

**Advanced Functional Materials and Cell-based Therapies for the Treatment of Ischemic Stroke and Post-Ischemic Stroke Effects**

*Christos Tapeinos\**, *Aitor Larrañaga*, *Francesca Tomatis*, *Joëlle Bizeau*, *Attilio Marino*,  
*Matteo Battaglini*, *Abhay Pandit*, *Gianni Ciofani\**

Dr. C. Tapeinos, Dr. A. Marino  
Istituto Italiano di Tecnologia, Smart Bio-Interfaces, 56025 Pontedera (Pisa), Italy

Dr. A. Larrañaga  
University of the Basque Country, Department of Mining-Metallurgy Engineering and  
Materials Science & POLYMAT, 48013 Bilbao, Spain

F. Tomatis  
Politecnico di Torino, Department of Mechanical and Aerospace Engineering, 10129 Torino,  
Italy

J. Bizeau, Prof. A. Pandit  
National University of Ireland Galway, CÚRAM, Centre for Research in Medical Devices,  
H91W2TY, Galway, Ireland

M. Battaglini  
Istituto Italiano di Tecnologia, Smart Bio-Interfaces, 56025 Pontedera (Pisa), Italy  
Scuola Superiore Sant'Anna, The Biorobotics Institute, 56025 Pontedera (Pisa), Italy

Prof. G. Ciofani  
Istituto Italiano di Tecnologia, Smart Bio-Interfaces, 56025 Pontedera (Pisa), Italy  
Politecnico di Torino, Department of Mechanical and Aerospace Engineering, 10129 Torino,  
Italy

E-mail: [christos.tapeinos@iit.it](mailto:christos.tapeinos@iit.it), [gianni.ciofani@iit.it](mailto:gianni.ciofani@iit.it)

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**Abstract**

Ischemic stroke is considered one of the most threatening neurological disorders with high percentages of mortality and morbidity worldwide. Although in recent years, several nanotechnological advances have improved the survival rates, severe untreated post-stroke side-effects continue to significantly influence the way of life of many. Tissue plasminogen activator and mechanical thrombectomy are considered the gold standards for the treatment of acute cerebral ischemia. These, however, fail to improve post-ischemic disorders. Herein, after a brief description of the pathophysiology of ischemic stroke and the following biochemical cascade, we review the most recent biomaterial and cell-based strategies for its treatment. We also present other therapeutics that have been proposed not only for the treatment of cerebral ischemia but also for the regeneration of the infarcted brain that is responsible for a variety of disorders, including cognitive, motor, and speech problems. Finally, a few reported studies on diagnostic and theranostic nanostructures are also provided.

## 1. Introduction

Ischemic stroke is a condition that occurs when brain arteries are narrowed or blocked, causing reduced blood flow to the brain. This occlusion is followed by an inadequate blood supply (ischemia), and a lack of oxygen (hypoxia) and nutrients to the brain. Subsequently, a cascade of biochemical reactions that leads to neuronal cell death and neuro-inflammation is initiated. <sup>[1]</sup> During this cascade, neurons depolarize and release glutamate, which subsequently leads to increased calcium influx inside neurons and the so-called excitotoxic cell death. Moreover, the overproduction of reactive oxygen and nitrogen species (ROS and RNS respectively), sustain further the neuro-inflammatory responses, resulting further to neuronal loss. Besides this direct damage, the increased proteolytic activity, the oxidative stress and the neuro-inflammation of the ischemic tissue, increase the permeability of the blood-brain barrier (BBB) and leukocyte's extravasation.

To date, the FDA-approved strategy for the treatment of ischemic stroke was the use of tissue plasminogen activator (tPA), while more recently, mechanical thrombectomy and specific catheters have also been approved. <sup>[2]</sup>

Fast restoration of the blood flow (reperfusion), followed by neuroprotection and neuroregeneration, is the primary goal of the current stroke-treatment strategies. Although these treatments increase the survival rates and partially ameliorate the symptoms derived from the neuronal cell death, they fail to cure the disease completely. Furthermore, one of the main complications of the ongoing clinical approaches is the increased risk of hemorrhagic transformation. <sup>[3]</sup> This can occur precisely when thrombolytic agents like tPA are used, preventing their use in a significant number of patients.

Several therapeutic approaches with angiogenic, neuroprotective, and neurogenic properties have been developed, aiming to overcome the limitations of the current clinical treatment strategies. <sup>[4]</sup> However, due to the high complexity of the brain physiology and partly to the inability of the proposed nanotherapeutics to reach the brain parenchyma, the clinical

translation of these devices is hindered.<sup>[5]</sup> Several studies have been reported that acknowledge the severity of this disease and the need for new treatment solutions that will allow higher efficiency and fewer side-effects. We seek to elucidate the recent advances in the development of nanotherapeutic approaches that were carried out during the last five years. Our aim here is to highlight for readers the advantages and disadvantages of early-stage-developed therapeutic nanosystems. To do so, we are going to summarize the ischemic stroke and the cascade of the neuropathological pathways that follow cerebral ischemia resulting in ischemic injury. We will then describe a variety of biomaterial- and cell-based methods that have been proposed in the literature. In this review, the advantages and disadvantages of various biomaterials will be discussed. This will include polymer and lipid nanoparticles, hydrogels, carbon-based structures, protein/glycan nanotherapeutics, as well as cell-based therapies such as the transplantation of progenitor and stem cells, and the use of exosomes and cell-mimetic nanoparticles. Finally, a few reported works, in which nanostructures are used either as diagnostics or theranostics, will also be discussed.

## 2. Ischemic stroke and post-ischemic stroke effects

Stroke is a complex medical condition classified into two major categories: ischemic, which derives from a lack of blood flow in a brain region, and hemorrhagic, which originates from bleeding due to the rupture of a blood vessel or an abnormal vascular structure.<sup>[6]</sup> During a stroke, the brain tissue at the infarct core rapidly dies, while in the following hours and days, apoptosis and necrosis occur in the surrounding hypo-perfused regions (*e.g.*, ischemic penumbra).<sup>[7]</sup> The main aim of the acute stroke treatments is to rescue the brain functions in the ischemic penumbra. The causes for ischemic stroke are incredibly diverse, including embolism, thrombosis, hypo-perfusion, and cerebral venous sinus thrombosis. Moreover, the loss of blood supply induces a complex multistep pathway known as an ischemic cascade.<sup>[8]</sup>

This series of biochemical events (**Figure 1**) that occurs during ischemia starts with the

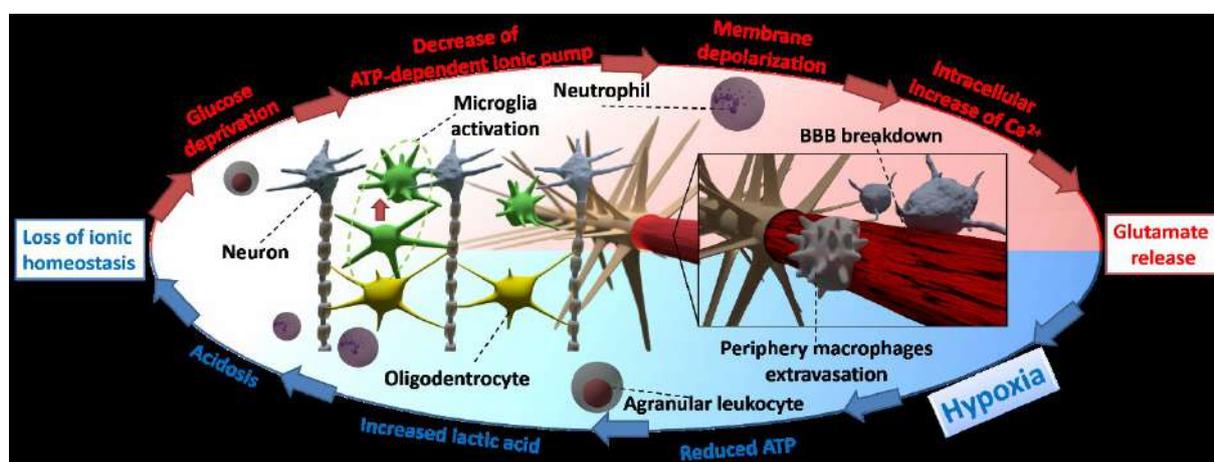
switch to anaerobic metabolism due to the lack of oxygen in the tissue. Anaerobic metabolism, compared to the aerobic one, is less efficient in producing the high energy phosphate compounds (*e.g.*, adenosine triphosphate, ATP), necessary for all the energy-dependent biochemical activities of the cell. This type of metabolism generates lactic acid increasing the tissue acidity, and subsequently leads to a concomitant loss of ionic homeostasis in neurons. The simultaneous glucose deprivation, resulting from a lack of nutrients, and the consequent drop of ATP levels, causes a decrease of the ATP-dependent ionic pump activity. This leads to a depolarization of the membrane and an increase of the intracellular  $\text{Ca}^{2+}$  concentration. Subsequently, this triggers uncontrolled neurotransmitter release (*e.g.*, glutamate) in neural cells. Glutamate is an excitatory neurotransmitter that induces a further  $\text{Ca}^{2+}$  influx in post-synaptic cells. This excitotoxic sub-pathway that promotes massive intracellular  $\text{Ca}^{2+}$  increments leads to the activation of proteolytic enzymes, endonucleases, and phospholipases. This activation results in severe cell damage and activation of apoptotic pathways. <sup>[9]</sup> Other glutamate excitotoxic effects include abnormal intracellular elevations of water and  $\text{Na}^+$ , cell swelling, and edema. <sup>[9]</sup>

The sustained elevations of  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and adeno phosphatase (ADP) concentrations in ischemic cells, induces overproduction of ROS at levels that antioxidant agents (*e.g.*,  $\alpha$ -tocopherol) and enzymes (*e.g.*, catalase, superoxide dismutase, glutathione peroxidase) cannot scavenge. Persistence of these oxidative conditions and the homeostatic imbalance can lead to apoptosis through extrinsic and intrinsic pathways, autophagy, and necrosis. <sup>[10]</sup> When necrosis occurs, cells release in the extracellular liquid, toxic biomolecules, and neurotransmitters, sustaining inflammation, and excitotoxicity. <sup>[11]</sup>

The blood flow can be restored after cerebral ischemia, and reperfusion can be promoted by recombinant tissue plasminogen activator-mediated thrombolysis, with significant improvements of the patient clinical conditions. Unfortunately, reperfusion can originate severe secondary injuries, including i) BBB integrity loss, ii) further ROS generation, iii) activation of

peripheral immune responses, and iv) inflammation. [8] For these reasons, additive therapies to reduce the side-effects of reperfusion are currently being investigated.

