeostasis (5). Prolonged activation of microglia, commonly observed in neurodegenerative diseases such as Alzheimer's and Parkinson's, can lead to chronic neuroinflammation. This persistent inflammation contributes to the progression of these diseases by causing tissue and cellular damage (1). Identifying and targeting specific pathways and molecules involved in microglial activation and the resultant neuroinflammation holds potential for developing therapeutic strategies to treat neurodegenerative diseases.

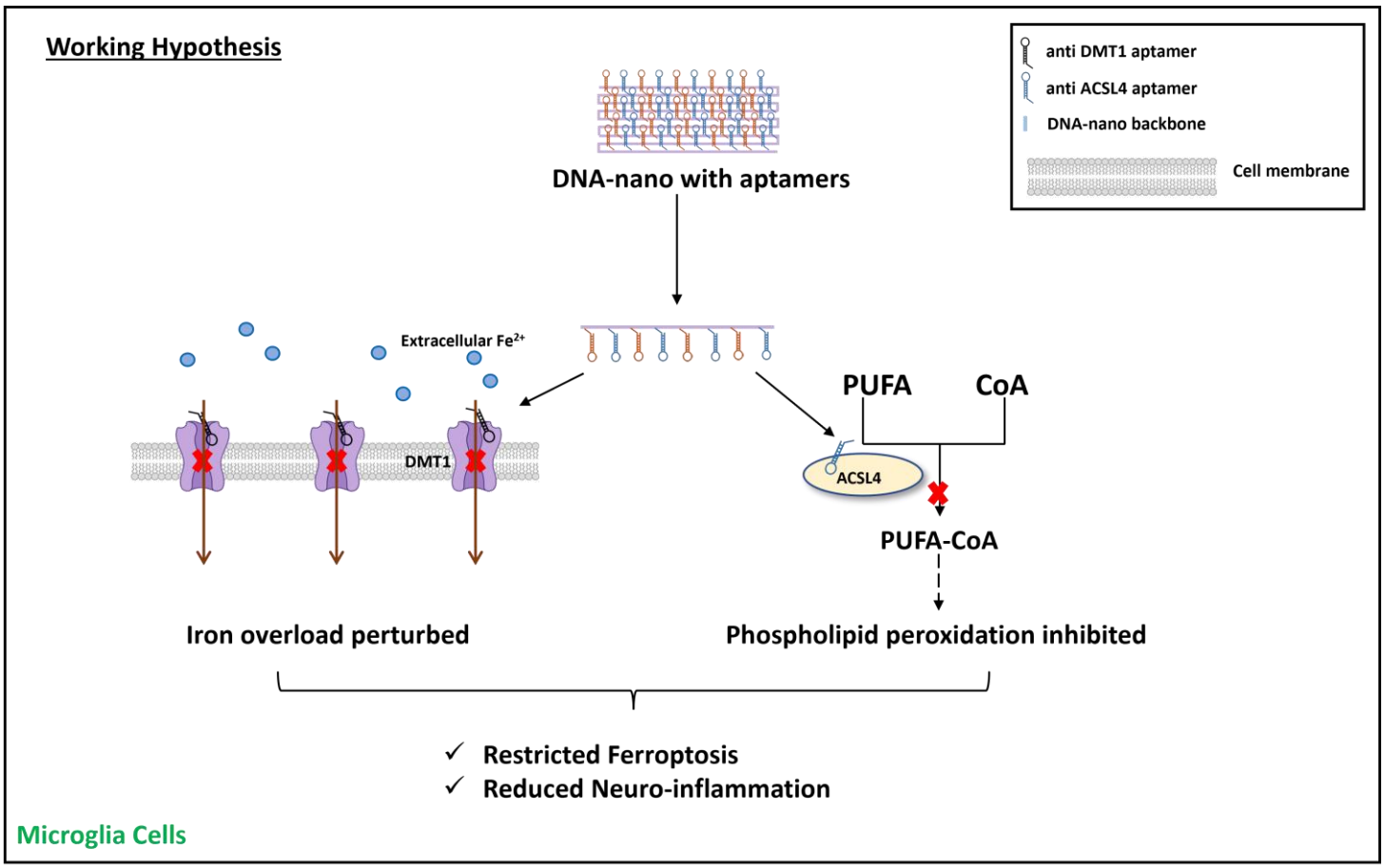
Ferroptosis, a newly identified form of cell death, is characterized by iron accumulation and lipid peroxidation within cells. It is distinct from necroptosis, apoptosis, and autophagy; as it is characterized by normal-sized nuclei, non-agglutinated chromatin, and significantly altered mitochondria (6). Microglial ferroptosis includes

