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REVIEW: “ISCHEMIC STROKE: from fibrinolysis to functional recovery”

Nanomedicine: emerging approaches to treat ischemic stroke

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HIGHLIGHTS

Nanoparticles are versatile agents able to treat all phases of the ischemic stroke

Thrombolysis and neuroprotection can be tackled with nanotechnologies

Many NPs have promising preclinical results, but only few reach the clinical stage

Robust and scalable production methods are needed to produce NPs for clinical use

Efficacy and biosafety tests must be standardized to improve nanosystem assessment

Abstract

Stroke is responsible for 11% of all deaths worldwide, the majority of which are caused by ischemic strokes, thus making the need to urgently find safe and effective therapies. Today, these can be cured either by mechanical thrombectomy when the thrombus is accessible, or by intravenous injection of fibrinolytics. However, the latter present several limitations, such as potential severe side effects, few eligible patients and low rate of partial and full recovery. To design safer and more effective treatments, nanomedicine appeared in this medical field a few decades ago. This review will explain why nanoparticle-based therapies and imaging techniques are relevant for ischemic stroke management. Then, it will present the different nanoparticle types that have been recently developed to treat this pathology. It will also study the various targeting strategies used to bring nanoparticles to the stroke site, thereby limiting side effects and improving the therapeutic efficacy. Finally, this review will present the few clinical studies testing nanomedicine on stroke and discuss potential causes for their scarcity.

Relevance of nanomedicine

Cardiovascular diseases account for approximately a third of global mortality, with stroke alone causing over 10% of annual deaths worldwide (Institute for Health Metrics and Evaluation, 2022). In most cases, the so-called ischemic stroke is triggered by the formation of a blood clot (thrombus) (Feske, 2021), which restricts blood flow to the brain. The thrombus formation is a complex

phenomenon which involves multiple players, from cellular (such as platelets, red blood cells, and neutrophils) to molecular elements (such as fibrin and Von Willebrand Factor (VWF)). Consequences may be severe if the vascular occlusion is not addressed promptly, thus demanding a rapid and efficient therapeutic intervention to alleviate possible permanent damages resulting from ischemic strokes. However, pharmacological and surgical treatment options for ischemic strokes are limited. On one hand, mechanical intervention through surgical thrombectomy (endovascular therapy, or EVT) is not always possible depending on thrombus accessibility and availability of competent surgeons and infrastructure. On the other hand, pharmaceutical action can be achieved mostly through fibrinolytic drugs (plasminogen activators (PA)), as well as anticoagulants and antiplatelets for the prevention of secondary strokes (Feske, 2021). However, these fibrinolytic treatments are intravenously injected and therefore have body-wide side effects and limited thrombolysis efficiency. For instance, the gold standard treatment, Alteplase® (a recombinant tissue PA, rtPA), suffers from low circulation half-life, risks of symptomatic intracerebral hemorrhage (sICH), neurotoxicity, and overall cannot achieve full recanalization in all the patients (only in 30%) (Bhatia et al., 2010; Mosconi and Paciaroni, 2022).

In summary, stroke treatments are not always administered (e.g., around 10% of ischemic stroke patients in the United States received rtPA in 2018) (de Havenon et al., 2021), partly due to a very short therapeutic window (4.5 hours from stroke onset), often with major side effects through systemic activity and lack of efficacy, thus requiring new directions to improve patients' outcome.

To overcome these limitations, nanomedicine applied to stroke treatment has been investigated for several years. In the early 19th century, the German Nobel prize Paul Ehrlich created the concept of "magic bullet" specifically directed against pathogens in the organism, like bullets fired from a gun that hit a specific target (Strebhardt and Ullrich, 2008). New treatments were inspired by this concept and are now designed to bring an active drug with poor availability and adverse toxicity profile to a specific target by encapsulating it into a controlled vector - the nanoparticle (NP). In this context, the encapsulation and targeted delivery of thrombolytic agents would benefit from: i) protection from the physiological environment, thus increasing the agent's half-life; ii) limitation of systemic adverse effects; iii) improvement in therapeutic efficacy by increasing its accumulation at the thrombus site (Blanco et al., 2015). This concept has also been adapted to stroke diagnosis. Indeed, instead of bringing an active drug to the targeted thrombus, nanoscale objects such as NPs can be used directly as contrast agents (e.g., iron oxide NPs for magnetic resonance imaging (MRI) or to transport imaging compounds such as radioactive markers). Thanks to their targeting capacity, they can accumulate at the cerebral blood clot and enhance the imaging signal of the stroke that can be obtained with MRI, computed tomography (CT), positron emission tomography (PET) or doppler ultrasonography (Sarmah et al., 2021).

In addition to resolving the thrombus and restore blood flow, neuroprotection is essential to limit tissue damage resulting from ischemia (Ghozy et al., 2022). This is why NP-based treatments are promising as they have the potential to simultaneously deliver both neuroprotective and thrombolytic agents and enhance overall therapeutic outcomes (Vos et al., 2022). In particular, NPs benefit from very specific characteristics due to their size which can be useful for imaging as well as therapeutic activity. First of all, their small size allows access to areas not available to other objects, such as the intracellular compartment or the crossing of the blood-brain barrier (BBB). Indeed, the BBB crossing capacity of NP has been largely documented in the case of brain tumors as well as for the treatment of various neurological disorders, including Krabbe, Alzheimer's or Parkinson's diseases (Waris et al., 2022; Moore et al., 2023), and this property can be essential for treating the insulted tissue post-ischemia (Ma et al., 2019).

Operating at the nanometric scale induces specific advantages in the circulation, as the vascular margination of blood-borne objects varies based on their size and shape (Müller et al., 2014; Lee et al., 2013). It has also been stated that NPs above 10 nm avoid the kidneys' filtration, and for those below the micron scale, clearance by phagocytosis is hindered: thus, NPs in a 10 nm - few hundreds of nm size window benefit from good circulation, though it also varies with material, shape, or coating (Duan and Li, 2013). Moreover, this size reduction is accompanied by an increasing surface/volume

ratio, which plays a critical role in functionalization, target recognition, as well as surface exchange for drug release (Navya and Daima, 2016). There is increasing evidence that at such scale NPs' behavior is critically affected by their shape, with consequences on cell interaction for instance (Navya and Daima, 2016). Modified responses to exogenous stimuli can also appear, which is for instance the case for ultrasound contrast enhancement upon ultrasound imaging (Pellow et al., 2018). Regarding inorganic NPs, specific properties emerge. For instance, quantum dots (QDs) present strong luminescence with tunable absorption and emission wavelengths, notably through NP size control. They can therefore be useful for imaging, as shown with the near-infrared emitting QDs for thrombus imaging from Imamura's group (Imamura et al., 2016). Plasmonic NPs such as gold materials can absorb light at different wavelengths depending on their size through localized surface plasmon resonance (LSPR), which emerges from the classical surface plasmon resonance at nano scales. This has been used in a proof-of-concept photothermal thrombolytic system based on gold NPs in 2016 (Singh et al., 2016). At the nanoscale, similar NPs show great thermal conversion properties due to their naturally high thermal conductivity combined to increased surface/volume ratio, which has allowed the development of such a photothermal approach (Pallavicini et al., 2021). In addition, ferro/ferrimagnetic NPs undergo a superparamagnetism phenomenon: when they are small enough, each NP is made of only one magnetic domain. Thus, in the absence of an external magnetic field, the magnetic moments of all NPs are differently oriented, as dictated by Néel relaxation, leading to zero magnetization. In the presence of an external magnetic field, instead, superparamagnetic NPs exhibit a stronger magnetization than paramagnetic ones (Farinha et al., 2021). Additionally, through their Néel and Brownian relaxation processes, under stimulation of an alternating magnetic field, superparamagnetic NPs can also be used to produce heat in order to help the dissolution of the clot (Abenojar et al., 2016; Zhang et al., 2021b).