The BBB is crucial to maintaining the homeostasis and the functions of the brain. During ischemic stroke, however, the BBB can be damaged and disrupted. In addition to this direct injury, increased BBB permeability and extravasation are induced by the combined effects of hypoxia, inflammation, angiogenesis, and proteolytic activity of matrix metalloproteinases. [12] The BBB dysfunction and the consequent disruption of extracellular fluid homeostasis in the brain parenchyma leads to the excess accumulation of fluid in the extracellular spaces of the brain (*e.g.*, vasogenic edema). This induces compression with damage to the brain tissue and may result in hemorrhagic transformation. [13] Moreover, leukocytes infiltration through the injured BBB, further aggravates inflammatory responses and brain damages. [14] Even though the loss of BBB integrity in ischemic stroke generates different harmful effects, a passive targeting of these brain areas with therapeutic nanotechnologies can be obtained through the disrupted BBB fenestrations. [15]



**Figure 1.** Representation of the ischemic cascade that occurs in the neurovascular unit after an acute ischemic stroke. The lack of oxygen (hypoxia), which initiates the ischemic cascade, and the lack of nutrients (glucose) activate excitotoxic pathways that lead to the disruption of the blood-brain barrier and to subsequent neuronal cell death.

Angiogenesis, the out-growth of pre-existing vasculature, occurs in the peri-infarct region after ischemic stroke. In humans, active angiogenesis occurs about three days after ischemic

stroke and continues for more than 21 days. <sup>[16]</sup> Pericytes deriving from both bone marrow pericyte progenitors and adjacent immature pericytes accumulate in the peri-infarct zone mediating angiogenesis and BBB repair. Interestingly, brain vascular pericytes display multipotential progenitor cell activity following ischemia and can differentiate towards both neural and vascular lineage cells. <sup>[17]</sup> In addition to angiogenesis, the development of new vasculature (vasculogenesis) occurs in post-ischemic tissue; vasculogenesis is mediated by circulating endothelial progenitor cells, which home and differentiate into endothelial cells in the region of neovascularization. <sup>[10]</sup>

Other endogenous compensatory responses to ischemic stroke are neurogenesis and neuroplasticity. Concerning neurogenesis, once the injury has stabilized, neural progenitors in the subventricular zone (SVZ) rapidly generate neuroblasts. These migrate to the ischemic boundaries in rodent models and differentiate into mature neurons. <sup>[18]</sup> Neurogenesis after ischemic stroke has also been observed in the adult human brain, even in the case of aged patients. <sup>[19]</sup> Many molecular pathways regulate neural stem/progenitor cell proliferation after stroke (a detailed description is reviewed by Lindvall *et al.* <sup>[20]</sup>), but microRNAs seem to play a prominent role for the stroke-induced neurogenesis. <sup>[21]</sup> These findings indicate novel therapeutic targets for rescuing the brain functions at the penumbra. Recent investigations highlighted that the brain-derived neurotrophic factor (BDNF) that is responsible for the physical rehabilitation after stroke is already upregulated at four hours after stroke through three different biochemical routes that operate in parallel. <sup>[22]</sup> Other additional endogenous modulators playing important protective roles for neural survival and plasticity are the erythropoietin, the insulin-like growth factor 1 (IGF-1), and the vascular endothelial growth factor A (VEGF-A).<sup>[23]</sup>

Having referred to the post-ischemic stroke effects and the cascade of events that takes place during the stroke and after reperfusion, various strategies based on biomaterials and cell-based approaches will now be described.

### 3. Biomaterial-based approaches

Several therapeutic agents, including growth factors, antioxidants, anti-inflammatory drugs, and neurotrophins (proteins responsible for the development and the function of neurons) have been studied as potential curatives for ischemic stroke. Although neuronal repair and regeneration are what the growth factors and the neurotrophins target for, the use of anti-inflammatory drugs and antioxidants can also be proven beneficial by reducing neuroinflammation and oxidative stress. Besides the use of therapeutic nanostructures, the localized delivery of contrast agents in the ischemic tissue can provide valuable information for the restoration/regeneration of the infarcted tissue. Nevertheless, the delivery of both therapeutic and imaging agents to the brain has proven to be very challenging due to presence of the blood-brain barrier. Aiming at overcoming this limitation, drug delivery systems (DDS) (*e.g.*, nanoparticles, hydrogels, nanotubes), either for systemic administration or localized therapy, have been developed. The non-invasive nature of the systemic administration makes these systems favorable in comparison to invasive localized therapies. In order to enhance the delivery of these DDS to the ischemic brain, surface functionalization with specific ligands is also performed. The development of these DDS increases the bioavailability of the delivered therapeutic agents and increases their circulation times, leading to an enhanced therapeutic outcome. On the other hand, localized therapies (*e.g.*, implantable hydrogels, stem cells transplantation) solves the problem of the BBB and the side effects of systemic circulation. However, the invasiveness of these procedures and the non-controllable dosage of the delivered therapeutic after implantation prevents their wide use. In the following paragraphs we describe the most significant biomaterial-based approaches for both systemic and localized treatment of the ischemic brain. **Figure 2** summarizes these described biomaterial-based approaches in terms of used materials, types of therapeutics and targeting ligands.

### 3.1 Lipid-based nanoparticles

Lipid-based nanostructures have been widely used for the treatment of central nervous system (CNS) diseases due to their low toxicity and low immunogenicity, as well as owing their inherent ability to cross the BBB. In addition, their biomimetic nature, the easiness in surface modification, their biodegradability, and the enhanced colloidal stability makes them good candidates for the encapsulation of several therapeutics. [4d] Taking into consideration these characteristics, several DDS encapsulating a variety of therapeutics have been reported. The main goal of these studies was the amelioration of the post-ischemic stroke symptoms, but each one of them was different in terms of the used therapeutic approach. For example, liposomes encapsulating immunosuppressant drugs like Tacrolimus<sup>®</sup> [24] or lipid-based nanostructures encapsulating natural antioxidants like Baicalin were shown to reduce oxidative damage and improve the survival rates on ischemic rats. In the former case, the therapeutic effect could be attributed to the reduction of the excessive influx of Ca<sup>2+</sup> into cells, while in the latter to the regulation of the amino acids produced during I/R. [25] Other approaches like the use of inhibitors, including the postsynaptic density protein (PSD-95) / neuronal nitric oxide synthase (nNOS), ZL006, [26] or the use of a Rho-kinase inhibitor like Fasudil, [27] showed the efficacy of this approach in several ways. Both methods showed a reduction of the infarct volume and a reduction of the neurological deficiencies, with the Rho-kinase inhibitor to additionally inhibit neutrophil infiltration and to improve the motor function. Additionally, both studies showed enhanced accumulation to the ischemic brain, with the difference that ZL006-loaded liposomes were functionalized with a specific peptide (HAIYPRH), named T7, against the transferrin receptor. As previously mentioned, specific ligands on the surface of various DDS are used, aiming at improving their accumulation. This was shown not only with the T7 peptide but also with the use of other peptides like the stroke homing peptide (SHp) [28] or the use of antibodies like OX26. [25] In certain instances the effect of the therapeutic lipid-based nanostructures was assessed with or without the use of the thrombolytic tPA. It is well known that although tPA

can lead to the lysis of the formed clot allowing reperfusion, it may create adverse effects like hemorrhagic transformation or further disruption of the BBB. Because of this, the aforementioned Rho-kinase inhibitor was tested along with tPA. The combination of these therapeutics resulted in reduced brain damage on ischemic rats compared to rats treated just with tPA. <sup>[29]</sup> An interesting approach for the treatment of the ischemic brain is the delivery of growth factors that have shown to exhibit neuroprotective and neurogenic properties. These properties were demonstrated by the delivery of the basic fibroblast growth factor (bFGF) <sup>[30]</sup> into an ischemic brain. This delivery led to a reduction of the infarction and improved functional recovery, potentially through the PI3-K/Akt pathway.

Details of the studies, as mentioned earlier, can be found in **Table 1**.

**Table 1.** Studies reporting on the therapeutic abilities of lipid-based nanoparticles in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
DSPC / DPPC / cholesterol / DSPE-PEG2000	FK506 Tacrolimus	Reduction of the infarct volume Improved motor functions Increased cell survival	Male Wistar rats	N/A	Oxidative stress reduction	[24]
Tetrandrine / stearylamine / soya phospholipids	Baicalin	Neuronal protection	Male SD rats	OX26 antibody	Regulation of excitatory and inhibitory amino acids	[25]
Cholesterol / DSPE-PEG2000 / soya lecithin	ZL006	Reduction of the infarct volume Amelioration of neuronal deficits	Male SD rats	T7 peptide (& SHp)	Inhibition of glutamate-induced cytotoxicity	[26, 28]
DSPC/cholesterol/ DSPE-PEG2000	Fasudil	Reduction of the infarct volume Improvement of motor function Inhibition of neutrophil migration to the ischemic area (enhanced accumulation to the brain)	Male Wistar rats	N/A	Protection from H <sub>2</sub> O <sub>2</sub> mediated apoptosis & suppression of neutrophil invasion	[27]
	Fasudil+tPA	Thrombolysis + Fasudil neuroprotective properties			[29]	
Hydrogenated Soya phosphatide/cholesterol	bFGF	Reduction of the infarct volume Improvement of motor functions	Female SD rats	N/A	Neuroprotection through the PI3-K/Akt pathway	[30]

\*bFGF: basic fibroblast growth factor, CHOL+: Cholesteryl 3 $\beta$ -N-(dimethylaminoethyl) carbamate hydrochloride, CHOL-PEG: cholesteryl-polyethylene glycol 600 sebacate, DLPC: 1,2-Didodecanoyl-sn-glycero-3-phosphocholine, DOPA:1,2-dioleoyl-sn-glycero-3-phosphoric acid monosodium salt, DOPE: 1,2-dioleoyl-sn-glycero-3-phospho ethanolamine, DPPC: dipalmitoyl phosphatidylcholine, DSPC: Distearoyl phosphatidyl choline, DSPE: 1,2-Distearoyl-sn-glycero-3-phospho ethanolamine, FK506: Tacrolimus, SHp: Stroke homing peptide, tPA: tissue plasminogen activator, TGF: tumor growth factor, ZL006: specific inhibitor.

### 3.2 Polymeric nanoparticles

Synthetic polymer-based micelles, vesicles (polymersomes), and nanoparticles have also been considered as therapeutics for ischemic stroke. Progress in polymer synthesis and surface modification enables the fabrication of nanodevices with tunable mechanical properties, degradation rates, and advanced functionalities. Additionally, hydrophilic/hydrophobic molecules (*e.g.*, enzymes, drugs, siRNA) and nanoparticles with specific features can be easily incorporated within the polymeric system *via* scalable and straightforward synthesis procedures. Among the available synthetic polymers, poly(L-lactide) (PLLA), poly(D, L-lactide) (PDLLA), poly(glycolide) (PGA), and their copolymers have attracted particular attention, thanks to their biocompatibility and biodegradability. Moreover, their ability to encapsulate both hydrophilic and/or hydrophobic molecules, as well as their ability to carry large payloads renders them good candidates as DDS. With respect to lipid-based nanostructures (*e.g.*, liposomes), polymeric nanomaterials present increased colloidal stability, but limited ability to cross the BBB, besides poor pharmacokinetics and fast clearance from the reticulo-endothelial system (RES). As in the case of the lipid nanostructures, their surface can also be modified overcoming these limitations and improving their therapeutic effect.