two key components: iron accumulation and phospholipid peroxidation (7). Excess cytoplasmic iron forms a pool of highly reactive ferrous ion (Fe^{2+}), catalyzing the formation reactive oxygen species (ROS) through Fenton reaction. The ROS, then initiate lipid peroxidation, leading to the formation of lipid hydroperoxides (PLOOHs) that disrupt cellular membrane integrity leading to cell death. This cell death triggers an inflammatory response, with microglia releasing pro-inflammatory cytokines like IL-1β, TNF-α, and IL-6, further exacerbating neuroinflammation (8). Thus, inhibiting microglial ferroptosis serves as an attractive and alternative target for treating neuroinflammation.

Neuroinflammation has been shown to be restricted by inhibiting either iron accumulation or phospholipid peroxidation during microglia ferroptosis. Extracellular free Fe^{2+} is transported into the cell by Divalent metal transporter 1(DMT1). Treatments like 6-hydroxydopamine, lipopolysaccharide, or β-amyloid induce DMT1 expression, leading to iron overload in microglia (9-11). This iron accumulation is exacerbated by inflammatory stimuli such as TNF-α and IL-6, which further promote DMT1 expression (12). This results in increased ROS, TNF-α, and inducible nitric oxide synthase, advancing neuroinflammation (13). Inhibition or knockdown of DMT1, along with iron chelation, significantly reduces pro-inflammatory cytokines, highlighting DMT1 as a potential therapeutic target to mitigate neuroinflammation and ferroptosis in neurodegenerative diseases (14) .

Phospholipid peroxidation, catalyzed by acyl-CoA synthetase long-chain family member 4 (ACSL4) and lipoxygenases, leads to the formation of PLOOHs (15, 16). The continuous accumulation of PLOOHs irreversibly damages the cell membrane, ultimately leading to cell death by compromising membrane integrity (15). Inhibiting phospholipid peroxidation during microglia ferroptosis prevent neuroinflammation. Knockdown of the ACSL4 gene in microglia decreases their sensitivity to ferroptosis and reduces the production of proinflammatory cytokines (17). Recently, caffeic acid has been reported to reduce neuroinflammation and resist ferroptosis in cerebral ischemia by downregulating ACSL4, as investigated using a primary microglia-neuron coculture system (18). This suggests that targeting ACSL4 could be an effective therapeutic strategy for mitigating ferroptosis in microglia and protecting against neurodegenerative diseases. Ferroptosis inhibitors such as Liproxstatin-1, Ferrostatin-1, and Deferoxamine offer potential treatments but, challenges like poor selectivity, limited bioavailability, and associated toxicities limit clinical use, necessitating advances in alternate therapeutics with high selectivity to enhance therapeutic outcomes (19-22).

Working Hypothesis:



We propose to develop Single-Stranded Deoxyribonucleic Acid (ssDNA) aptamers targeting DMT1 and ACSL4 as novel inhibitors of ferroptosis and neuroinflammation. These aptamers will also be arranged on DNA nanostructures for dual targeted inhibition. Aptamers, the single-stranded DNA or RNA oligonucleotides, have garnered attention as promising therapeutics due to several advantages, including cost-effectiveness and high specificity. DNA aptamers are highly stable and least immune-reactive with negligible off-target effects. These are highly specific for the target proteins. They can effectively mask active and allosteric sites and perturb the activity of target proteins (23). The key strength of aptamers lies in the availability of a reliable and efficient development method known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX), also known as in vitro selection, in which target molecules are incubated with a highly diverse library of ssDNA or RNA molecules with each oligonucleotide folded into a unique three-dimensional structure (23). This vast repertoire of single-stranded oligonucleotides enables the development of aptamers with exceptional affinity, avidity, and specificity for their target molecules. We plan to develop DNA aptamers that specifically bind to the

transmembrane Domain 1/6/10 of DMT1 (AlphaFold: P49281). DMT1 has 12 predicted transmembrane domains. The critical regions involved in the formation of the ion transport pathway are transmembrane domain 1, transmembrane domain 6, and transmembrane domain 10. These domains are highly conserved and form the central part of the ion transport pore (24). Targeting these domains may inhibit Fe^{2+} influx and iron overload in activated microglia.