Finally, NPs exhibit high versatility as carriers capable of delivering various drugs to a wide range of tissues. Their ability to either remain in the vascular compartment or cross the BBB depending on their size and shape provides great flexibility and a tailored approach to tackle the different phases of ischemic stroke. For example, during the hyperacute phase of stroke (first 6 hours), when the BBB becomes leaky in the ischemic area, thrombolysis and neuroprotection will be the main therapeutic objectives. NPs enable the targeting of the blood clot for thrombolytics delivery or to reach the ischemic tissue through the more permeable BBB to limit neuronal damage with neuroprotectants. In the subacute and chronic phases (>48 hours after stroke onset), neoangiogenesis and neurogenesis will be the main targeted processes. Thus, growth factors or miRNAs will be delivered to the infarcted region, notably via targeting agents on the NPs surface that promote the passage of BBB, which is less permeable than in the early stage of ischemic stroke (Bernardo-Castro et al., 2021).

Nanotechnological platforms developed to tackle stroke

Over the last two decades, different nanotechnologies have been proposed in combination with tPA or other therapeutics, to broaden the portfolio of stroke treatments and diagnostic tools. Here are described the diverse nanotechnological platforms that have been tested: liposomes, polymeric NPs, inorganic NPs, and miscellaneous NPs (*Figure 1*).

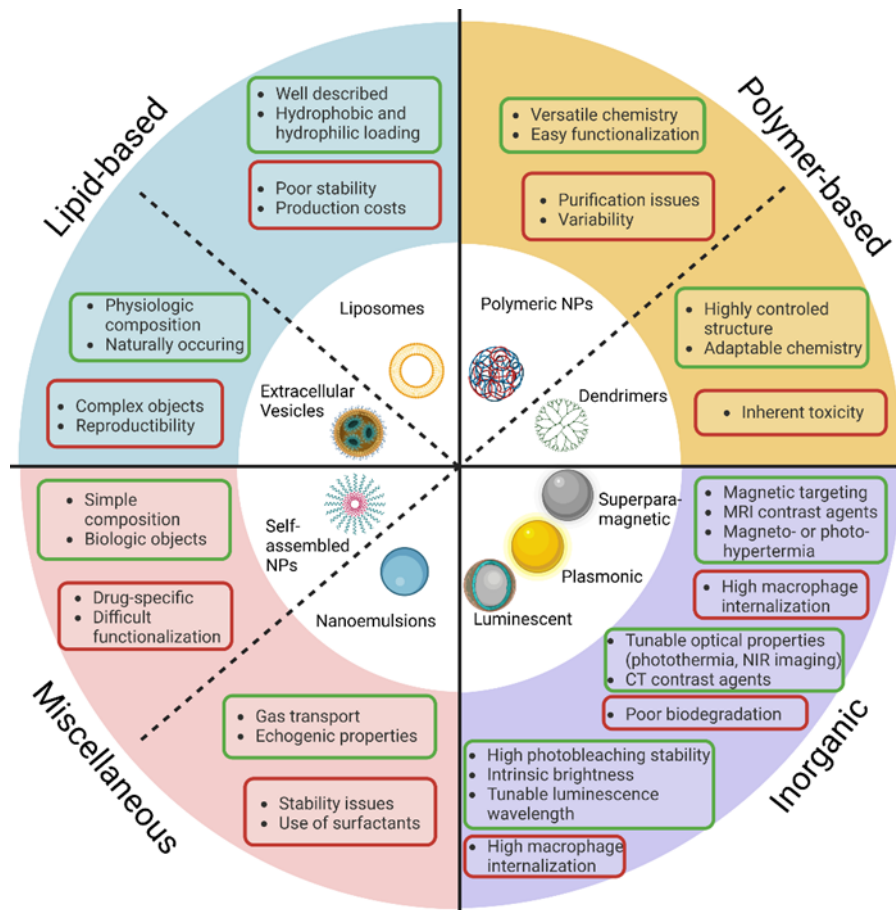


Figure 1: Main NP types used for stroke treatment and imaging, and the associated advantages (green frame)/drawbacks (red frame). NP: nanoparticles; MRI: magnetic resonance imaging; CT: computer tomography; NIR: near infrared. Respective examples of superparamagnetic, plasmonic and luminescent NPs are iron oxide NPs, gold NPs and quantum dots.

Liposomes

Liposomes are nano-micrometer sized particles, made of a phospholipid bilayer which encloses a hydrophilic core, that can be loaded with both hydrophobic and/or hydrophilic molecules. For stroke applications, thrombolytic drugs or contrast agents were principally incorporated in the hydrophilic region. This idea was applied successfully in the work of Heeremans *et al.* in 1995, where rt-PA (Actilyse®) loaded liposomes showed a significantly higher thrombolytic efficacy than free rt-PA. However, the incomplete and suboptimal encapsulation efficiency led to a fast removal of more than 50% of the circulating drug (Heeremans *et al.*, 1995).

To further increase the systemic circulation of the thrombolytic agents, Kim *et al.* tested polyethylene glycol (PEG)-ylated liposomes to conceal the particles from the innate body defense. Both *in vitro* and *in vivo* rtPA release rates were slower and more sustained in the presence of the PEG, with a significant improvement (up to 20-times) of the drug half-life. Furthermore, rtPA entrapment efficiency was constant over 45 days of storage at 4°C (Kim *et al.*, 2009). Echogenic liposomes (EL), carrying gas in the core, are ultrasound-responsive, and were tested as contrast and thrombolytic agents, alone or in combination with tPA. The selective and controlled release of the liposome content was driven by ultrasonic pulses that trigger cavitation and the destruction of gas bubbles, thus further affecting clot integrity and tissue permeability. These advantages were confirmed in several studies, where rtPA-loaded EL showed an increased thrombolytic activity in presence of ultrasound, compared to the free drug (Refaat *et al.*, 2021). Nonetheless, several studies highlighted that the rtPA encapsulation efficiency was limited in this technology, in addition to the limited targeting properties. Overall, further optimization steps are needed before reaching a clinically valuable product (Wang *et al.*, 2020).

By using different lipids and their relative ratios, the liposome composition can be tailored, for instance, with the addition of surface targeting moieties or stimulus responsive molecules to increase selectivity. Furthermore, the possibility to load multiple and different types of drugs allows one to combine the thrombolytic function with neuroprotection. An example is given by the design developed by Quan and colleagues who encapsulated tPA and aspirin (anti-inflammatory drug) in liposomes associated to cryo-shocked platelets (CsPLTs). In this novel approach, the binding of CsPLTs with liposomes was mediated by an “host-guest” interaction between adamantane, a reactive oxygen species (ROS)-responsive molecule on the liposomes, and cucurbit[7]uril on the platelet membrane. This technology showed high affinity with clots as demonstrated *in vitro* and *in vivo* in a murine model of mesenteric artery thrombosis. *In vivo*, in a stroke mouse model induced via photothrombosis, a significant improvement of neurological scoring, volume of stroke damage, and BBB leakage was documented. This technology showed a safe profile with no negative effects as assessed by histological analyses, coagulation molecular indicators, liver and kidney functions (Quan et al., 2023). In 2023, Sun *et al.* proposed a smart liposomal nanocarrier, aiming to tackle neutrophil extracellular traps (NET) formation (NETosis): this phenomenon, that occurs in several pathological conditions including stroke, is regulated by the neutrophils and is responsible for the immune system overactivation in the brain. Their liposomal system (C-Lipo/CA) was intercalated with ROS-responsive polymers (for ROS scavenging and drug release) and coated with CREKA peptide sequences (for fibrin binding). C-Lipo/CA were loaded with Cl-amidine, a peptidylarginine deiminase 4 (PAD4) enzyme inhibitor, to block the NETosis process. *In vitro*, C-Lipo/CA showed efficient binding to microthrombi. *In vivo* experiments displayed a good safety profile, with no impairments in organ architecture and biochemical indicators. In addition, biodistribution tests displayed high retention of the particles in the ischemic brain area. In the middle cerebral artery occlusion and reperfusion (MCAO/R) stroke animal model, C-Lipo/CA showed positive effects on animal behavior, a lesion size reduction from 46.7% (saline group) to 12.1%, and BBB leakage, resulting from the suppression of NETosis and the cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes (cGAS-STING) pathway, both overactivated in ischemic area (Sun et al., 2023).