One of the most promising approaches for the treatment of ischemia is the use of antioxidant substances and/or enzymes. The regulation of the overproduced reactive oxygen and nitrogen species (ROS and RNS, respectively) after reperfusion results in a reduction of the oxidative stress and a subsequent reduction to the ROS/RNS-mediated apoptosis. Furthermore, a potential amelioration of inflammation by limiting the production of several pro-inflammatory cytokines can also be achieved. <sup>[31]</sup> Both antioxidant substances, including curcumin, <sup>[32]</sup> quercetin, <sup>[33]</sup> and edaravone, <sup>[34]</sup> as well as antioxidant enzymes (superoxide dismutase and catalase) <sup>[35]</sup> were encapsulated inside polymer matrices and were used as curatives for cerebral ischemia. Their encapsulation inhibited limitations like short half-life, fast RES clearance, and non-specific selectivity towards brain tissues, enhancing, as in the case of lipid-based nanostructures, their

therapeutic effect. In all the reported studies, these antioxidants resulted into a reduction of ROS and to therapeutic outcomes such as BBB preservation and reduction of neuro-inflammation, <sup>[32c]</sup> activation and mobilization of progenitor stem cells, <sup>[33]</sup> improved axonal remodeling, <sup>[34]</sup> and inhibition of apoptosis with simultaneous induction of angiogenesis. <sup>[35]</sup> It has to be noted that surface functionalization with specific moieties has also been performed. Ligands like the triphenylphosphonium (TPP) cation that targets the mitochondria, <sup>[33]</sup>, or the adenosine 2A receptor agonistic agent that has the ability to open the BBB tight junctions, <sup>[34]</sup> have been attached to PLGA and PEG-PLA nanoparticles, respectively. Another targeting moiety worth to be mentioned is the chlorotoxin, that has the ability to target matrix metalloproteinase-2 (MMP-2), which is overexpressed in ischemic stroke. <sup>[36]</sup> In this study, the used PLGA nanoparticles (~20 nm) were loaded with lexiscan and modified with chlorotoxin aiming at modulating BBB permeability. Interestingly, the paracellular tight junction openings (~25 nm) were large enough to allow the passage of the nanoparticles, while limiting the passage of erythrocytes and leucocytes.

Gene delivery has also been accomplished using polymeric nanostructures (PEG-PDLLA). Delivered C3-siRNA resulted in the reduction of the infarction, the inhibition of the neuronal apoptosis, and an improved functional recovery supporting the potential use of siRNAs for cerebral ischemia. <sup>[37]</sup> Aiming at reducing the side-effects of tPA, polymeric nanoparticles (poly(isobutyl cyanoacrylate)) were used for its delivery to the thrombus. In this study it was shown that these nanoparticles after their functionalization with a P-selectin molecule (fucoidan), <sup>[38]</sup> enhanced thrombolysis. However, no data concerning ischemia were presented. Finally, polymeric nanostructures based on poly(urethane amino sulfamethazine) have also been used for the delivery of growth factors. Polymeric micelles were used to deliver the stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) to the ischemic brain, aiming at recruiting endothelial progenitor cells. <sup>[39]</sup> The delivery was successful, and this was proven by the enhanced neurogenesis and angiogenesis.

Details of the studies, as mentioned earlier, can be found in **Table 2**.

**Table 2.** Studies reporting on the therapeutic abilities of polymeric nanoparticles in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
PEG-PDLLA	Curcumin	Preserve BBB integrity Reduction of pro-inflammatory cytokines Reduce the number of activated M1-microglia Reduce infarct size Improve function recovery	<i>In vitro</i> : microvascular endothelial cells <i>In vivo</i> : male C57BL/6J mice	N/A	Reduction of oxidative stress and inflammation	[32c]
PLGA	Quercetin	Reduce oxidative stress Restore the activity of antioxidant enzymes Preserve mitochondrial integrity	Wistar rats	TPP	Reduction of mitochondrial ROS	[33]
PEG-PLA	Edaravone	Enhance BBB permeability Reduce oxidative stress Reduce infarct size & apoptosis Prolong survival of injured animals/ Improve axonal remodeling	<i>In vitro</i> : bEnd.3 and RAW264.7 cells <i>In vivo</i> : male ICR mice	A <sub>2A</sub> R	Reduction of oxidative stress	[34]
PLGA	SOD, CAT	Promote endogenous repair process Reduce infarct size & apoptosis	Male SD rats	N/A	Reduction of oxidative stress	[35]
PLGA	Lexiscan/NEP1-40	Enhance BBB permeability Prolong the survival of injured animals Reduce infarct size	Male C57BL/6J mice	Chlorotoxin	Enhanced BBB permeability	[36]
PEG-PDLLA	C3 siRNA	Reduce microglia C3 expression Reduce neutrophils, microglia & macrophages Attenuate the expression of pro-inflammatory cytokines / Neuroprotection / Reduce infarct size	<i>In vitro</i> : Primary microglial and neuronal cells <i>In vivo</i> : male C57BL/6J mice	N/A	Suppression of the expression of C3	[37]
Polysaccharide-PIBCA	tPA	Improve the thrombolysis efficiency	Male C57BL/6J mice	P-selectin	Degradation of the fibrin network	[38]
PUASM	SDF-1 $\alpha$	Improve neurogenesis and angiogenesis	Male SD rats	N/A	Recruitment of EPCs	[39]

\*A<sub>2A</sub>R: Adenosine 2A receptor, CAT: Catalase, EPCs, Endothelial progenitor cells, PEG: Poly (ethylene glycol), PIBCA: poly (isobutyl cyanoacrylate), PLGA: Poly (lactic-co-glycolic) acid, PUASM: Poly (urethane amino sulfamethazine), ROS: Reactive oxygen species, SOD: Superoxide dismutase, SDF-1 $\alpha$ : Stromal derived factor 1 $\alpha$ , tPA: tissue plasminogen activator.

### 3.3 Protein and glycan-based nanotherapeutics

Protein and glycan-based nanostructures represent a biomimetic approach with reduced immunogenicity and negligible cytotoxicity, suitable for CNS diseases. These nanotherapeutics present several advantages, including, among others, high biocompatibility, tailorable release by varying the cross-linking degree and the surface-to-volume ratio, ability to encapsulate growth factors, cells, and plasmid DNA,<sup>[40]</sup> and most importantly the ability to be administered intranasally. The latter provides a practical and non-invasive method for the delivery of therapeutics into the brain, bypassing the BBB. As a result, a few studies in which proteins like gelatin,<sup>[41]</sup> or polysaccharides<sup>[42]</sup> like chitosan have been used for the delivery (intravenous or intranasal) of therapeutics to ischemic tissues. As in the previous cases of lipid and polymeric nanotherapeutics, both antioxidants (rutin<sup>[42b]</sup> and acetyl-11-keto- $\beta$ -boswellic acid<sup>[42a]</sup>) and silencing RNA (inducible nitric oxide synthase siRNA<sup>[41]</sup>) have been studied. The results showed that either after intravenous or intranasal administration the infarct volume was reduced, and in certain cases enhanced neuroprotection was observed.<sup>[42a]</sup>

Details of the aforementioned studies can be found in **Table 3**.

**Table 3.** Studies reporting on the therapeutic abilities of protein and glycan-based nanotherapeutics in ischemic stroke.

Material	Therapeutic Substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
Gelatin	iNOS siRNA	Reduction of the infarct volume	Male SD rats	N/A	iNOS inhibition using siRNA	[41]
Chitosan	Acetyl-11-keto- $\beta$ -boswellic acid	Anti-inflammatory Antioxidant	Primary neurons / Male SD rats	N/A	Pathway modulation (Nrf2, HO-1, NF- $\kappa$ B, 5-LOX)	[42a]
Chitosan	Rutin	Neuro-protection / Infarct volume reduction	Wistar rats	N/A	N/A	[42b]

\* iNOS: inducible nitric oxide synthase, Nrf2: nuclear erythroid 2-related factor 2, HO-1: heme oxygenase-1, NF- $\kappa$ B: nuclear factor-kappa B, 5-LOX: 5-lipoxygenase, NMDARs: N-methyl-D-aspartate receptors, PSD-95: postsynaptic density protein.

### 3.4 Peptide-based nanotherapeutics

A large number of publications on peptide-based therapeutics for the treatment of a variety of CNS diseases show an undeniable interest in the use of these nanostructures. Peptides can act as signaling molecules able to regulate cellular responses, resulting in the pathogenesis or treatment of major neurodegenerative diseases. Peptides are widely used against CNS diseases, and some of the major reasons are the following; a) high target-specificity and potency, b) lack of accumulation in tissues, c) metabolization by endogenous enzymes to non-toxic amino acids and d) ability to act both as targeting moieties as well as therapeutic molecules. In this section, we are going to present some of the most recent works using peptides as therapeutics, while throughout the manuscript we are going to present the functionalization of other nanotherapeutics with peptides that act as targeting ligands. Peptides can lead to a reduction of the infarct volume and to improved neuroprotection through several mechanisms including, modulation of neuro-inflammation <sup>[43]</sup> as well as inhibition of several apoptotic pathways <sup>[43b, 43e, 44]</sup> (*e.g.*, caspase family). The presented peptide-based nanotherapeutics can act as antioxidants <sup>[44c]</sup>, as inhibitors of specific ion channels <sup>[45]</sup> and as inhibitors for chemokine heterodimer's formation <sup>[46]</sup>. In addition, peptides can lead to functional recovery through improved circulation, <sup>[47]</sup> enhanced angiogenesis, <sup>[48]</sup>, and finally through endogenous neural stem cells' mobilization and remyelination. <sup>[43d]</sup> Peptides have also been combined with tPA, <sup>[43f, 43g]</sup>, achieving the reduction of the tPA-induced hemorrhage. Moreover, in several studies <sup>[43e, 43f]</sup> it was shown that some therapeutic peptides could also protect the BBB, providing further protection to the damaged brain. Other peptide-based therapeutics that are worth mentioning include several isoforms of polyarginine peptides, <sup>[49]</sup> which lead to enhanced neuroprotection through the reduction of the glutamate-induced cytotoxicity.

Details for the aforementioned studies can be found in **Table 4**.