We also plan to develop specific aptamers that binds to the C-terminal structural domain of ACSL4 (AlphaFold: O60488). This domain of ACSL4 is crucial for catalyzing the acylation reaction, forming the catalytic active center of the enzyme (25). Thus, targeting the C-terminal domain may reduce the formation of phospholipid peroxides in activated microglia.

Real-World Impact: The study has the potential to develop a novel approach for treating and diagnosing neurodegenerative diseases, reduce the economic burden on healthcare systems, and advance scientific understanding in the realm of neuroinflammation and ferroptosis.

Objectives:

Objective 1- Development of DNA aptamers against DMT1 and ACSL4.

Objective 2- Identification of the most enriched aptamers using Next Generation Sequencing.

Objective 3- Systemic screening of aptamers with specific and high affinity binding to selective cell types using flow cytometry.

Objective 4-Testing the efficacy of selected aptamers using DNA nanostructures on neuroinflammation.

Work Plan:

Objective 1 (8 weeks).

A ssDNA library containing a central random sequence of 30 nucleotides, flanked by specific sequences of 20 nucleotides at the 5' and 3' termini, will be designed and obtained from a commercial facility. The aptamers will be folded by heating the library for 5 minutes, followed by snap cooling. The negative selection will be performed by incubating the ssDNA pool with streptavidin-coated magnetic beads (SCMB), followed by the collection of unbound aptamers. These aptamers will be incubated with target protein, purified using immune-purification methods from the cell lines. After washing, ssDNA-peptide complexes on SCMB will be denatured by heating and collected by magnetic separation. The eluted ssDNA will be amplified by Polymerase Chain

Reaction (PCR) using a 5’ phosphorylated reverse primer. The obtained ssDNA aptamer pool will be used for the subsequent rounds of negative and positive selections. The selection cycle will be repeated 8-10 times till enrichment is observed using real-time PCR melting curve analysis (26). This will result in a pool of ssDNA aptamers having high specificity for the target peptides.

Objective 2 (6 weeks).

The enriched ssDNA aptamers will be analyzed by next-generation sequencing. The abundance of each aptamer in the pool will be calculated as a percentage of each number of reads within the total reads. The 10 most abundant ssDNA aptamers that bind to the peptides will be utilized in objective 3.