Interestingly, empty liposomes with a specific lipid composition, but without any encapsulated drug, could also be effective. A brief report published in 2022 explained that empty nanoliposomes (<100 nm size) made of phosphatidylcholine, cholesterol and monosialogangliosides, have a positive effect against stroke. The authors showed an antioxidant protective response *in vitro* on human neuroblastoma (SH-SY5Y) and human microvascular endothelial cells exposed to oxygen-glucose deprivation-reoxygenation (OGD/R). Similarly, nanoliposomes improved neurological scoring, reduced infarct, and edema size in MCAO mice model (Ahmad et al., 2022).

Noteworthy, Al-Ahmady *et al.* found two temporal windows to maximize liposomes localization into the brain after stroke: during the hyperacute phase (0.5 h post-stroke), and the sub-acute/chronic phase (48h post-stroke). This discovery has opened up new avenues for the development of therapeutic options, allowing for the targeting of various aspects of the pathology (Al-Ahmady et al., 2019).

Taking into consideration all these studies, drug encapsulation into liposomes increases efficacy, stability, and therapeutic index of the thrombolytic treatment, reducing at the same time toxic and off-site effects. Adverse effects can be further reduced by adding stimulus-responsive molecules to the liposome composition, thus releasing the drug only at the thrombus site. Nevertheless, their use is still limited due to high production costs, poor stability and short half-life after administration or during the storage (Akbarzadeh et al., 2013).

Polymeric nanoparticles

Polymeric NPs (PNPs) are versatile drug delivery systems made of particulate dispersions of polymers and have a size range between 10 and 1,000 nm. Their size, shape and mechanical properties can be precisely designed, thanks to several easy fabrication techniques, and a wide list of natural or synthetic biocompatible and biodegradable monomers/polymers that can be used as brick for their synthesis. PNP surface can be tailored with the insertion of targeting moieties, to increase the particle

selectivity to the therapeutic target. For these reasons, PNPs were suggested for different biomedical applications, including stroke treatment. Chauvierre's group in 2018 developed fucoidan-labeled PNPs carrying rtPA adsorbed on the surface. PNPs were made of polysaccharide-poly(isobutylcyanoacrylate) by a redox radical emulsion polymerization (RREP). With RREP, the polysaccharides were distributed on the surface of the PNPs to allow rtPA (Actilyse®) adsorption. The addition of dextran modified with amino groups increased the stability of the drug. This technology showed, both *in vitro* and *in vivo*, increased affinity to blood clot and enhanced thrombolytic efficacy (Juenet et al., 2018). Lately, the same research group developed and tested other PNPs named fucoidan-functionalized dextran particles (Fuco-NPs). These PNPs were obtained by green chemistry starting from FDA-approved biodegradable and biocompatible polysaccharides. They were successfully loaded with rtPA, with high encapsulation efficiency of about 65% and 80% for control-NPs and Fuco-NPs, respectively. *In vivo*, Fuco-NPs loaded with rtPA could improve the angiographic scores in murine stroke models: Fuco-NPs loaded with rtPA showed a 67% total recanalization rate versus only 33% for free rtPA treated mice (Zenych et al., 2021).

PNPs shape can be finely tailored, to enable specific hemo-dynamic performance after systemic administration. For example, Moore *et al.* designed discoidal polymeric nanoconstructs (DPNs) that have a diameter of 1,000 nm and a height of 400 nm, and are composed with a mixture of poly(lactico-glycolic acid) (PLGA) and PEG. DPNs were successfully tested *in vitro* and *in vivo* for vascular delivery of imaging and therapeutic molecules against tumors (Moore et al., 2022). Colasuonno *et al.* tested rtPA-loaded DPNs as a thrombolytic agent, demonstrating a clot lytic activity similar to the free drug in static experimental conditions, while it was increased in microfluidic experiments with dynamic conditions. *In vivo* experiments on the mouse mesenteric vein thrombosis model confirmed the clot lytic efficacy, where rtPA-DPN outperformed free rtPA for occlusion resolution (Colasuonno et al., 2018). Another approach based on hemo-dynamic performance was proposed by Korin *et al.*, in 2012. Shear-activated nanotherapeutics (SA-NTs) produced by spray-drying concentrated solutions of small PLGA NPs (~180 nm) to form microaggregates (~3.8 µm). These microaggregates are stable in aqueous solution due to the PLGA NPs' hydrophobicity and remain intact under physiological blood flow conditions. Interestingly, they break when exposed to high shear stress, similarly to what occurs in proximity to an occluded vessel, enabling the release of tPA near the occlusion. *In vitro*, in presence of a simulated clot, SA-NTs showed increased thrombolytic activity in comparison to free tPA. *In vivo*, SA-NTs induced progressive thrombolysis, increased efficacy, and animal survival in two different preclinical animal models (Korin et al., 2012).

A combined approach mixing targeting, thrombolytics and ultrasound sensitivity was proposed by Marsh *et al.*, in 2007. They tested a fibrin-specific liquid perfluorocarbon (perfluoro-octylbromide) NP loaded with the thrombolytic agent streptokinase (SK). Perfluoro-octylbromide is a biocompatible molecule with echogenic properties. The authors confirmed the efficacy of this technology as an acoustic contrast agent, useful for clot imaging and volumetric analysis. Furthermore, the clot lysis was efficaciously increased in comparison to free SK thanks to the NPs' targeting ability, and could be monitored through ultrasound visualization (Marsh et al., 2007). Similarly, Kawata *et al.*, combined basic gelatin and zinc ions to carry and control the activity of rtPA Montepase (Cleactor®). In this complex, rtPA activity is reduced to 45%, but it can be fully restored after ultrasound exposure. This approach allowed the rtPA protection from serum proteins inactivation, and the control of off-site effects of the treatment (*i.e.*, hemorrhagic transformation). rtPA-loaded gelatin-zinc particles have an intrinsic targeting ability due to the affinity between gelatin and VWF, as highlighted by *in vitro* and *in vivo* experiments in the mouse arterial thrombosis model. Animal experiments confirmed the efficacy of the treatment and the survival rate dramatically increased in comparison to free-rtPA treatment in the swine acute myocardial infarction model (Kawata et al., 2012).

Multifunctional polymers with specific features, such as microenvironment responsiveness, were adopted for PNP crafting. An example is given by the technology proposed by Wu *et al.* in 2022: PNP made with a modified version of poly(ethylene glycol) (PEG), having in the structure a ROS-degradable poly(2,2'-thiodiethylene 3,3'-thiodipropionate or PTT) polymer with an antioxidant

activity, and a thrombin-cleavable peptide (NH₂-norleucine-TPRSFL-C-SH). This tailored polymer is cleaved in the presence of thrombin, with a resulting particle rearrangement and shrinkage, to improve brain penetration. The synthesized particles made with a modified co-polymer (PTT NP) showed in biodistribution experiments the efficient localization into the ischemic area 24h after stroke induction and treatment. PTT NPs showed positive effect in the MCAO/R mouse model, with a reduction of the infarct area from about 60% (PBS group) to 30% (PTT NPs). To further increase the brain penetration, the surface of the PTT NPs was decorated with AMD3100 for its specific affinity with a chemokine receptor, CXCR4, particularly expressed in the ischemic tissue. The conjugation with AMD3100 enabled a significant enhancement in NP localization into the ischemic area, as confirmed by whole-animal fluorescence analysis. Lastly, AMD3100-conjugated shrinkable PTT NPs (ASPTT) were loaded with glyburide, an anti-edema drug useful in stroke condition, as determined in the clinical trial GAMES-RP, where its administration resulted in an increased patient survival (Sheth et al., 2016). ASPTT-glyburide showed a controlled release of the drug, which happens only in presence of thrombin and ROS. *In vivo*, in the MCAO/R model, ASPTT-glyburide outperformed the control conditions (ASPTT NPs without glyburide or free glyburide) in terms of reduced infarcted area, neurological scoring, survival and BBB leakage (Wu et al., 2022).