**Table 4.** Studies reporting on the therapeutic abilities of peptide-based nanotherapeutics in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Mechanism of action	Ref.
Apelin-13	N/A	Reduction of the infarct volume/neuronal death	Male C57BL/6J mice	Reduction of TNF- $\alpha$ , IL-1 $\beta$ , MCP-1 Increase in VEGF & MMP-9	[43a]
15-epi-lipoxin A <sub>4</sub> / AnxA1 <sub>Ac2-26</sub>	N/A	Regulation of neutrophil-platelet aggregate formation	Wild-type (WT) C57BL/6J mice	Inhibition of the Fpr2/lipoxin A4 receptor	[43c]
NCAM-FGL	N/A	Inflammation modulation Neuro-regeneration	Male Wistar rats	NSCs mobilization Modulation of the number and M1/M2 polarization of microglia	[43d]
Vasculotide	N/A	Reduction of the infarct volume, BBB permeability, and neuro-inflammation	Type I diabetic rats	Decrease of RAGE, MCP-1, TNF- $\alpha$ , and TLR4 expression	[43e]
HBHP combined with tPA	N/A	Amelioration of BBB damage and inflammation	Male SD rats	Blocking of HMGB1	[43f]
MHP1-AcN & tPA	N/A	Reduced inflammation and inhibition of hemorrhagic transformation	C57Bl6/J mice	N/A	[43g]
Apelin-13		Reduction of the infarct volume Anti-apoptotic effect	Male ICR mice	AMP-activated protein kinase pathway	[43b]
PDZ1	N/A	Neuroprotection	Male SD rats	Inhibition of FasL, DISC, BID, cas-3, cas-8, cas-9 through inhibition of GluK2-PSD-95	[44a]
Irisin	N/A	Reduction of brain edema and apoptosis	Male Swiss albino mice	Upregulation of Bcl-2 Downregulation of Bax and cas-3 Increase of BDNF	[44b]
ND13	N/A	Improved motor functions	Male wild-type C57BL/6J mice	Increase in SDHAF4 & decrease in COMMD, MIRO2, and USP35	[44c]
Tat-NTS	N/A	Reduction of the infarct volume / apoptosis Improvement of motor / cognitive functions	Male C57Bl6/J mice	Inhibition of p53 and cas-3 pathway / BID decrease Inhibition of translocation of ANXA1	[44d]
PcTx1 / Hi1a	N/A	Reduction of the infarct volume Amelioration of motor and neuronal deficits Anti-apoptotic effect	Male SHR rats	Inhibition of ASIC1a	[45]

**Table 4 (continue).** Studies reporting on the therapeutic abilities of peptide-based nanotherapeutics in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Mechanism of action	Ref.
MKEY	N/A	Reduction of the infarct volume Improved neurological deficit scores	Male C57BL/6J mice	Inhibition of CXCL4-CCL5 heterodimer formation Inhibition of MoMΦ-mediated Neuro-inflammation	[46]
Apelin-17 Apelin-36	N/A	Reduction of the infarct volume	Male SD rats	Potential apelin-17-induced cerebral artery dilation through the NO-cGMP pathway	[47]
VIP	N/A	Reduction of the infarct volume Angiogenic/Neurogenic properties	Male SD rats	Increment of VEGF levels	[48]
Poly-arginine (R12-R15-R18) / TAT-NR2B9c	N/A	Reduction of the infarct volume and the brain damage	Male SD rats	Reduction of excitotoxic glutamic acid-induced calcium influx	[49]

\* ASIC1a : acid-sensing ion channel 1a, AMP: 5'-adenosine monophosphate, APLNR: Apelin receptor, CCL5: chemokine (C-C motif) ligand 5, CXCL4: chemokine (C-X-C motif) ligand 4, DISC: death-inducing signaling complex, Bax: B-cells leukemia 2-associated X, Bcl-2: B-cell leukemia 2, BDNF: brain-derived neurotrophic factor BID: BH3 interacting domain death agonist, cas: caspase, COMMD: copper homeostasis protein, FasL: First apoptotic signal ligand, Fpr2: Formyl peptide receptor, GluK2: ionotropic glutamate receptor kainate type subunit 2, Hi1a: disulfide-rich venom peptide, HMGB1: high mobility group box 1 protein, HBHP: HMGB1 binding heptamer peptide, IL-1β: Interleukin 1β, MCP-1: Monocyte chemoattractant protein-1, MHP1-AcN: N-terminal acetylation and the C-terminal amidation of the microglia healing peptide 1, MIRO2: mitochondrial Rho GTPase 2, MKEY: peptide inhibitor, MoMΦs: Monocyte-derived macrophages, NCAM-FGL: Neural Cell Adhesion Molecule-Derived Peptide FG Loop, ND13: protein deglycase-based peptide, NO-cGMP: Nitric oxide cyclic guanosine monophosphate, NSCs: Neural stem cells, PcTx1: psalmotoxin, PDZ1: neuroprotective peptide, PSD-95: postsynaptic density protein 95, RAGE: Receptor for advanced glycation end products, SDHAF4: mitochondrial protein succinate dehydrogenase assembly factor 4, Tat-NTS: trans-activator of transcription (Tat) domain conjugated with the non-transcribed spacer, TLR-4: Toll-like receptor-4, TNF-α: Tumor necrosis factor-α, USP35: ubiquitin-specific peptidase 35.

### 3.5 Carbon-based nanostructures

Carbon-based nanostructures such as carbon nanotubes and fullerenols have also been reported as potential therapeutics for the treatment of ischemic stroke. As the previously described materials, carbon-based nanostructures are characterized by attractive properties like high loading efficiency, biodegradation, good biocompatibility, increased mechanical strength, and ability to penetrate the BBB due to their lipophilic character, that renders them suitable for the treatment of CNS diseases. However, the two most important characteristics that differentiate these nanostructures from others described in this review are firstly the modulation of synaptic plasticity and the promotion of neurite outgrowth in the case of nanotubes,<sup>[50]</sup> and secondly the inherent radical scavenging ability and the anti-inflammatory properties in the case of fullerenes.<sup>[51]</sup> Although not all the carbon-based nanostructures present the aforementioned characteristics, the studies that are presented in this review and are summarized in Table 5 were based on these.

Details for the aforementioned studies can be found in **Table 5**.

**Table 5.** Studies reporting on the therapeutic abilities of carbon-based nanotherapeutics in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
Fullerenol	Glucosamine	Reduction of the infarct volume	Wistar-Kyoto (WKY) normo- & hypertensive	N/A	Attenuation of IL-1 $\beta$ /TLR-4	[51a]
Fullerenol	N/A	Improved brain I/R mediated neuronal death Reduction of the infarct volume	Male Wistar rats	N/A	NMDA/nitrate reduction & GSH/SOD increase	[51b]
					Reduction of CGT	[51c]

\*IL-1 $\beta$ : Interleukin 1 $\beta$ , CGT:  $\gamma$ -glutamyl transpeptidase, GSH: Reduced glutathione, NMDA: N-Methyl-D-aspartate, SOD: Superoxide dismutase, TLR-4: Toll-like receptor 4.

### 3.6 Hydrogels

One of the widely-studied strategies for the treatment of ischemic stroke involves the use of hydrogels due to the versatile properties that they present. These include tailored mechanical properties, controlled delivery of growth factors, and/or other therapeutic substances, as well as the delivery, survival, and growth of progenitor or stem cells. Hydrogels started to be developed with the aim of overcoming the limitations of current systemic administration strategies. Among these, the inability of the therapeutic substances to cross the BBB, <sup>[52]</sup> the failure to deliver cells without a proper scaffold, and the failure of the current systems to fill the formed cavity at the infarcted brain <sup>[52]</sup> are the most important.

Hyaluronic acid (also called hyaluronan, HA), is a biocompatible and bioresorbable linear polymer belonging to the family of glycosaminoglycans. It is abundant in the brain and has been reported to reduce the inflammatory response of tissues and the glial scar formation, as well as to promote cell survival. These properties make it a desirable material for the development of hydrogels for stroke. It is noteworthy that a specific formulation of HA (HyStem<sup>®</sup>-C) is commercially available and has notably been used to deliver growth factors (*e.g.*, BDNF) in the stroke cavity. <sup>[52]</sup>

BDNF, as well as other growth factors like SDF-1 $\alpha$  and bFGF, have been encapsulated inside HA-based hydrogels aiming at ameliorating the post-stroke effects. The amelioration was achieved by promoting the migration and proliferation of neuronal stem cells, and <sup>[52]</sup> by regulating neuro-inflammation, that subsequently led to neuro-regeneration and functional recovery. <sup>[53]</sup> Immunosuppressants like cyclosporine A (CsA) were also encapsulated inside PLGA microparticles that were subsequently embedded in an HA-based hydrogel. The modified hydrogel demonstrated the increased proliferation of neural stem progenitor cells (NSPCs) and a decrease of the stroke cavity. It is noteworthy that a decrease in the stroke infarct was also observed even in the absence of CsA, suggesting that CsA role is mostly related to cell proliferation. <sup>[54]</sup> Except growth factors and immunosuppressants, HA-based hydrogels have

been used for the delivery of various stem cells <sup>[55]</sup> with or without the use of growth factors (*e.g.*, VEGF), <sup>[56]</sup> proteins (*e.g.*, bone morphogenetic protein/BMP-4), and various peptides (*e.g.*, cathepsin K, glutamine, arginyl-glycyl-aspartic acid -RGD- and laminin-derived). <sup>[57]</sup> Using all these approaches, neurogenesis and axogenesis were promoted through modulation of inflammation and through the migration of neural progenitor cells in the damaged tissue.

Other types of hydrogels that have been used for the treatment of ischemic stroke were based on a variety of proteins, including laminin (main cerebral extracellular matrix protein), sericin, and fibroin. The latter two proteins derive from silk and demonstrate an inherent ability to promote axogenesis and neurogenesis, suggesting their potential use as scaffolds for proliferation and growth of stem cells. <sup>[58]</sup> Similar results were also presented when differentiated human embryonic stem cells were encapsulated inside a laminin-derived hydrogel. The hydrogel showed survival and maturation of the transplanted cells, as well as axonal growth. <sup>[59]</sup>

Extracellular matrix (ECM)-based hydrogels have also been used due to their high cytocompatibility, as well as to their ability to provide differentiation stimuli for neural stem cells, making it an optimal material for CNS diseases. In particular, urinary bladder matrix ECM (UBM-ECM) promotes a better neurite outgrowth than that of CNS-derived ECM. <sup>[60]</sup>

Details of the studies, as mentioned earlier, can be found in **Table 6**.