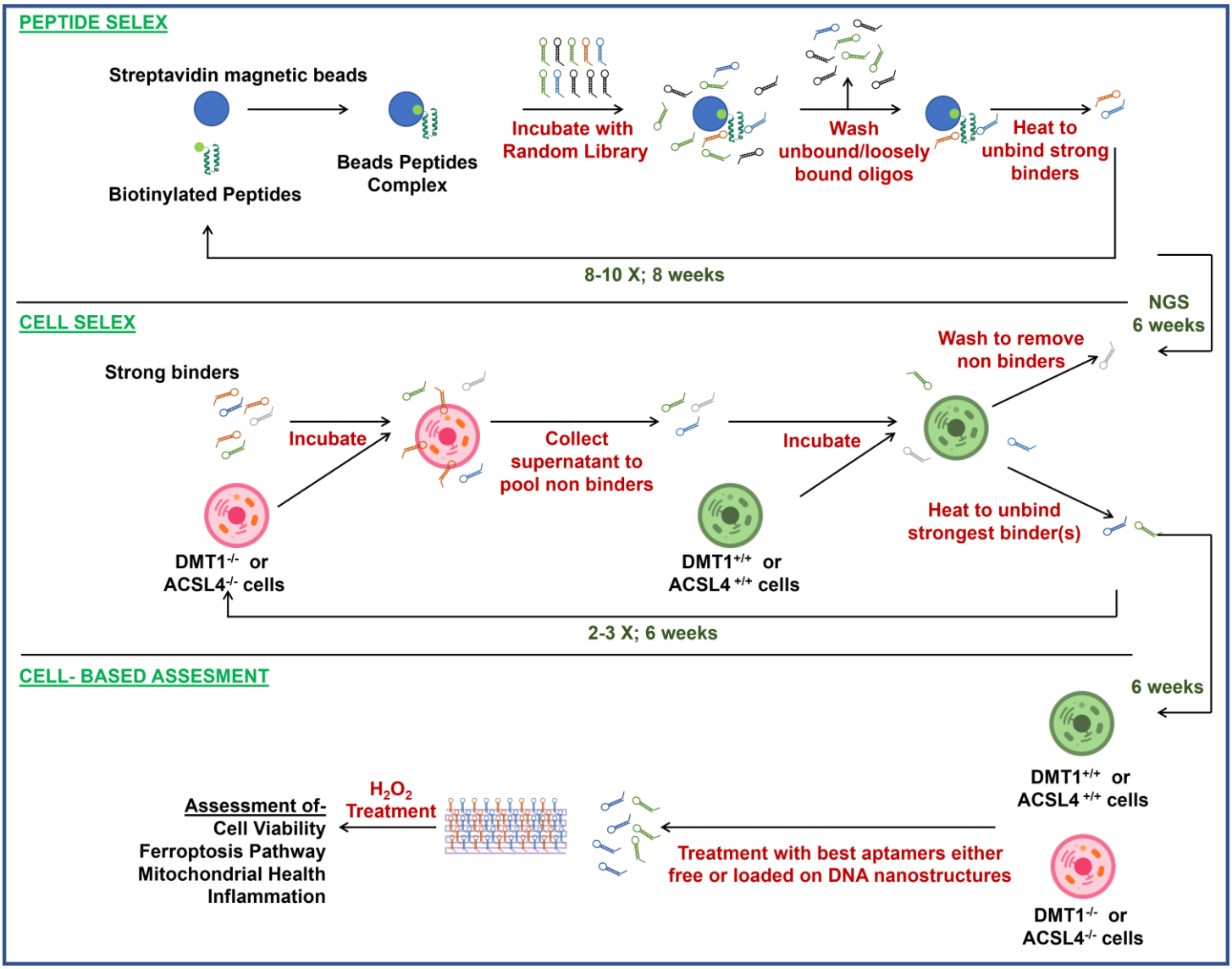
Objective 3 (6 weeks)

To monitor the binding of enriched ssDNA pool in live cells, FAM-labeled ssDNA pools will be procured from a commercial facility and incubated with wild-type microglial cell lines like BV2 and with knock-down lines of target proteins ((ACSL4-/-) or (DMT11-/-)) or cell lines not expressing the proteins naturally. Cells will be washed twice after incubation, and the fluorescence intensity will be determined by flow cytometry. This will be repeated at least three times. The equilibrium dissociation constant of the aptamer-cell interaction will also be determined by performing the cell- based SELEX in a concentration-dependent manner (27). This will help determine DNA aptamers that strongly bind to the target proteins in live cells.

Objective 4 (6 weeks)

The H_2O_2-stimulated microglial cells will be treated with the best aptamers, and subsequently, cellular viability, mitochondrial health and signaling pathways will be analyzed using qPCR and flow cytometry. The aptamers will be used free in solution or arranged on DNA nanostructures such as the DNA-Net and the M13 origami to determine any multi-valency associated improvement (28, 29).

Working Strategy:



Significance of the Proposal

Aptamers are revolutionizing the fields of diagnostics and therapeutics due to their unique physiochemical properties. Exceptional specificity and customizability make aptamers invaluable tools for precisely targeting disease-associated targets, effectively minimizing off-target effects and enhancing treatment efficacy. Notably, these molecules exhibit low immunogenicity and can be mass-produced cost-effectively, offering a safer and more economical alternative to traditional drugs (30). For example, the groundbreaking discovery of MACUGEN, the first FDA-approved RNA-based therapy that targets vascular endothelial growth factor for the treatment of wet age-related macular degeneration, demonstrates the real-world potential of these molecules in clinical applications (31). Moreover, the innovative strategy of loading multiple aptamers onto DNA nanostructures holds great promise, allowing for selective interaction with multiple sites on a single target as well as multiple targets (29, 32-36). This approach opens new possibilities for highly selective and efficient applications in clinical and basic research, and beyond.