Similarly, Cheng *et al.* tested a pH-sensitive theranostic rapamycin (RAPA)-loaded NP system (RAPA/Gd³⁺@NPs), able to selectively release the payload (drug and contrast agent) in the ischemic tissue. RAPA/Gd³⁺@NPs were synthesized by self-assembly of the pH-sensitive amphiphilic block copolymer (mPEG-b-P(DPA-co-HEMA)-Ce6) in aqueous solution with RAPA and Gd³⁺, obtaining spherical particles with a mean diameter of 20–30 nm. The biodistribution in the MCAO/R rat model showed high accumulation of RAPA/Gd³⁺@NPs inside the ischemic area 6h-post administration, as displayed by MR T1-imaging and ex-vivo NIR fluorescence imaging. Also, neurological and behavioral tests showed an amelioration of ~50% of the scores after treatment with RAPA/Gd³⁺@NPs, in comparison to the saline control group. Similar benefits were found evaluating lesion size and BBB integrity. BBB integrity was also preserved with a significant edema reduction in the ischemic hemisphere. The molecular analysis of inflammatory markers (IL-6 and TNF- α) suggested the neuroprotective role of these NPs by helping the BBB heal after the ischemic event. Finally, these technologies showed a good safety profile, with no obvious damage detected by histological analysis of the main organs (Cheng et al., 2021).

A subtype of highly ordered multibranching polymeric NPs, called dendrimers, has also been investigated for drug deployment in stroke, as it is possible to precisely tune their size distribution, architecture, and functionalization. For example, Lee *et al.* described early-generation polyamidoamine dendrimers (PG2HR), which have been tested as gene carriers in MCAO/R stroke animal models and injected together with heme oxygenase-1 (HO-1) plasmid. The complex pHO-1/PG2HR reduced apoptosis and infarct sizes (from 40% to 30% total infarct brain area) in ischemic brains, suggesting a potential use as gene therapy of inflammatory diseases including ischemic stroke, also due to the high gene delivery efficiency and low cytotoxicity (Lee et al., 2021).

Even though PNPs have a great potential as nanomedicines due to their versatility, highly controlled chemistry, tunable architecture, and the broad range of drugs and targeting moieties they can be equipped with, several limitations remain to be overcome. PNPs synthesis often requires the use of catalyzers, organic solvents, photo-polymerizers, which are all potentially toxic reagents for cells and tissues. Therefore, extensive purification steps are needed to limit the presence of impurities. Several biocompatible and biodegradable polymers have a limited use due to batch-to-batch variations. Finally, to assure suitability for biomedical application, it is crucial to outline a standardized array of characterization techniques in order to guarantee a complete quantitative analysis.

Inorganic nanoparticles

Inorganic NPs have been investigated for their intrinsic material features, such as catalytic, magnetic, thermal or optical properties, which sustained their application as a diagnostic and therapeutic tool, notably for stroke application. Among them, Mn₃O₄ nanoenzymes stabilized by human serum albumin (HSA-Mn₃O₄) have been studied as an antioxidative treatment to address

ischemic stroke reperfusion injury. Mn_3O_4 NPs can release manganese ions in circulation, which promotes the activity of superoxide dismutase 2 in the body. The positive *in vitro* results against oxygen-glucose deprivation, endoplasmic reticulum (ER) damage and cell apoptosis were further confirmed in animal investigations. The presence of the HSA on the surface enabled prolonged circulation time (~72h), preserving the ROS scavenging ability from off-site activation. *In vivo*, HSA- Mn_3O_4 showed a strong neuroprotective effect, with reduction in stroke volume and improvement in behavioral score. In addition, the highest dose of HSA- Mn_3O_4 (0.4 mg/kg) reversed the ER stress pathways activation to normal level, counteracting the activation of pro-apoptotic signals (Huang et al., 2022).

Inorganic NPs also enable theranostic approaches, as shown by Kim *et al.*, with glycol-chitosan-coated gold NPs, conjugated to fibrin-targeting peptides (fib-GC-AuNP). Gold NPs especially show desirable optical properties due to the surface plasmon resonance effect, enabling photothermal treatment. This optical effect is localized from the visible to the infrared region, depending on the particle size, shape, and structure (Khlebtsov, 2008). The particles from Kim's group hold targeting features and the capacity to visualize cerebrovascular thrombi by CT-based imaging. Thus, fib-GC-AuNP allowed quantification of cerebral thrombi over the tPA treatment to directly evaluate its thrombolytic effect. Remarkably, fib-GC-AuNP acted as CT contrast agent and enabled the image analysis on recurrent thrombosis by mCT for up to 3 weeks, with a single administration and without neurobehavioral or systemic toxicity (Kim et al., 2015).

Among magnetic NPs, iron oxide NPs (IONPs), made of magnetite (Fe_3O_4), maghemite ($\gamma-Fe_2O_3$) or mixed ferrites (MFe_2O_4 where $M=Co, Mn, Ni$ or Zn) have been proposed and tested for several different applications. IONPs are non-toxic, biodegradable, can modulate cell differentiation and eventually become a source for *in vivo* iron supplements, useful as anemia treatment (Dadfar et al., 2019). A clear example is the approach proposed by Voros *et al.*, that aimed to increase thrombolytic activity of rtPA (Alteplase®). They tested clusters of iron oxide nanocubes, coated and stabilized by rtPA (rtPA-NCs), to have a combined pharmacological and thermal therapy of blood clots. Thermal therapy is a strategy already approved by the FDA against cancer, to increase the efficacy of chemotherapy. For stroke, this strategy involves heating local areas through magnetic hyperthermia, in order to improve rtPA clot lysis. *In vitro*, rtPA-NCs showed higher dissolution rates compared to free rtPA, in absence of heating (around 100 times). The exposure to thermal treatment increased even more the efficacy of the rtPA-NCs (10 times) compared to unexposed NPs. *In vivo* experiments on the mouse mesenteric thrombosis model confirmed rtPA-NCs efficacy, leading to a complete clot dissolution in less than 3 min, while the same concentration of free-rtPA was ineffective even after 60 min. Noteworthy, rtPA-NCs displayed high values of transverse relaxivity, suggesting the possible application of these particles for MRI imaging and magnetic targeting of the clot (Voros et al., 2015).

Similarly, nanoscale superparamagnetic IONPs (SPIO), with a 20-50 nm core size, were already exploited as a contrast agent for T2 weighted MRI. Further investigations on IONP magnetic properties revealed that by reducing the NP size, the longitudinal T1 relaxation effect was enhanced. For this reason, ultrasmall superparamagnetic iron oxide NPs (USPIOs), and extremely small-sized iron oxide NPs (ESIONs) characterized by a core size below 4 nm and a paramagnetic behavior, were suggested as T1 contrast agents for MRI (Kim et al., 2011). This is mainly due to the high surface-to-volume ratio of the NPs and the predominance of a magnetically disordered spin-canted shell (Richard et al., 2016).