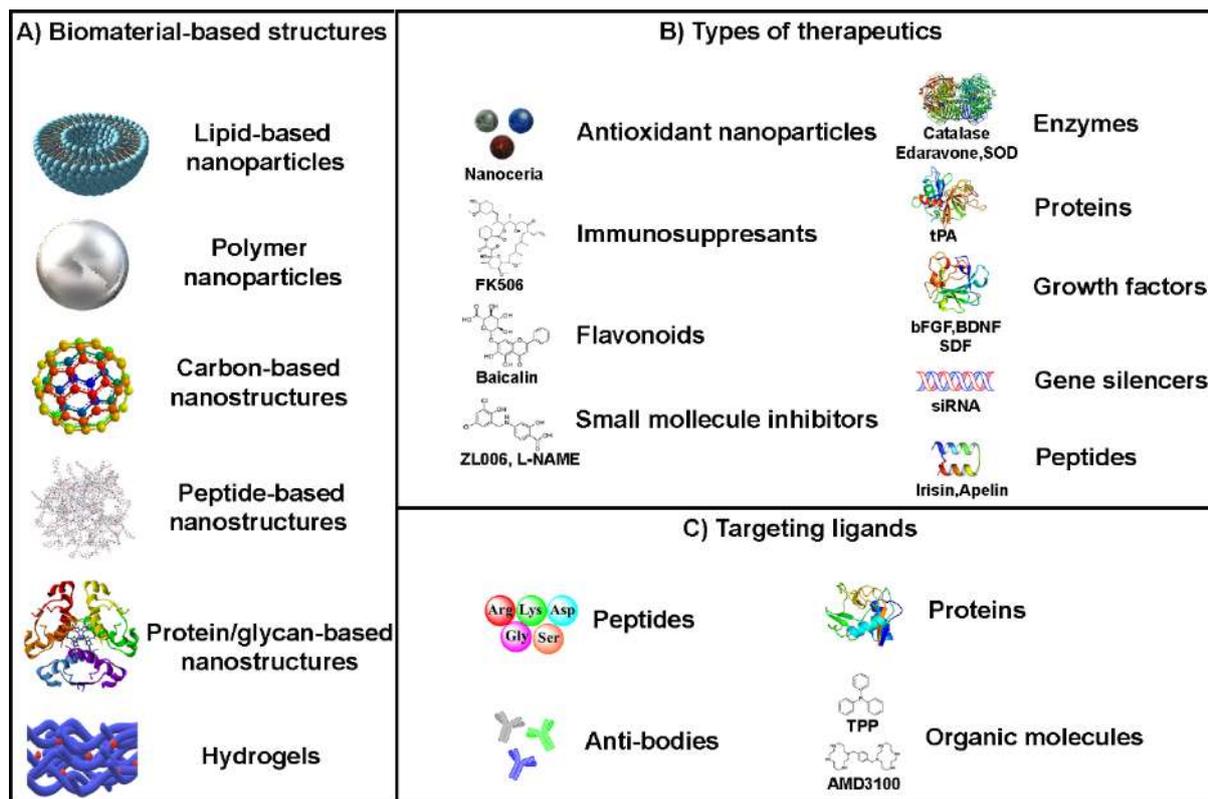
**Table 6.** Studies reporting on the therapeutic abilities of hydrogels in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Mechanism of action	Ref.
HyStem®-C hydrogel	BDNF	Functional recovery in the motor system and neurogenesis	Male C57BL/J6 mice and pDCX-DSRed2 mice / male <i>Macaca fascicularis</i> monkeys	Axonal sprouting and neurogenesis through BDNF release	[52]
HyStem®-C hydrogel	BDNF	Improved sensorimotor function and attenuation of neuro-inflammation	Male SD rats	Reduction of neuro-inflammation through BDNF release (reduction of Iba1, CD68, and GFAP levels)	[53a]
HA	PCN, SDF-1 $\alpha$ bFGF	Tissue repair and functional recovery via the promotion of NSCs migration Promotion of proliferation	<i>In vitro</i> : rat neuronal stem cell used: semi-adhesive HCN-A94-2 <i>In vivo</i> : male SD rats	Neurogenesis and angiogenesis via SDF-1 $\alpha$ and bFGF pathways	[53b]
PLGA / HAMC	CsA	Controlled delivery of CsA and increment in the number of proliferating NSPCs	Neural stem and progenitor cells in male Long-Evans rats	Stimulation of endogenous NSPCs and attraction of migratory neuronal progenitor cells	[54]
HAMC	Early-, mid- and late-differentiated iPSC-cNEPs cells	Behavioral recovery <i>via</i> the transplantation of neurons	Male SD rats	N/A	[55]
HA	VEGF and heparin nanoparticles	Functional recovery <i>via</i> revascularization of the cavity site in stroke, promoting the formation of a neuronal structure	Male C57BL/6J mice	Neurogenesis and axonogenesis via the anti-inflammatory effect of heparin nanoparticles (reduction of TNF- $\alpha$ ) Angiogenesis through the activation of VEGF-2 receptor	[56]
HA functionalized with RGD, YIGSR, and IKVAV, cross-linked with MMP-degradable peptide	Heparin, BDNF, BMP-4, iPS-NPCs	Promotion of iPS-NPCs proliferation and differentiation into neurons once implanted in the stroke cavity	C57BL/6J mice and Nod-Scid-Gamma (NSG) mice	Proliferation and differentiation of iPS-NPCs with a cocktail of adhesion peptides and growth factors	[57a]

**Table 6 (continue).** Studies reporting on the therapeutic abilities of hydrogels in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Mechanism of action	Ref.
HA functionalized with three peptides (RGD, cathepsin, and glutamine peptides)	N/A	Migration of neural progenitor cells from the SVZ inside the stroke cavity Revascularization	Male C57BL/6J mice	Reduction of astrogliosis and microgliosis, resulting in an attenuation of inflammation	[57b]
Sericin	N/A	Protection against hypoxia-induced death Enhanced axon extension and branching Increased survival rate and proliferation	<i>In vitro</i> : primary cortical neurons & SH-SY5Y <i>In vivo</i> : nude mice	Activation of the Lkb1-Nuak1 kinase pathway	[58a]
Silk Fibroin	N/A	Good biocompatibility Support of endogenous cell proliferation <i>in vivo</i>	Male SD rats	N/A	[58b]
Laminin	N/A	Promotion of neuroblasts migration from the V-SVZ to the stroke site	Neuroblasts in normal and Itgb1-knockout IRC mice	Laminin / integrin $\beta$ 1 signaling	[59a]
Laminin-derived peptides	N/A	Long-term survival (9 months) High vascularization Reduced cortical atrophy Functional electrophysiological properties	<i>In vitro</i> : HES3-ENVY <i>In vivo</i> : athymic nude rats	Increased cas-3 expression and potentially through the 'Phoenix Rising' pathway	[59b]
UBM-ECM	N/A	<i>In situ</i> gelation with a displacement of the extracellular fluid of the stroke cavity and retention in the cavity	Male SD rats	N/A	[60]

\*BDNF: Brain-derived neurotrophic factor, bFGF: basic fibroblast growth factor, BMP4: Bone morphogenetic protein 4, cas: caspase, CD68: Cluster differentiation 68, CsA: Cyclosporin A, GFAP: Glial fibrillary acidic protein, HA: Hyaluronic acid, HAMC: Hyaluronic acid methylcellulose, Iba1: ionized calcium-binding adaptor molecule 1, IKVAV: Laminin-derived peptide, iPS-NPCs: induced pluripotent stem cells differentiated into neural progenitor cells, iPSC-cNEPs: induced pluripotent stem cells cortically-specified neuroepithelial progenitor cells, NSPCs: Neural stem progenitor cells, PCN: polyelectrolyte complex nanoparticles, PLGA: Poly (lactic-co-glycolic) acid, RGD: Arginine-glycyl-aspartic acid, SDF-1 $\alpha$ : Stromal derived factor 1 $\alpha$ , SVZ: Subventricular zone, TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ , VEGF: Vascular endothelial growth factor, UBM-ECM: Urinary bladder matrix-extracellular matrix, YIGSR: Laminin-derived peptide.



**Figure 2.** Summary of A) biomaterial-based structures, the types of B) therapeutics, and C) targeting ligands that are used for the fabrication of nanotherapeutics for the treatment of ischemic stroke.

## 4. Cell-based approaches

### 4.1 Stem cells

The use of stem cells in ischemic stroke was suggested as a method to replace the different cell populations that die during cerebral ischemia. Stem cells can differentiate to several specific cells rendering them an apt candidate for the remodeling of the neurovascular unit after I/R injury. Although a variety of stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells can be used, the most widely used are neural stem cells (NSCs) and mesenchymal stem cells (MSCs). Concerning the transplantation technique, three methods are usually utilized, and these are the intracerebral (such as intrastriatal and intraventricular), the intravenous, and the intra-arterial transplantation. Each one of these methods offers advantages and disadvantages in terms of targeting, invasiveness, and pulmonary circulation (in the case of intravenous injection).

#### 4.1.1 Neural stem cells

Primary NSCs do not represent a distinctly feasible route to follow. This is because of the complex protocol of isolation, the limited number, and the ethical problems arising from the use of fetal brains. However, several works have been reported, in which neural stem cells are used. NSCs derived from different sources, including murine cerebellum, <sup>[61]</sup> autologous pluripotent from mini-pigs, <sup>[62]</sup> and human embryos <sup>[63]</sup> demonstrated an enhanced therapeutic outcome. Briefly, the transplantations improved the proliferation both of the transplanted and of the endogenous cells, <sup>[61]</sup> promoted migration, <sup>[63]</sup> and demonstrated neuroprotection, neurogenesis and enhanced blood perfusion to the brain. <sup>[62]</sup> It has to be noted that in the case of embryonic stem cells the enhanced therapeutic efficacy is also attributed to the simultaneous use of BDNF.

#### 4.1.2 Mesenchymal stem cells

MSCs are part of the family of the stromal cells, and they can be isolated from different sources. They are usually exploited in ischemic stroke treatment due to their inherent properties,

including the ability i) to self-renew, ii) to differentiate in non-hematopoietic cells, iii) to secrete useful molecules with therapeutic effect, and finally, iv) to be easily isolated.

The delivery of human bone marrow mesenchymal stem cells (hBM-MSCs) leads to enhanced proliferation, <sup>[64]</sup> neuroprotection, <sup>[64b]</sup> angiogenesis, <sup>[64b]</sup> neurogenesis <sup>[64b]</sup> and functional recovery <sup>[65]</sup> by various mechanisms. These mechanisms include, among others, the reduction of autophagy, which is one of the side effects during I/R injury <sup>[66]</sup> and the anatomical restoration of cortical interhemispheric connections. <sup>[65]</sup> In fact, it has been demonstrated that there is no difference among the intra-cerebral, intravenous and intra-arterial delivery of these cells concerning the functional recovery of the ischemic brain. <sup>[67]</sup> However, intra-arterial delivery may provide more evident results. **Figure 3** summarizes several of the mechanisms that are involved in the neuro-restoration of the brain after ischemic stroke, as well as the types of cells used, along with various therapeutic molecules.

Although in the above-mentioned studies, the transplantation of MSCs showed a positive outcome, a factor that was not taken into consideration was the time frame of the transplantation. To answer this question, a work in which the timing of the intra-arterial transplantation of MSCs was related to their therapeutic efficacy was performed. <sup>[68]</sup> The results showed that after 24 h of transplantation, a significantly higher amount of integrated MSCs was detected, and the infarct volume was significantly decreased. Additionally, the levels of both the neuroprotective bFGF and of SDF-1 $\alpha$  increased considerably.

As in the case of hydrogels, the therapeutic effect of the BM-MSCs has been enhanced by the simultaneous use of various growth factors, including BDNF and VEGF, as well as by the use of proteins like noggin (NOG). The combinational use of BDNF and noggin led to enhanced angiogenesis and to a reduction of inflammation, oxidative stress, and apoptosis, <sup>[69]</sup> while the use of VEGF promoted neuronal differentiation and led to better engraftment. <sup>[70]</sup> The latter effect was also observed after the combination of recombinant BM-MSCs with a palmitic acid peptide, which led to a higher cell number in the infarcted tissue. <sup>[71]</sup>

Except for BM-MSCs, MSCs of different origin were also reported in some works. MSCs derived from the human umbilical cord have been used in a study showing that the adverse effects of stroke in mice after MCAO can be limited by reducing the peripheral inflammation. [72] Moreover, adipose-derived MSCs (AD-MSCs) combined with exosomes, showed a significant improvement after ischemia. This combination also led to a reduction of the inflammation, oxidative stress, apoptosis, fibrosis, DNA-damage, and brain edema levels. [73]

Finally, enhanced angiogenesis was also observed by injecting the human MSC cell line (B10) into rats 24 h after MCAO. [74] Angiogenesis was proved by analyzing the expression of several factors including the placenta growth factor (PGF), the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), angiopoietin1, VEGF, tumor growth factor- $\beta$  (TGF- $\beta$ ) and IL-1 $\beta$

Details of the aforementioned studies can be found in **Table 7**.