Neuroinflammation is a key player in a wide range of neurological disorders that are significant health concerns in India (37). Addressing neuroinflammation is vital for potentially decelerating or even halting the progression of these conditions, ultimately improving the quality of life in affected individuals, simultaneously reducing the socioeconomic burden on healthcare systems. Emerging research also suggests neuroinflammation may play a role in neuropsychiatric disorders like depression and anxiety, highlighting the potential for treatments in the realm of mental health (38). The development of effective therapies for neuroinflammation stands at the intersection of scientific discovery, clinical care, and improved human well-being.

Targeting ferroptosis in microglia holds immense significance in the context of neuroinflammation and neurodegenerative diseases. Microglia, the resident immune cells of the brain, play a central role in neuroinflammatory responses (39). Ferroptosis is an iron-dependent and phospholipid peroxidation-driven process to regulate cell death (15). Previous studies have shown that ferroptosis is closely related to neuroinflammation (17, 19, 40). DMT-1- mediated iron overload and ACSL4-mediated accumulation of phospholipid peroxides in microglia releases many inflammatory factors that exacerbate neuroinflammation. Thus, inhibiting microglia ferroptosis holds promise for the prevention and treatment of neuroinflammation.

Utilizing aptamers for targeting ferroptosis in microglia presents a distinct advantage in pursuing therapeutic

precision. Aptamers can be strategically engineered for multi-valent binding to enhance their efficacy further by arranging on various DNA nanostructures to achieve a multi-valent and pattern-matched interaction, ensuring a comprehensive restoration of cellular homeostasis. This approach can simultaneously inhibit the activity of the two proposed targets, DMT-1 and ACSL4 in cells. It will help develop a potential tool to restrict ferroptosis with the high specificity and optimize the potential for successful intervention in neuroinflammatory processes.

Scientific Benefit: The overseas host, Dr. Wang has a well-established SELEX pipeline that will be applied to select proposed aptamers. Dr. Wang's research primarily focuses on designing and synthesizing functional DNA nanostructures (28, 35, 41-43). These nanostructures are aimed at developing highly potent therapies and rapid and cost-effective diagnostics for various diseases. Dr. Wang has also developed protocols to reliably test the biostability of different DNA origami nanostructures in cell culture media and living cells and the elucidation of the molecular basis for protein-free dsDNA homologous pairing. (https://bioengineering.illinois.edu/people/xingw). Research Experience from Dr. Wang's lab will help the applicant gain access to state-of-the-art equipment and resources and hands-on experience in aptamer and DNA nanotechnology. The applicant is a young researcher who joined CDRI in June 2021. and is working in the area of neuroscience and ageing biology. By performing cutting-edge research, she aims to identify novel targets and develop innovative therapeutics for neuroinflammation, neurodegeneration and age-associated diseases. She has experience in various molecular biology techniques that will help her to complete the study within stipulated time period.

References:

1. Gao C, Jiang J, Tan Y, Chen S. Microglia in neurodegenerative diseases: mechanism and potential therapeutic targets. Signal Transduct Target Ther. 2023;8(1):359.
2. Mohan S, Alhazmi HA, Hassani R, Khuwaja G, Maheshkumar VP, Aldahish A, et al. Role of ferroptosis pathways in neuroinflammation and neurological disorders: From pathogenesis to treatment. Heliyon. 2024;10(3):e24786.
3. Xu Y, Jia B, Li J, Li Q, Luo C. The Interplay between Ferroptosis and Neuroinflammation in Central Neurological Disorders. Antioxidants (Basel). 2024;13(4).
4. Arcuri C, Mecca C, Bianchi R, Giambanco I, Donato R. The Pathophysiological Role of Microglia in Dynamic Surveillance, Phagocytosis and Structural Remodeling of the Developing CNS. Front Mol Neurosci. 2017;10:191.
5. Guo S, Wang H, Yin Y. Microglia Polarization From M1 to M2 in Neurodegenerative Diseases. Front Aging Neurosci. 2022;14:815347.
6. Seibt T, Wahida A, Hoeft K, Kemmner S, Linkermann A, Mishima E, et al. Ferroptosis Biology. Nephrol Dial Transplant. 2024.
7. Liu S, Gao X, Zhou S. New Target for Prevention and Treatment of Neuroinflammation: Microglia Iron Accumulation and Ferroptosis. ASN Neuro. 2022;14:17590914221133236.
8. Keles E, Kazan HH, Aral A, Bolay H. Microglial iron trafficking: new player in brain injury. Turk J Med Sci. 2022;52(5):1415-24.
9. McCarthy RC, Sosa JC, Gardeck AM, Baez AS, Lee CH, Wessling-Resnick M. Inflammation-induced iron transport and metabolism by brain microglia. J Biol Chem. 2018;293(20):7853-63.
10. Olmedo-Diaz S, Estevez-Silva H, Oradd G, Af Bjerken S, Marcellino D, Virel A. An altered blood-brain barrier contributes to brain iron accumulation and neuroinflammation in the 6-OHDA rat model of Parkinson's disease. Neuroscience. 2017;362:141-51.
11. Xu M, Li Y, Meng D, Zhang D, Wang B, Xie J, et al. 6-Hydroxydopamine Induces Abnormal Iron Sequestration in BV2 Microglia by Activating Iron Regulatory Protein 1 and Inhibiting Hepcidin Release. Biomolecules. 2022;12(2).
12. Urrutia P, Aguirre P, Esparza A, Tapia V, Mena NP, Arredondo M, et al. Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells. J Neurochem. 2013;126(4):541-9.
13. McIntosh A, Mela V, Harty C, Minogue AM, Costello DA, Kerskens C, et al. Iron accumulation in microglia triggers a cascade of events that leads to altered metabolism and compromised function in APP/PS1 mice. Brain Pathol. 2019;29(5):606-21.
14. Nnah IC, Lee CH, Wessling-Resnick M. Iron potentiates microglial interleukin-1beta secretion induced by amyloid-beta. J Neurochem. 2020;154(2):177-89.
15. Jiang X, Stockwell BR, Conrad M. Ferroptosis: mechanisms, biology and role in disease. Nat Rev Mol Cell Biol. 2021;22(4):266-82.
16. Kagan VE, Mao G, Qu F, Angeli JP, Doll S, Croix CS, et al. Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. Nat Chem Biol. 2017;13(1):81-90.
17. Cui Y, Zhang Y, Zhao X, Shao L, Liu G, Sun C, et al. ACSL4 exacerbates ischemic stroke by promoting ferroptosis-induced brain injury and neuroinflammation. Brain Behav Immun. 2021;93:312-21.
18. Li XN, Shang NY, Kang YY, Sheng N, Lan JQ, Tang JS, et al. Caffeic acid alleviates cerebral ischemic injury in rats by resisting ferroptosis via Nrf2 signaling pathway. Acta Pharmacol Sin. 2024;45(2):248-67.
19. Cao Y, Li Y, He C, Yan F, Li JR, Xu HZ, et al. Selective Ferroptosis Inhibitor Liproxstatin-1 Attenuates Neurological Deficits and Neuroinflammation After Subarachnoid Hemorrhage. Neurosci Bull. 2021;37(4):535-49.
20. Huang L, Zhang Y, Zhao L, Chen Q, Li L. Ferrostatin-1 Polarizes Microglial Cells Toward M2 Phenotype to Alleviate Inflammation After Intracerebral Hemorrhage. Neurocrit Care. 2022;36(3):942-54.
21. LeBlanc RH, 3rd, Chen R, Selim MH, Hanafy KA. Heme oxygenase-1-mediated neuroprotection in subarachnoid hemorrhage via intracerebroventricular deferoxamine. J Neuroinflammation. 2016;13(1):244.
22. Zilka O, Shah R, Li B, Friedmann Angeli JP, Griesser M, Conrad M, et al. On the Mechanism of Cytoprotection by Ferrostatin-1 and Liproxstatin-1 and the Role of Lipid Peroxidation in Ferroptotic Cell