Another approach appraised in the last decade consists of labeling cells with NPs for *in vivo* tracking of the transplanted cells by imaging techniques (e.g., MRI, CT, and bioluminescence BMI). This has been applied to stem cells which represent a promising therapy for different pathologies, including stroke, but lack a way for *in vivo* tracking (Bliss et al., 2007). Wen and collaborators tested the efficacy of NPs to track neural stem cells (NSC) in presence of stroke: before injection, the cells were labeled with nano-sized cationic polymersomes loaded with SPION and QDs. QDs are 1–10 nm sized nanocrystals with enhanced optical properties. Their first generation has a core made of elements from groups II to VI of the periodic table, surrounded by a second semiconductor, usually zinc sulfide, making them brighter and much more stable than common fluorophores. Labeled cells were

effectively visualized *in vivo* by dual-modal imaging (magnetic resonance and fluorescence imaging). In the rat stroke model, NPs-labeled NSC were detected by T2- and T2*-weighted MRI, until 6 weeks post-injection. In fluorescence imaging, the signal was recorded 1 week after transplantation, with a gradual decline until 5 weeks (Wen et al., 2014). These QDs were followed by the development of next-generation structures, such as carbon dots (CDs), which are useful as MRI and optical probes. Nanohybrids, formed by the covalent linkage between thrombolytic agents and CDs, have been reported to emit a fluorescence signal in the brain of transient MCAO murine models and for retaining PA activity. Such nanohybrids could also be a promising indicator of early BBB damages and intracranial hemorrhage after thrombolysis, favoring early intervention (Niu et al., 2020).

Inorganic NPs were also applied as agents or carriers of neuroprotective drugs. For instance, Cerium oxide (CeO₂) NPs have several intrinsic antioxidant and neuroprotective effects, in addition to BBB crossing, and were therefore already proposed as drug delivery systems for neurological disorders (Portioli et al., 2013). For instance, Li *et al.* investigated CeO₂ NPs loaded with dl-3-n-butylphthalide (NBP-CeO₂ NPs). NBP acts on the ischemic area microcirculation promoting angiogenesis, neurogenesis and neuroplasticity, reducing inflammation and apoptosis, with overall neuroprotective effects. For these reasons, it was FDA-approved for a phase II trial in the treatment of ischemic stroke. Here, NBP-CeO₂ NPs reduced ROS levels in brain microvascular endothelial cells (BMVECs) and hippocampal neurons after OGD/R. In the MCAO/R mouse model, NBP-CeO₂ NPs preserved BBB integrity and reduced lesion volume in comparison to control conditions (damaged area of 39.8% in the saline treated group, reduced to 10.6% in the NBP-CeO₂ NPs group). Neuroinflammation was also reduced in the NBP-CeO₂ group, in comparison to the saline condition. Interestingly, empty CeO₂ NPs also showed positive effects on BBB leakage, lesion size reduction and neuroinflammation, supporting the development of this nanotechnology for new stroke therapies (Li et al., 2022b).

Even though possible toxicity issues related to inorganic NPs have been highlighted and debated (Soenen et al., 2011), the literature still does not offer a definitive conclusion on this matter (Sani et al., 2021; Bhatti et al., 2022). Like organic nanomaterials, their biodegradation depends mostly on NP material and coating, and for instance, clear degradation issues have been shown with certain inorganic nanomaterials such as gold (Balfourier et al., 2020). Other inorganic technologies showed good biocompatibility and reduced risk of toxic effects (Ma et al., 2009; Kim et al., 2015; Hu et al., 2018; Huang et al., 2022). All things considered, further pre-clinical and clinical investigations focusing more on safety and toxicological aspects will be needed to elucidate the potential risks for human health.

Miscellaneous nanoparticles

Nanoemulsions

Nanoemulsions (NEs) are dispersions of small droplets in the range of 0.1 – 100 µm, generated with two immiscible phases (liquid in liquid emulsions), and stabilized by emulsifying agents (surfactants and cosurfactants). The size of the droplets depends on the composition of the system and the homogenization method. Among NEs, dodecafluoropentane nanoemulsion (DDFPe) is a neuroprotectant perfluorocarbon that was initially proposed as ultrasound contrast agent. Additional investigations highlighted the *in vivo* potential as oxygen and carbon dioxide cargo. Preclinical studies in the rabbit embolic stroke model demonstrated >80% reduction in acute infarction. Further, the repeated administration of DDFPe over a 24-hour period showed positive improvement in vital signs and cerebral tissue, as highlighted by histological analysis. Different MRI markers of infarct improved in the early hours following vascular occlusion (Brown et al., 2014; Fitzgerald et al., 2015), with extension in the time window for tPA intervention (Culp et al., 2015).

Natural compound-derived nanoparticles

Several natural compound-derived NPs were tested against stroke, with the advantage of using natural materials with intrinsic therapeutic effects, combined to an efficient brain delivery. Zhang *et al.* screened a collection of natural nanomaterials, identifying betulinic acid (BA) as a preferential compound against stroke. BA has intrinsic antioxidant properties, useful to counteract the reperfusion injury damage and oxidative stress triggered by stroke. A chemically modified form of BA, named

betulinic amine (BAM), was used for the NP preparation. BAM NPs were synthesized using a standard emulsion procedure, with solvent evaporation over the emulsion. After the purification, BAM NPs were conjugated to AMD3100, a CXCR4 antagonist, which increased the brain penetration of the particles. Intravenous administration showed an increased survival of mice, reduced infarct volume by 57%, and improved neurological scores. Furthermore, the neuroprotective peptide NA1, already in clinical trial (phase III) for its neuroprotective activity against NMDA receptor-mediated toxicity, was bound on the surface of BAM NPs. The additional conjugation of NA1 further increased the mice survival and reduced the infarct volume by 69.8%, in comparison to the control condition (Zhang et al., 2022).

Extracellular vesicles

Extracellular vesicles (EVs) are released by living cells and transfer lipids, nucleic acids, proteins, and metabolites within and between organs in an endocrine process. The heterogeneous population of EVs produced *in vivo* play crucial roles in physiological and pathogenetic mechanisms. EVs include exosomes produced through the endosome pathway and released into the extracellular space, and ectosomes formed by directly budding of the cell plasma membrane (Cocucci and Meldolesi, 2015). Wang *et al.* observed that serum exosomes from young healthy rats, administered into aged ischemic rats, improved short- and long-term functional outcomes, and reduced synaptic loss. Conversely, exosomes from aged healthy rats did not have positive effects (Wang et al., 2023). In the work of Zhang *et al.*, they observed that the levels of pro-inflammatory mediators in serum exosomes increase with age. This mechanism is associated with worsened stroke outcomes, through excessive activation of C3aR-dependent microglial phagoptosis (Zhang et al., 2021a). Recent discoveries showed the therapeutic potential of EVs from specific cell types. Exosomes released from adipose-derived mesenchymal stem cells (ADSC-Exo) were tested against cerebral ischemia/reperfusion injury. ADSC-Exo carried miR-760-3p (miRNA) which inhibits with high efficiency the CHAC1 gene expression, upregulated by ischemic stroke ferroptosis. *In vivo*, intranasal administration of ADSC-Exo improved mice neurobehavior after ischemia/reperfusion injury (Wang et al., 2023). Similarly, cortical astrocytic derived EVs accelerated recovery after stroke, in the rat ischemia/reperfusion model after a single intracerebroventricular administration (Heras-Romero et al., 2022). EVs exert neuroprotective effects, modulating the recruitment of circulating immune cells into the damaged area. In Han *et al.* study, small EVs obtained from Mesenchymal Stem Cells (MSC), after intravenous administration in young and aged ischemic mice, reduced neurological deficits, infarct volume, brain edema, and neuronal injury, with a decreased count of immune cells in the ischemic brain of aged mice (Han et al., 2023).