**Table 7.** Studies reporting on the therapeutic abilities of transplanted stem cell-based approaches in ischemic stroke.

Cell-based approach	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
Mouse-derived NSCs	N/A	Functional recovery Enhanced cells proliferation	Male SD rats	N/A	Enhanced migration probably due to SDF-1 and CXCR4	[61]
Human-derived iNSCs	N/A	Functional recovery Neuroprotection & neurogenesis Anti-inflammation ability	Male castrated Landrace pigs	N/A	Stimulation of the migration of DCX+ neuroblasts from the SVZ to the infarcted area	[62]
Human embryonic-derived NSCs	BDNF	Improvement of cell engraftment and survival	Non-obese diabetic/severe combined immunodeficient mice	N/A	Increment of the expression of chemokine receptors and adhesion molecules	[63]
Human-derived BM-MSCs	N/A	Enhanced neurogenesis	Male SD rats	N/A	Endogenous erythropoietin production	[64a]
Rat-derived BM-SCs	N/A	Improvement of locomotion, neurogenesis, and angiogenesis	Male SD rats	N/A	Paracrine factor secretion Differentiation of endothelial cells	[64b]
Rat-derived BM-MSCs	N/A	Preservation of interhemispheric cortical connections Functional recovery Reduction of the infarct volume	Male SD rats	N/A	Preservation of interhemispheric cortical connections	[65]
Rat-derived BM-MSCs	N/A	Reduction of autophagocytosis	Male SD rats	N/A	Reduction of autophagy-associated proteins / Activation off the PI3K/Akt pathway	[66]
Rat-derived BM-MSCs	N/A	Functional recovery Enhanced neurogenesis	Male SD rats	N/A	Paracrine secretion and integration of the stem cells in the infarcted area	[67]

**Table 7 (continue).** Studies reporting on the therapeutic abilities of transplanted stem cell-based approaches in ischemic stroke.

Cell-based approach	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
Allogeneic MSCs	N/A	Behavioral improvement Reduction of the infarct volume	Male Wistar rats	N/A	Increased levels of bFGF and SDF-1 $\alpha$	[68]
Rat-derived BM-SCs	BDNF/ Noggin	Functional recovery	Male SD rats	N/A	Inhibition of apoptosis Anti-inflammatory ability	[69]
Rat-derived BM-MSCs	VEGF	Functional recovery, angiogenesis & MSCs engraftment	Male SD rats	N/A	Enhanced VEGF, BDNF, and MAP2 expression	[70]
Rat-derived BM-MSCs	miR-133b	Enhanced MSCs engraftment	<i>In vitro</i> : Neuro-2a & Primary astrocytes <i>In vivo</i> : male SD rats	Palmitic acid- modified peptide	N/A	[71]
Human umbilical cord-derived MSCs	N/A	Reduction infarct volume Attenuation of peripheral immune-inflammation	Male mice	N/A	Attenuation of IL-1, TNF- $\alpha$ , IL-23, IL- 17, and IL-10 Decrement of T-helpers 17 Increment of T-reg cells/TGF- $\beta$	[72]
Mini-pig adipose- derived MSCs (AD-MSCs)	N/A	Functional recovery Reduction of the infarct volume, apoptosis, fibrosis, DNA-damage, brain edema Promotion of angiogenesis	Male SD rats	N/A	Modulation of inflammation, oxidative stress, and immunomodulation	[73]
Human-derived MSCs	N/A	Promotion of angiogenesis	<i>In vitro</i> : B10 & HMO6 cells <i>In vivo</i> : male Wistar rats	N/A	Increased expression of HIF-1 $\alpha$ and other angiogenesis factors (PGF, angiopoietin1, VEGF, TGF- $\beta$ , IL-1 $\beta$ )	[74]

\*bFGF: Basic fibroblast growth factor, BDNF: Brain-derived neurotrophic factor, BM-SCs: Bone marrow stromal cells, BM-MSCs: Bone marrow mesenchymal stem cells, CXCR4: C-X-C motif chemokine receptor 4, DCX+: Doublecortin-expressing cells, HIF-1 $\alpha$ : Hypoxia-inducible factor 1 $\alpha$ , IL: Interleukin, iNSCs: induced pluripotent stem cells-derived neural progenitor cells, MAP2: Microtubule-associated protein 2, MSCs: Mesenchymal stem cells, PGF: Placental growth factor, SDF-1: Stromal derived factor 1, SVZ: Subventricular zone, TGF- $\beta$ : Tumor growth factor  $\beta$ , VEGF: Vascular endothelial growth factor.

## 4.2 Progenitor cells

Progenitor cells (PCs), a unipotent type of cells, have also been suggested as potential therapeutics for cerebral ischemia. These cells are inactive or present low activity in the tissues in which they reside, but they can be activated in the case of a tissue injury and/or damaged/dead cells through the secretion of cytokines and growth factors. In addition, PCs can promote neurogenesis, angiogenesis, and revascularization. Based on these characteristics, several studies in which progenitor cells were used to treat ischemia have been presented in the last few years. However, in this review, we are going to selectively report the studies in which exogenous progenitor cells were used for stroke treatment.

PCs derived either from peripheral blood, or bone marrow have been used due to their inherent neurogenic and angiogenic properties that they present. In all the cases where PCs were delivered into the brain, a neuroprotective effect, followed by modulation of inflammation<sup>[75]</sup> and an anti-apoptotic effect<sup>[75a]</sup> were observed. This demonstrated the potential of these cells as stroke therapeutics. In several cases, transplanted PCs were shown to reduce oxidative stress<sup>[75a]</sup> as well as to inhibit the generation of nitric species through a specific nitric oxidase inhibitor (L-NAME).<sup>[76]</sup>

In one of the reported studies, the therapeutic effect of multipotent adult PCs was assessed in relation to immune responses. In this study, MAPCs were transplanted in rats with and without a spleen, and it was observed that functional recovery and modulation of inflammation could be achieved only in rats with a spleen.<sup>[77]</sup> This suggests that stroke recovery is closely related to the responses of the immune system. Finally, in order to improve functional recovery and increase angiogenesis and neurogenesis, PCs were combined with SDF-1 $\alpha$ ,<sup>[78]</sup> resulting in increased proliferation and migration of oligodendrocyte progenitor cells (OPCs).

Details of the aforementioned studies can be found in **Table 8**.

**Table 8.** Studies reporting on the therapeutic abilities of transplanted progenitor cells in ischemic stroke.

Cell-based approach	Therapeutic Substance	Therapeutic ability/feature	Biological models	Mechanism of action	Ref.
PBDEPCs	G-CSF	Reduction of the infarct size Improved neurological functions	Male SD rats	Enhanced angiogenesis Oxidative stress reduction Amelioration of cellular apoptosis and DNA damage	[75a]
EPCs/HEN6	N/A	Amelioration of neurological and motor functions	In vitro: HEN6 In vivo: SD rats	Downregulation of BRM, I $\kappa$ B, Foxf1, ITIH-5, PMCA2 & Upregulation of RECA1	[75b]
BMEPCs	L-NAME	Increased angiogenesis, neurogenesis & axonal growth	Male C57BL/6J mice	eNOS/BDNF related angiogenesis	[76]
MAPCs	N/A	Immunomodulation of spleen responses favors brain recovery	Male Long-Evans rats	Immunomodulation of the spleen	[77]
EPCs	CXCL12	Reduction of brain atrophy Protection of myelin sheath integrity	Male ICR mice	Enhanced neurogenesis/angiogenesis/proliferation and migration of OPCs	[78]

\* BMEPCs: Bone marrow endothelial progenitor cells, BRM: stroke-associated Brahma gene, CXCL12: C-X-C motif chemokine 12, eNOS: endothelial nitric oxide synthase, EPCs: Endothelial progenitor cells, Foxf1: Forkhead Box F1, G-CSF: Granulocyte-colony stimulating factor, HEN6: Human cerebral endothelial cells, I $\kappa$ B: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, ITIH5: Inter-Alpha-Trypsin Inhibitor Heavy Chain 5, L-NAME: N(gamma)-nitro-L-arginine methyl ester, MAPCs: Multipotent adult progenitor cells, OPCs: Oligodendrocyte progenitor cells, PBDEPCs: Peripheral blood-derived endothelial progenitor cells, PMCA2: Plasma membrane Ca<sup>2+</sup>, RECA1: DNA repairing protein.

### 4.3 Exosomes / cell-mimetic nanoparticles

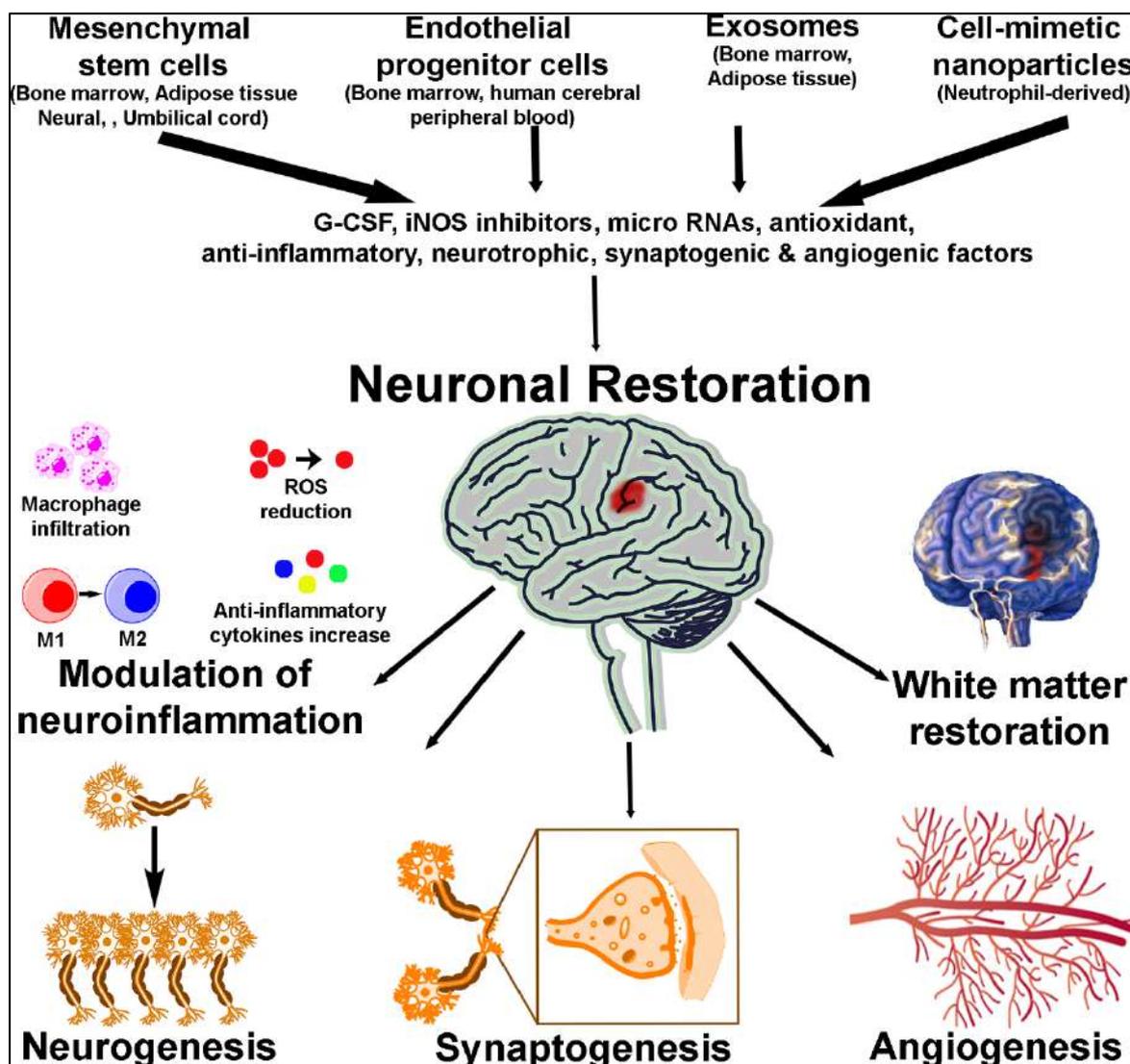
Cell-derived extracellular vesicles, also known as exosomes, have also been studied as biomimetic nanotherapeutics for ischemic stroke. Their particular composition, a combination of lipids and proteins, renders them with specific characteristics including colloidal stability, high biocompatibility, no cytotoxicity, good immunocompatibility if they derive from an autologous source, enhanced ability to pass the BBB, and specific organotropism (targeting ability).<sup>[79]</sup> These characteristics allow them to be used in a significant number of biomedical applications as highly biocompatible drug/gene delivery systems.