Death. ACS Cent Sci. 2017;3(3):232-43.
23. Zhu C, Feng Z, Qin H, Chen L, Yan M, Li L, et al. Recent progress of SELEX methods for screening nucleic acid aptamers. Talanta. 2024;266(Pt 1):124998.
24. Chaloupka R, Courville P, Veyrier F, Knudsen B, Tompkins TA, Cellier MF. Identification of functional amino acids in the Nramp family by a combination of evolutionary analysis and biophysical studies of metal and proton cotransport in vivo. Biochemistry. 2005;44(2):726-33.
25. Shimbara-Matsubayashi S, Kuwata H, Tanaka N, Kato M, Hara S. Analysis on the Substrate Specificity of Recombinant Human Acyl-CoA Synthetase ACSL4 Variants. Biol Pharm Bull. 2019;42(5):850-5.
26. Teng IT, Li X, Yadikar HA, Yang Z, Li L, Lyu Y, et al. Identification and Characterization of DNA Aptamers Specific for Phosphorylation Epitopes of Tau Protein. J Am Chem Soc. 2018;140(43):14314-23.
27. Sefah K, Shangguan D, Xiong X, O'Donoghue MB, Tan W. Development of DNA aptamers using Cell-SELEX. Nat Protoc. 2010;5(6):1169-85.
28. Gallego I, Grover MA, Hud NV. Folding and imaging of DNA nanostructures in anhydrous and hydrated deep-eutectic solvents. Angew Chem Int Ed Engl. 2015;54(23):6765-9.
29. Chauhan N, Xiong Y, Ren S, Dwivedy A, Magazine N, Zhou L, et al. Net-Shaped DNA Nanostructures Designed for Rapid/Sensitive Detection and Potential Inhibition of the SARS-CoV-2 Virus. J Am Chem Soc. 2023;145(37):20214-28.
30. Kumar Kulabhusan P, Hussain B, Yuce M. Current Perspectives on Aptamers as Diagnostic Tools and Therapeutic Agents. Pharmaceutics. 2020;12(7).
31. Ng EW, Shima DT, Calias P, Cunningham ET, Jr., Guyer DR, Adamis AP. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat Rev Drug Discov. 2006;5(2):123-32.
32. Tian C, Zhang C. Three-Dimensional DNA Nanostructures Assembled from DNA Star Motifs. Methods Mol Biol. 2017;1500:11-26.
33. Rothenbuhler S, Iacovache I, Langenegger SM, Zuber B, Haner R. Complex DNA Architectonics horizontal line Self-Assembly of Amphiphilic Oligonucleotides into Ribbons, Vesicles, and Asterosomes. Bioconjug Chem. 2023;34(1):70-7.
34. Lee H, Wang W, Chauhan N, Xiong Y, Magazine N, Valdescruz O, et al. Rapid detection of intact SARS-CoV-2 using designer DNA Nets and a pocket-size smartphone-linked fluorimeter. Biosens Bioelectron. 2023;229:115228.
35. Kwon PS, Ren S, Kwon SJ, Kizer ME, Kuo L, Xie M, et al. Designer DNA architecture offers precise and multivalent spatial pattern-recognition for viral sensing and inhibition. Nat Chem. 2020;12(1):26-35.
36. Umrao S, Zheng M, Jin X, Yao S, Wang X. Net-Shaped DNA Nanostructure-Based Lateral Flow Assays for Rapid and Sensitive SARS-CoV-2 Detection. Anal Chem. 2024;96(8):3291-9.
37. Muzio L, Viotti A, Martino G. Microglia in Neuroinflammation and Neurodegeneration: From Understanding to Therapy. Front Neurosci. 2021;15:742065.
38. Zheng ZH, Tu JL, Li XH, Hua Q, Liu WZ, Liu Y, et al. Neuroinflammation induces anxiety- and depressive-like behavior by modulating neuronal plasticity in the basolateral amygdala. Brain Behav Immun. 2021;91:505-18.
39. Feldman RA. Microglia orchestrate neuroinflammation. Elife. 2022;11.
40. Ko CJ, Gao SL, Lin TK, Chu PY, Lin HY. Ferroptosis as a Major Factor and Therapeutic Target for Neuroinflammation in Parkinson's Disease. Biomedicines. 2021;9(11).
41. Rodriguez A, Gandavadi D, Mathivanan J, Song T, Madhanagopal BR, Talbot H, et al. Self-Assembly of DNA Nanostructures in Different Cations. Small. 2023;19(39):e2300040.
42. Ren S, Fraser K, Kuo L, Chauhan N, Adrian AT, Zhang F, et al. Designer DNA nanostructures for viral inhibition. Nat Protoc. 2022;17(2):282-326.
43. Chakraborty B, Das S, Gupta A, Xiong Y, T-V V, Kizer ME, et al. Aptamers for Viral Detection and Inhibition. ACS Infect Dis. 2022;8(4):667-92.