Targeting strategies

While the structure and composition of NPs can vary considerably, most recent nanoplatforms include some sort of targeting. Indeed, this is critical to ensure that the payload, or desired effect, can be delivered at the expected location: namely, the clot itself or the ischemic tissue. As discussed above, the main explored class of thrombolytics and the only approved one for ischemic stroke is formed by plasminogen activators (PAs), which suffer from limited recanalization rate and high deleterious side effects (Zenych et al., 2020). Thus, targeting the drug delivery only to the thrombus can limit systemic side effects, while ensuring a local high concentration of active agents. Here are described various targeting strategies, from the single molecular targeting to multiple molecular targeting methods, as well as magnetic and passive targeting (**Figure 2**). Notably, we focused on quantification of targeting capacities rather than therapeutic efficacy to illustrate the potential of the various methods described hereafter.

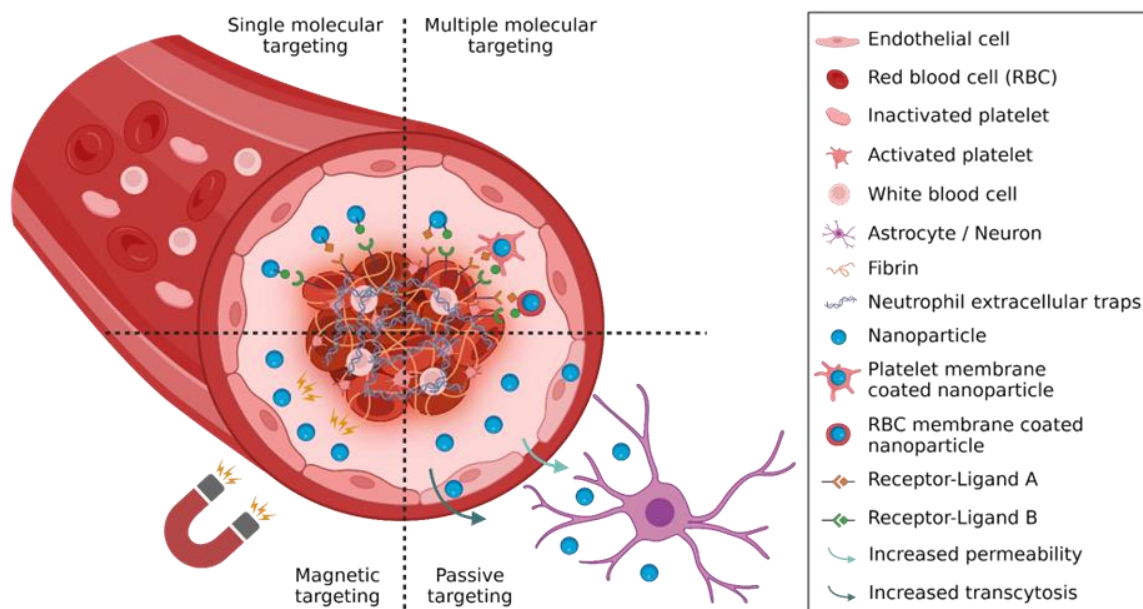


Figure 2: The different targeting strategies for NP-related stroke treatment and diagnosis.

Single molecular targeting

Multiple biomarkers have been identified to recognize either the blood clot, or the ischemic region. Regarding the former, some NPs aim to target activated platelets. The peptide cRGD is among the well-known binding motifs used for NPs, as it was characterized for stroke application, to bind the GPIIb/IIIa receptor, which is expressed on platelets, and play a critical role during their activation and aggregation through fibrinogen and VWF binding (Liao et al., 2019). For instance, Liao *et al.* described in 2018 a trimethyl chitosan NP, loaded with lumbrokinase (LK) and functionalized with cRGD. This system showed improved thrombolysis *in vitro* and *in vivo* on rat thrombus models, compared to free drug (around 60% reduction in clot weight for targeted NPs vs. no difference for untargeted NPs) (Liao et al., 2019). More complex designs were also described, such as the Janus-rod micromotors from Cao *et al.*, in 2021, in which the cRGD targeting allowed the US-responsive objects to accumulate at the thrombus (3.4-fold improved accumulation at the thrombus vs. no cRGD), where enhanced penetration and thrombolysis (74%) was achieved (Cao et al., 2021). Other biomarkers exist for activated platelets, such as the P-selectin, a transmembrane protein expressed on activated platelets and endothelium, which mediates leukocyte adhesion through the Sialyl Lewis x (SLe x) motif on PSLG-1 ligand. A natural polysaccharide, fucoidan, was found to mimic this motif with high affinity and specificity (Bachelet et al., 2009), and can thus be used as targeting moiety: for instance, the technology developed in 2021 by Zenych *et al.* used it as a targeting agent. Here, fucoidan was crosslinked to PNPs, and the resulting Fuco-NPs could effectively target P-selectin and aggregated platelets *in vitro*, with a significant increase in aggregated platelets' targeting in a microfluidic setup compared to untargeted NPs (Zenych et al., 2021). Interestingly, some authors took into account the time-wise development of thrombi in their targeting, such as Pan *et al.* who described in 2017 micelles which could be loaded with LK and functionalized with Annexin V. Annexin V have high affinity for phosphatidylserine, a lipid presented on the exterior membrane of apoptotic cells, as well as on platelets during their activation. This allowed a significant accumulation of targeted micelles to the clots (compared to non-targeted) and significantly enhanced thrombolysis in FeCl₃ murine thrombosis model, when compared to free LK or non-targeted micelles (Pan et al., 2017).

Other groups chose to target components of the thrombus, such as the fibrin molecule. For instance, Marsh *et al.* described in 2011 SK-loaded perfluorocarbon NPs decorated with *ad hoc* antibodies against fibrin (10⁻¹⁰ M affinity, with no reactivity toward fibrinogen). These NPs retained good SK-mediated *in vitro* thrombolysis (20-30% increase in clot dissolution when compared to empty

NPs). Moreover, in the canine thrombosis model, an accumulation of targeted NPs into clots was observed when compared to the non-targeted NPs. However, they lack evidence of enhanced thrombolysis *in vivo* (Marsh et al., 2011). Recent literature focused on small peptides to target fibrin, notably CREKA (Zhao et al., 2020), or interestingly *ad hoc* designed peptides (Seo et al., 2018). For example, Seo *et al.* leveraged peptides, selected through phage display, that could only recognize fibrin-fibronectin complexes (and not fibrin alone), to allow the specific targeting of thrombi. This was incorporated in a transferrin-based nanocage to deliver C-terminal encoded microplasmin (and produced thanks to a single transgene expression). This system was validated *in vitro* for their clot-binding properties. In the murine arterial thrombus or deep vein thrombosis models, they showed a 50% reduction of thrombosis, in comparison to non-targeted, non-loaded nanocages, or targeted microplasmin without the nanocage construct (Seo et al., 2018).

Nevertheless, some approaches aim not at dissolving the clot, but rather alleviating the damage of ischemia, therefore requiring targeting to the ischemic tissue. While the leakiness of the BBB can be exploited to deliver certain drugs with NPs (Yuan et al., 2021), some authors increase the specificity of drug delivery to the damaged tissue by adding targeting moieties to ischemic regions (Wang et al., 2015), such as Zhang *et al.* who described in 2022 antioxidant NPs targeting CXCR4, a biomarker of ischemic cells in the brain, thanks to a small antagonist molecule, AMD3100. The resulting NPs were shown to significantly accumulate in the ischemic region with high reduction (50%) of the infarct region and significant neurological recovery compared to non-targeted NPs in mice stroke models (Zhang et al., 2022).

Therefore, there is no single gold standard biomarker for thrombus targeting. Instead, multiple biomarkers have been identified. Appropriate targeting strategies should be carefully chosen by identifying the target area: the clot itself, the ischemic tissue, or a single cell type for example.