Since exosomes derive from cells, their origin varies depending on the cell type they are generated from. Based upon this, exosomes derived from mesenchymal stem cells from embryos,<sup>[80]</sup> bone marrow<sup>[81]</sup> and adipose tissue<sup>[82]</sup> have been reported. In these studies, exosomes were loaded with various therapeutics, including antioxidants like curcumin<sup>[80-81]</sup> and the pigment epithelium-derived factor (PEDF),<sup>[82]</sup> or small noncoding RNAs like the miR-124.<sup>[81b]</sup> In the cases of the antioxidant therapeutics, amelioration of the post-ischemic stroke effects was achieved by repression of apoptosis and regulation of inflammation through the inhibition of pro-inflammatory cytokines' production. It has to be noted that in the case of the embryonic MSC-derived exosomes, increased neurogenesis, as well as BBB restoration, was also observed.<sup>[80]</sup> On the other hand, the delivery of miR-124 led to enhanced cortical neurogenesis that was demonstrated by the increase in SOX2 (sex-determining region Y-box 2) and nestin markers. Although exosomes exhibit targeting abilities towards autologous cell types, functionalization with various targeting groups like the cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide [c(RGDyK)]<sup>[81a]</sup> or proteins like the rabies virus glycoprotein (RvG) fused with an exosomal protein lysosome-associated membrane glycoprotein 2b (Lamp2b)<sup>[81b]</sup> have also been used aiming at improving their accumulation in ischemic tissues.

In a different approach, neutrophils were used to fabricate nanovesicles able to target the ischemic brain.<sup>[83]</sup> It was demonstrated that the neutrophil-derived nanovesicles could

specifically target the ischemic lesion through integrin  $\beta 2$  and P-selectin glycoprotein ligand-1 (PSGL-1). In parallel, they can alleviate inflammation through the encapsulated resolvin D2 (RvD2), thus improving neurological deficits. Moreover, real-time imaging of the neutrophil-derived nanovesicles binding to the inflamed vasculature was achieved through intravital microscopy.

Descriptive details of the aforementioned studies can be found in **Table 9**.



**Figure 3.** Neuronal restoration can be achieved either by the delivery of various therapeutics using cell-based approaches or due to the inherent ability of specific cell types (e.g., MSCs) to activate restorative mechanisms. The mechanisms involved in this restoration include modulation of neuro-inflammation, white matter restoration, neurogenesis, synaptogenesis, and angiogenesis.

**Table 9.** Studies reporting on the therapeutic abilities of exosomes and cell-mimetic nanoparticles in ischemic stroke.

Cell-based approach	Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
Exosomes	MSCs	Curcumin	Reduction of the infarct volume, brain edema & inflammation, enhanced neuroprotection	Male wild-type C57BL/6J	N/A	Reduction of NMDA & GFAP Restoration of vascular junctions	[80]
			Reduction of inflammation and cellular apoptosis	Male C57BL/6J mice	c(RGDyK)	Reduction of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6	[81a]
	BM-MSCs	miR-142	Cortical neurogenesis	Male C56BL/6J mice	RvG-Lamp2b	Gene regulation through miRNA delivery	[81b]
	Adipose MSCs	PEDF	Amelioration of the brain injury	<i>In vitro</i> : SH-SY5Y <i>In vivo</i> : male SD rats	N/A	Activation of autophagy and suppression of neuronal apoptosis	[82]
Cell-membrane particles	Neutrophils	RvD2	Amelioration of inflammation and neurological deficits	<i>In vitro</i> : HUVECs / fibroblasts <i>Ex vivo</i> : mouse brain tissues <i>In vivo</i> : male C57BL/6J mice	Integrin $\beta$ 2 & PSGL-1	Suppression of inflammation through the delivery of RvD2	[83]

\*BM-MSCs: Bone marrow mesenchymal stem cells, MSCs: Mesenchymal stem cells, GFAP: Glial fibrillary acidic protein, HUVECs: human umbilical vein endothelial cells, I: Interleukin, Lamp2b: lysosome-associated membrane glycoprotein, NMDA: N-Methyl-D-aspartate, PSGL-1: P-selectin glycoprotein ligand 1, PEDF: Pigment epithelium-derived factor, RvD2: Resolvin D2, RvG: Rabies virus glycoprotein, TNF- $\alpha$ : Tumor necrosis factor  $\alpha$ .

## 5. Combinational approaches

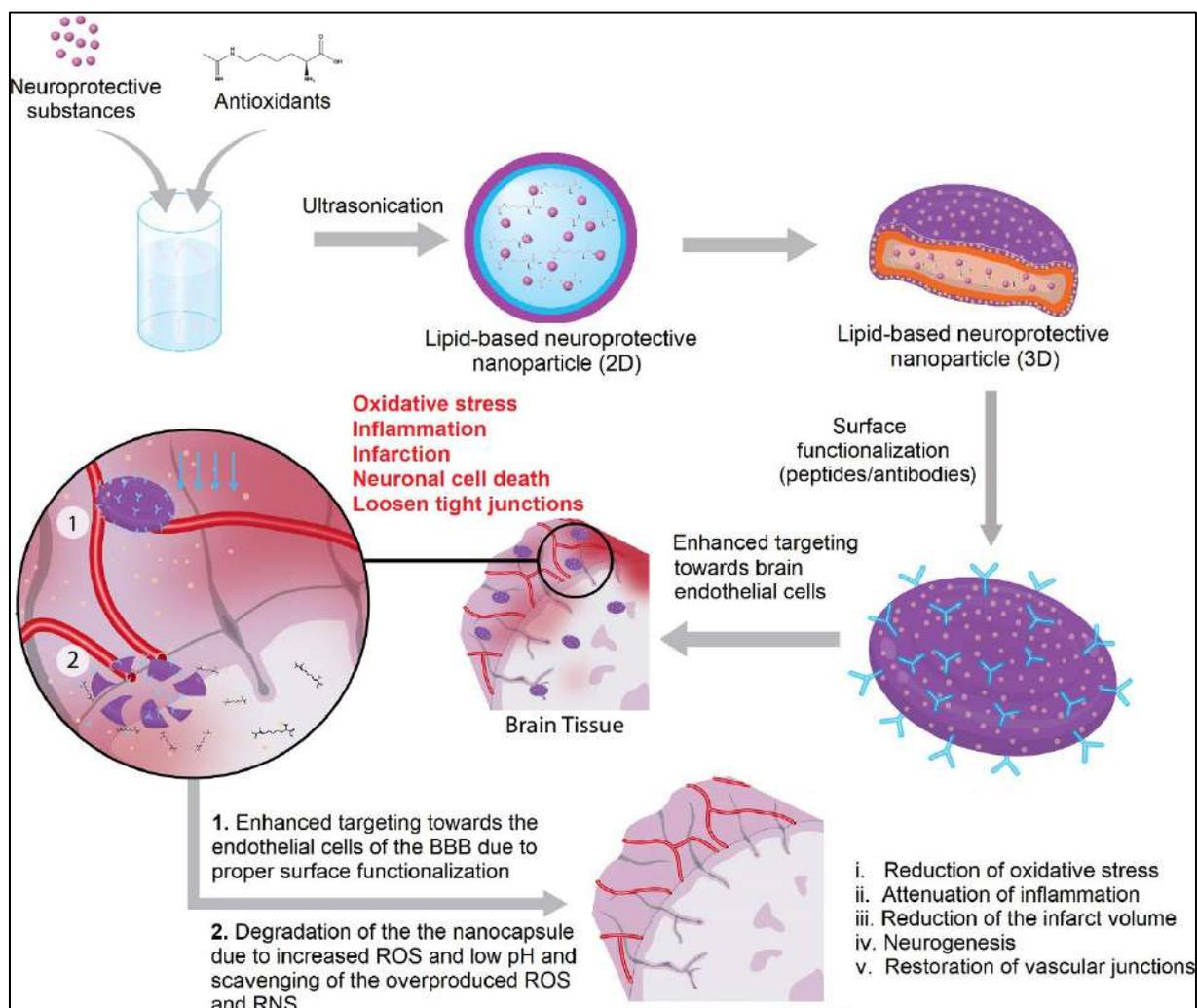
Essential advances have been presented in the literature concerning the use of biomaterial and cell-based strategies for the treatment of ischemic stroke. Nonetheless, there is still a pressing need for the development of treatment approaches with enhanced therapeutic effect. Each of the aforementioned described methods comes with advantages and disadvantages that should be taken into consideration before designing new therapeutics. Considering this, combinational methods that make use of biomaterial and cell-based therapies that try to overcome the current limitations have been developed, aiming at a tailor-made nanotherapeutic approach.