Multiple molecular targeting

Multiple molecule grafting

All the targeting strategies mentioned above involve only one targeting agent and one biomarker. However, recent papers proposed alternative strategies, including grafting more than one targeting moiety at the NP surface to increase either their specificity or their accumulation at the thrombus site (**Figure 2**). Sun *et al.* designed liposomes coated with either platelet-binding peptides (PBP), fibrin-binding peptides (FBP) or a combination of them (PBP + FBP), to compare their thrombus targeting efficacy (Sun et al., 2020). *In vitro*, and on the mouse carotid artery thrombus model, they showed that with a lower total peptide concentration, combination targeting allowed a greater accumulation to the clot than targeting with PBP or FBP only, which can be explained by the possible multiple interaction with receptors or clot components. This approach may also enhance the targeting specificity to acute ischemic stroke thrombi which are made up of a dense outer shell containing mainly fibrin, VWF, and aggregated platelets (Di Meglio et al., 2019). In addition, their results proved that targeting can be impaired when ligand density is too high, and they suggested a role of the steric hindrance between the targeting agent and its target. Rather than targeting different components of the thrombus, several articles proposed to target only platelets with two different peptides grafted, RGD (GPIIb/IIIa binding) and EWVDV (P-selectin binding). In two different articles for respectively thrombus treatment and thrombus imaging, the combination of these peptides increased the accumulation of NPs at the thrombus site, compared to RGD or EWVDV decoration only (Pawlowski et al., 2017; Zhang et al., 2019). Indeed, Pawlowski *et al.* demonstrated *in vitro* in a microfluidic dynamic flow experiment that the peptide combination enhanced the targeting to platelet-rich thrombus, doubling the retention onto platelets, compared to either one (Pawlowski et al., 2017). However, instead of targeting platelet-rich clots, fibrin or erythrocyte-targeted NPs would be preferable in the case of stroke, considering they are the major components of the associated clots (Jolugbo and Ariëns, 2021; Mereuta et al., 2021).

Grafting several molecules can also be a strategy to sequentially reach different targets in ischemic stroke (Zhao et al., 2016; Xu et al., 2019). For instance, by decorating liposomal nanocarriers with T7 peptide and stroke homing peptide (SHp), Zhao *et al.* aimed to cross the BBB, and then target

ischemic neurons to deliver ZL006, a novel promising neuroprotective agent (Zhao et al., 2016). Their coating combination enabled nanocarriers to accumulate 1.5 times more in the ischemic brain than with T7 alone and reduced the infarction volume by approximately 30% compared to coating with T7 or SHp alone, in a MCAO/R model on rats.

All things considered, grafting several targeting molecules can be performed to improve thrombus homing, to selectively select certain thrombi, or reach sequentially different targets.

Cellular membrane coating

Some groups have described a simple solution for NP targeting without having to settle for one or more biomarkers: by leveraging the cellular membrane of critically chosen cells, they addressed both the circulation stabilization (stealth) of their NPs and homing to the clot or the ischemic area.

Based on the process behind platelet aggregation and clot formation, the platelet membrane coating of NPs was assessed (Xu et al., 2019, 2020; Yu et al., 2022). For instance, Xu *et al.* described polymeric NPs coated with murine platelet membrane to deliver rtPA (PcNP) and compared them to erythrocyte-membrane coated control NPs (RcNP). They showed clear thrombus targeting of PcNPs vs. RcNPs in multiple murine thrombotic disease models, e.g., evidenced in a pulmonary embolism model with a significant fluorescence accumulation to the lungs compared to RcNPs. Moreover, they evidenced that while rtPA alone had a similar thrombolysis rate to their rtPA-loaded PcNP in mesenteric artery thrombosis mice models during thrombus formation, only the targeted NPs could elicit thrombolysis once the clot was fully formed. Finally, they demonstrated a clear enhancement of neurological and survival outcomes of PcNP-PA in comparison to free rtPA in transient stroke murine models (Xu et al., 2020). Others took advantage of the homing properties of platelet membranes to accumulate at the stroke site. Xu *et al.* developed in 2019 a nanoconstruct where rtPA was delivered after the thrombin-mediated cleavage of its linker to the NP, which in turn facilitated the exposure of the Tat peptide, secondary targeting of the endothelium, and crossing of the BBB for the antioxidant delivery. These NPs consisted of neuroprotectant (ZL006e) and dextran. It was shown that these NPs could accumulate on blood clots and have *in vitro* and *in vivo* enhanced thrombolysis compared to free drugs: the infarct volume was reduced to 20% compared to approximately 60% for non-cleavable NPs or free drug. Furthermore, the sequential targeting and thrombin dependent delivery significantly reduced the infarct area in mice stroke models in comparison to the free drugs (Xu et al., 2019).

Notably, other cellular coatings could be used to reach different targets. For instance, Ma *et al.* described in 2019 polymeric NPs coated with neural stem cell (NSC) membrane to deliver an anti-edema drug through the BBB membrane at the stroke site. However, they further strengthened the design by using CXCR4-overexpressing NSCs membranes and showed that it relied on the interaction between CXCR4 and SDF-1 (enriched in the ischemic microenvironment) to further enhance drug delivery and improve neurological recovery in a mouse stroke model compared to classic NSC membrane-coated NPs (Ma et al., 2019). Comparable approaches relied for example on cellular membrane from highly brain-metastatic tumor 4T1 cells (from a breast tumor cell line) to target the brain ischemic tissue and noted a significant neurological improvement, in comparison to non-membrane-coated NPs in murine models. These coated NPs accumulated three times more in the brain ischemic region than untargeted NPs (He et al., 2021). Macrophage membrane coating has also been used to increase targeting and disguise from immune system cells. For instance, macrophage membrane coated manganese dioxide NPs targeted endothelial cell adhesion molecules, thus allowing ROS scavenging, neuroprotection, and macrophage-type polarization (Li et al., 2021).

However, key aspects must be taken into account when resorting to cellular targeting: as discussed by Xu *et al.*, provenance of the cell membrane can play a major role in their tolerability by the host body, and human leukocyte antigen (HLA) matching could be an issue for scaling-up these therapies (Xu et al., 2020). On the other hand, using platelet membrane to deliver NPs with platelet-affecting drugs can prove ineffective: as demonstrated by Yi *et al.* in 2020, who had to use erythrocyte membrane functionalized with CREKA peptide (for fibrin targeting) instead of platelet membrane since they aimed at delivering tirofiban, a GPIIb/IIIa antagonist which prevents platelets aggregation in thrombi and thus impaired the targeting of platelet membrane coated NPs (Zhao et al., 2020).

Magnetic targeting

Over the past two decades, magnetic NPs have been increasingly used for biomedical applications (**Figure 2**). This can be explained by their numerous interesting properties, including their magnetic manipulability. Indeed, by applying an external magnetic field near the brain ischemic region, the circulating magnetic NPs can accumulate there. Ma *et al.* tested the effects of rtPA linked through carbodiimide-mediated amide bond to polyacrylic acid-coated magnetite (PAA-MNP) NPs. Positive results were obtained *in vitro* and *in vivo*, in a rat embolic model of iliac artery occlusion. The PAA-MNP-rtPA under magnetic guidance along the artery restored the iliac blood flow, while control groups and free rtPA showed no improvement on hemodynamics (Ma *et al.*, 2009). Hu *et al.* designed rtPA-coated iron oxide microrods (rtPA-MRs) to target the cerebral thrombus and dissolve it through combined fibrinolysis and mechanical stimulation by an external rotating magnetic field (RMF) (Hu *et al.*, 2018). On a distal MCAO stroke mouse model, the authors found that the microrods could efficiently target the brain clot by placing a magnet near the distal middle cerebral artery area. Further, the blood flow was restored in 25 minutes with rtPA-MRs at 0.13 mg/kg rtPA accompanied by RMF, compared to the 85 minutes needed with free rtPA at 10 mg/kg. Thus, their nanosystem could perform a more efficient thrombolysis with approximately 100 times fewer rtPA. In addition to fibrinolytic agents, magnetic guidance can be used to deliver neuroprotectants to the stroke region and thus limit the cerebral infarction. In their work, Kim *et al.* tested MSC-derived nanovesicles encapsulating iron oxide NPs (IONPs) (Kim *et al.*, 2020). In MCAO/R rat models, they obtained a 5.1-fold higher accumulation of their NPs at the ischemic lesion by magnetic stimulation in comparison to unstimulated ones. Furthermore, IONPs enabled a significant reduction of the infarcted volume through the angiogenic, anti-apoptotic and anti-inflammation properties of the MSC nanovesicles. Nonetheless, the clinical translatability of this approach needs further improvements: in this study, the helmet which enabled the magnetic guidance was kept 3 days on mice. Consequently, it is essential to refine and enhance this technique before considering its application in human use. To further increase the targeting efficacy to stroke lesions, Li's group loaded IONPs and L-arginine (for stimulation of NO generation) in platelet-membrane-based nanovesicles. Their results on a Rose Bengal induced mouse stroke model demonstrated that the magnetic guidance of NPs could increase the targeting compared to platelet membrane alone and allowed a faster blood flow recovery through NO-mediated vasodilation (Li *et al.*, 2020). Despite the efficacy of magnetic targeting, one should be aware of the remaining challenges for the transfer to humans: for example, the type of magnet to use and how to implement its use with the human body. Optimized magnets and mathematical models are necessary to predict the movement of magnetic NPs in biological fluids, besides the regulatory approvals that will be required (Shapiro *et al.*, 2015).

Passive targeting

In most stroke patients, there is a loss of the BBB integrity due to damaged tight junctions, which results in enhanced BBB permeability (Nian *et al.*, 2020). This pathological state can be used to increase the BBB crossing of circulating NPs in the ischemic region compared to the healthy one. This is the case in Yuan *et al.* study, where they showed a 33% higher accumulation of oligosaccharide NPs in the ischemic brain than in the healthy tissue in a MCAO/R mouse model, only through passive targeting (Yuan *et al.*, 2021) (**Figure 2**). This strategy is particularly interesting to deliver neuroprotective agents.

In addition to this enhanced permeability, the BBB presents a higher rate of transcytosis in stroke conditions which can additionally enhance the passive targeting of ischemia (Knowland *et al.*, 2014; Yuan *et al.*, 2021). Although these strategies are less effective than active targeting, they don't require any specific coating, thus limiting the complexity of the nanosystem injected to the patient and making their production and approval easier.

Clinical studies

As described above, there is a broad amount of *in vitro* and pre-clinical studies using therapeutic agents combined with NPs, to tackle ischemic stroke (Blanco *et al.*, 2015; Sarmah *et al.*, 2021).

However, to our knowledge, only clinical studies based on the use of USPIOs have been performed as detectors of stroke lesions compared to the conventional gadolinium-enhanced MRI (Saleh et al., 2004; Tang et al., 2009). Based on their size, SPIOs are phagocytosed by cells of the reticuloendothelial system in the liver and the spleen leading to rapid clearance from the peripheral circulation, while USPIOs are taken up by cells of the mononuclear phagocyte system and can circulate longer, promoting the interaction with phagocyte populations in the blood and lymph nodes. Among USPIOs' specificities, one can also mention T2-weighted tissue hypo-enhancement and the possibility to localize in macrophage-mediated inflamed areas, increasing the potential use in targeted imaging of inflammatory disease states (Farinha et al., 2021). USPIOs as contrast agents have been tested to enhance MRI for macrophage imaging in human ischemic stroke lesions (Saleh et al., 2004). USPIOs were infused one week after symptom onset, and 2 follow-up scans were performed 24 hours and 48 hours post-infusion. The presence of USPIOs in macrophages allowed the visualization of MRI signal variations such as vessel changes (signal loss in T2 images) and brain parenchymal enhancement (T1 images). USPIO-enhanced MRI may provide an *in vivo* indication of cellular inflammation in stroke and other neuroinflammatory diseases, with neurological effects, that involve microglial activation and blood macrophage recruitment. Another clinical trial tested the effects of 2 doses (10 mg and 80 mg) of atorvastatin on carotid plaque inflammation, determined by USPIO-enhanced carotid MRI (Tang et al., 2009). Patients with carotid stenosis and intraplaque accumulation of USPIO on MRI were treated with atorvastatin daily for 12 weeks. The primary endpoint was represented by a change from baseline in MRI signal intensity at 6 and 12 weeks. A significant reduction in USPIO-defined inflammation was observed in the 80 mg group at both time points. Additionally, this dose significantly reduced total cholesterol and low-density lipoproteins at 12 weeks. These trials support the USPIO-enhanced MRI methodology as a successful and timely imaging detection for stroke diagnosis with the first trial, and for the screening and assessment of the therapeutic response to anti-inflammatory interventions in patients with atherosclerotic lesions, typical in stroke patients.

With the goal of improving functional recovery and limiting ischemic damage, clinical trials for stroke patients tested the potential neuroprotection of DDFPe for hemorrhagic shock and/or traumatic brain injury (Li et al., 2022a). After promising results obtained in preclinical studies in rat and rabbit stroke models (Culp et al., 2015; Fitzgerald et al., 2015), DDFPe was intravenously injected in acute ischemic stroke patients, starting within 12 hours of symptom onset, and appeared to be safe at all doses tested. Although earlier DDFPe treatment seemed promising and clinical outcomes were observed, the small sample size limited the significance of differences (Culp et al., 2019).

Other clinical trials on stroke focused on EVs as potential biomarkers (Gualerzi et al., 2021; Bang et al., 2022, 2023). The detection of cell-derived structures, such as EVs, is based on the application of surface plasmon resonance imaging-based biosensors. Blood EVs from stroke patients could reveal differences in cell types and may be useful to identify new biomarkers for the profiling of stroke patients with the final aim of reaching a rehabilitation and regenerative personalized therapy (Gualerzi et al., 2021; Bang et al., 2023). Investigating the composition of EVs from stroke patients could also fuel the improvement and the characterization of targeting moieties for NPs, reducing the immune system trapping and increasing the half-life of drugs (Duong et al., 2023).

Recent clinical trials investigated tenecteplase, the emergency treatment of myocardial infarction and pulmonary emboli, as potential stroke therapy. Interestingly, compared to the approved form alteplase, already used for ischemic stroke, it has a longer half-life, higher fibrin specificity, less systemic depletion of circulating fibrinogen, and higher resistance to plasminogen activator inhibitor (see for e.g. (Bivard et al., 2022; Li et al., 2022a; Rehman et al., 2023)). In further studies, since it may be soon approved for stroke therapy, tenecteplase could be a promising agent for the development of new nanomedicines that must meet quality and safety requirements before definition and starting of clinical trials.

Conclusion

Nanomedicine appears suited to answer the current limitations in the clinical care for stroke patients. Over the years, multiple approaches have demonstrated how a broad range of NPs could be

manufactured with different configurations, including inorganic and organic materials, as well as biologically derived components, and boost imaging efficiency and therapeutic efficacy in thrombolysis and neuroprotection. However, to date, these promising results remain mostly limited to preclinical studies with few successful translations to clinical trials. From the articles described here, some key aspects seem to emerge and may be taken into account to overcome this gap. Standardization of both efficacy and biosafety assessments might improve visibility among the numerous approaches explored. For the former, several groups resort to simple, non-brain specific animal models such as the mouse mesenteric vein, while the objective is to prove efficacy for stroke application, where some more adequate models exist. As for biosafety, it draws major challenges related to NPs' degradation and accumulation in the body, especially since NPs often form complexes which might be more difficult to bring through clinical approval than simpler designs. Consequently, in order to breach these obstacles, NPs with robust production pathways, scalable and made using GRAS (Generally Recognized As Safe) materials, while preferring green chemistry when possible, may have the best chances to finally reach clinical applications.

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