One of the most used biomaterial-based nanostructures in the combinational studies are the polymeric ones. These nanostructures find several uses as supportive scaffolds for the delivery of MSCs, <sup>[84]</sup> as nanoparticles for drug and gene delivery <sup>[85]</sup> or as coatings for inorganic nanoparticles. <sup>[86]</sup> Each of these nanostructures is able to encapsulate a variety of therapeutics, including antioxidants like catalase, <sup>[85a]</sup> nanoceria, and edaravone <sup>[86]</sup>, growth factors like VEGF, <sup>[84]</sup> and other neuro-protectants like glyburide <sup>[85b]</sup> and the NR2B9C therapeutic peptide. <sup>[85c]</sup> In addition, these nanostructures can be modified with specific targeting ligands, including peptides like angiopep-2, <sup>[86]</sup> N-acetyl Pro-Gly-Pro (Ac-PGP), <sup>[85a]</sup> organic compounds like AMD3100, <sup>[85b]</sup> and proteins like wheat germ agglutinin (WGA) <sup>[85c]</sup> and laminin, <sup>[84]</sup> aiming at improving their uptake both by the BBB cells as well as by the ischemic tissue. An example of a combinational approach in which a surface functionalized lipid-based nanostructure is loaded with neuro-protectant and antioxidant therapeutics is presented in **Figure 4**.

Other combinational approaches made use of glycan-based nanostructures like chitosan <sup>[87]</sup> and dextran, <sup>[88]</sup> that were respectively loaded with growth factors <sup>[87]</sup> and apoptosis inhibitors (Z-DEVD-FMK), <sup>[87]</sup> as well as neuro-protectants, as the previously mentioned NR2B9C peptide. <sup>[88]</sup> Surface functionalization was also performed using antibodies against the transferrin receptor <sup>[87]</sup> or peptides like the stroke homing peptide (SHp). <sup>[88]</sup> It has to be noted,

that some of these nanotherapeutics were designed in a way to respond to factors that are characterizing ischemic stroke conditions, such as the overproduction of thrombin, MMP-9<sup>[85b]</sup> and ROS.<sup>[88]</sup> This responsiveness allowed for a controlled release of the encapsulated therapeutics leading to improved therapeutic effects.

Details of the studies above can be found in **Table 10**.



**Figure 4.** Schematic representation of a lipid-based nanostructure (LPN) encapsulating neuroprotective agents as well as antioxidant molecules through a simple ultra-sonication procedure. After the encapsulation, the LPN is functionalized with a specific ligand aiming at enhancing its internalization by endothelial and neuronal cells. I/R causes cerebral damage characterized by the creation of an infarction, oxidative stress, inflammation, neuronal cell death, and BBB dysfunction. The functionalized LPN can ameliorate the post-ischemic effects by reduction of the infarcted area, oxidative stress, and inflammation, and by promoting neurogenesis and restoration of vascular junctions.



**Table 10.** Studies reporting on the therapeutic abilities of various combinational approaches in ischemic stroke.

Biomaterial/Cell-based approach	Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
Polymer nanoparticles and cells	PLGA-MSCs	VEGF	Neurite outgrowth Increase of neuronal/trophic factors	<i>In vitro</i> : MSCs <i>In vivo</i> : Male SD rats	Laminin	Increase of BDNF and TGF $\beta$ 1 due to MSCs	[84]
Polymer nanoparticles, peptides, and cells	Poly-L-Lysine/PGP/Neutrophils	CAT	Reduction of the infarction Retention of CAT activity	<i>In vitro</i> : HUVEC/PC12/ Differentiated HL-60 <i>In vivo</i> : male BALB/c nude or C57BL/6J mice	PGP	Inhibition of ROS-mediated apoptosis	[85a]
Polymer nanoparticles functionalized with therapeutic peptides	PEG-PCL/Thrombin peptide/MMP-9 peptide	Glyburide	Reduction of the infarction Improved survival rates / Improved neurological scores	Male C57BL/6J mice	AMD3100	Inhibition of CXCR4	[85b]
Polymer & protein nanoparticles loaded with therapeutic peptides	PLGA / WGA	NR2B9C	Reduction of the infarct Amelioration of neurological deficits Enhanced neuroprotection	<i>In vitro</i> : Calu-3 cells & primary cortical neurons <i>In vivo</i> : normal rats	WGA	Protection against NMDAR-triggered excitotoxicity	[85c]
Polymer and inorganic nanoparticles	PEG-coated nanoceria	Edaravone	BBB protection Reduction of the infarct volume	Male SD rats	Angiopep-2	ROS scavenging	[86]
Glycan-based nanoparticles loaded with therapeutic peptides	Chitosan	bFGF/ Z-DEVD-FMK	Reduction of the infarct Restoration of the Akt-dephosphorylation	Male Swiss albino mice	Antibody against transferrin receptor-1	Cell death suppression and regeneration through the delivery of bFGF	[87]
Glycan-based nanoparticles loaded with therapeutic peptides	Dextran	NR2B9C	Antioxidant Reduction of the infarct volume	PC-12 cells / Male SD rats	Stroke homing peptide	Inhibition of NO production through interruption of NMDARs/PSD-95	[88]

\*AMD3100: organic compound against CXCR4, bFGF: Basic fibroblast growth factor, CAT: Catalase, CXCR4: C-X-C motif chemokine receptor 4, HL-60: promyelocytic leukemia cells, HUVEC: Human umbilical vein endothelial cells, NMDARs: N-methyl-D-aspartate receptors, NO: Nitric oxide, NR2B9C: Neuroprotective peptide, PC12: Neuronal cell line, PGP: Pro-Gly-Pro, PLGA-MSCs: Poly (lactic-co-glycolic) acid-Mesenchymal stem cells, PSD-95: postsynaptic density protein, Z-DEVD-FMK: caspase-3 inhibitor.

## 6. Therapy and diagnosis

In an effort to develop improved nanomedicine strategies for the treatment of stroke, nanostructures that combine both the therapeutic as well as the diagnostic ability have been developed. These nanostructures, entitled theranostics, allow studying not only their delivery and biodistribution on the diseased tissues but also the real-time effect of the attached therapeutics. One of the most used and non-invasive methods that are currently used is magnetic resonance imaging (MRI). Because of this, the probes that are currently developed for this type of imaging are inorganic nanoparticles like superparamagnetic iron oxide nanoparticles (SPIONs) or other probes based on gadolinium. Other methods have also been developed for the non-invasive imaging during ischemic stroke, some of which are later described.

Although inorganic nanoparticles have been used both as therapeutics and as diagnostics for cancer,<sup>[89]</sup> atherosclerosis,<sup>[90]</sup> intervertebral disc degeneration,<sup>[91]</sup> and others,<sup>[92]</sup> their use in ischemic stroke is limited. Except for their therapeutic role, inorganic nanoparticles find robust exploitation as imaging agents using various techniques such as MRI, magnetic particle imaging (MPI), and computed tomography (CT). Inorganic nanoparticles of iron oxide have been widely used as MRI contrast agents for several diseases. Unfortunately, not many studies have been reported in the last five years for ischemic stroke. This is probably due to limitations such as the low resolution of the current imaging techniques, as well as the high amounts of iron oxide nanoparticles needed for high-quality imaging. Nevertheless, in the few reporting studies, iron oxide nanoparticles, either in the form of Fe<sub>2</sub>O<sub>3</sub> (hematite) nanoparticles<sup>[93]</sup> or of SPIONs (magnetite),<sup>[94]</sup> have been used for the imaging of live apoptotic cells in the infarcted area after cerebral ischemia.

Due to their low stability in biologically relevant media, the fast RES clearance, and the non-controlled biodistribution, inorganic nanoparticles need to be encapsulated or coated with biomaterials that counteract these limitations. The most common materials used at this aim are polymers,<sup>[94-95]</sup> lipids,<sup>[96]</sup> silica,<sup>[97]</sup> proteins,<sup>[98]</sup> and cells.<sup>[97, 99]</sup>

SPIONs encapsulated or coated with polymeric materials have been used either for theranostic<sup>[95a]</sup> or diagnostic<sup>[94]</sup> purposes. The encapsulation of SPIONs inside a polymer matrix along with therapeutic agents like siRNA, that targets the Nogo-66 receptor (NgR), and a subsequent encapsulation inside neuronal stem cells (NSCs) showed the ability of the system to be used as a theranostic. The therapeutic outcome was proved through enhanced neuronal differentiation and functional recovery, while imaging was achieved using MRI.<sup>[95a]</sup>

In a different approach, SPIONs coated with silica and functionalized with gold nanorods were encapsulated inside MSCs. The fabricated system was able to be magnetically guided to the tissue of interest and to provide diagnostic information through MRI and photoacoustic imaging (PAI).<sup>[97]</sup> Encapsulation of SPIONs along with the nerve growth factor (NGF) and the mitogen-activated kinase inhibitor, U0126, inside an apolipoprotein E (ApoE) modified albumin, is another theranostic approach. This system resulted in neurite outgrowth and reduction of the infarct volume, while in parallel non-invasive MRI was used.<sup>[98]</sup>

Although SPIONs were mostly used as MRI agents,<sup>[94a]</sup> currently their use in MPI,<sup>[94b]</sup> a new technique that provides shorter acquisition times and higher spatial resolution compared to MRI, allowed for enhanced imaging abilities, offering an alternative imaging method for ischemic stroke.

Another type of inorganic nanoparticles that have been used for diagnostic purposes is represented by PEGylated barium-holmium-fluoride (BaHoF<sub>5</sub>) nanoparticles. These nanoparticles cannot work as MRI contrast agents, but they can be visualized in the ischemic brain using computed tomography angiography (CTA) and computed tomography perfusion (CTP).<sup>[95b]</sup>

The use of lipids as a coating material increases the colloidal stability and the systemic circulation both of inorganic nanoparticles and of other therapeutic agents encapsulated inside the matrix. The encapsulation of neuroprotective agents like citicoline<sup>[96a]</sup> or angiogenic factors like VEGF,<sup>[96b]</sup> respectively improves the functional recovery and increases angiogenesis after

ischemic stroke. If this therapeutic approach is properly combined with MRI <sup>[96a]</sup> or fluorescence and positron emission tomography imaging, <sup>[96b]</sup> additional information concerning the effect of the delivered nanotherapeutics in the ischemic tissue can be acquired.

An example of a theranostic approach based on imaging (fluorescence) and pharmaceutical (antioxidant) molecules is depicted in **Figure 5**. In the presented figure, the administered nanostructures change their fluorescence properties due to the overproduced ROS, which at a later point are reduced to their physiological level, due to scavenging by the same theranostic nanostructure.

A non-invasive imaging technique using a multi-functional nanoprobe modified with paramagnetic chelators and fluorophores has been used to monitor the homing of transplanted endothelial progenitor cells (EPCs) in a stroke model of diabetic rats. <sup>[99a]</sup> This approach, one of the first to report non-invasive techniques, showed that it is possible to image the homing of EPCs using MRI and near-infrared fluorescence imaging (NIRFI).

A recent development concerning biomimetic nanotherapeutics is the use of cell membrane-derived nanoparticles. These are used either as a coating for other inorganic and/or organic particles or as independently self-assembled nanoparticles. In one of these studies, self-assembled platelet-derived bio-nanobubbles (PNBs) were used as potential nanotheranostics for cerebral ischemia. <sup>[99b]</sup> The authors of this study demonstrated the ability of the PNBs to accumulate to the ischemic tissue promoting local revascularization of the injured vessels and restoration of the blood. Furthermore, they showed that PNBs could enhance ultrasound imaging on the ischemic tissue.

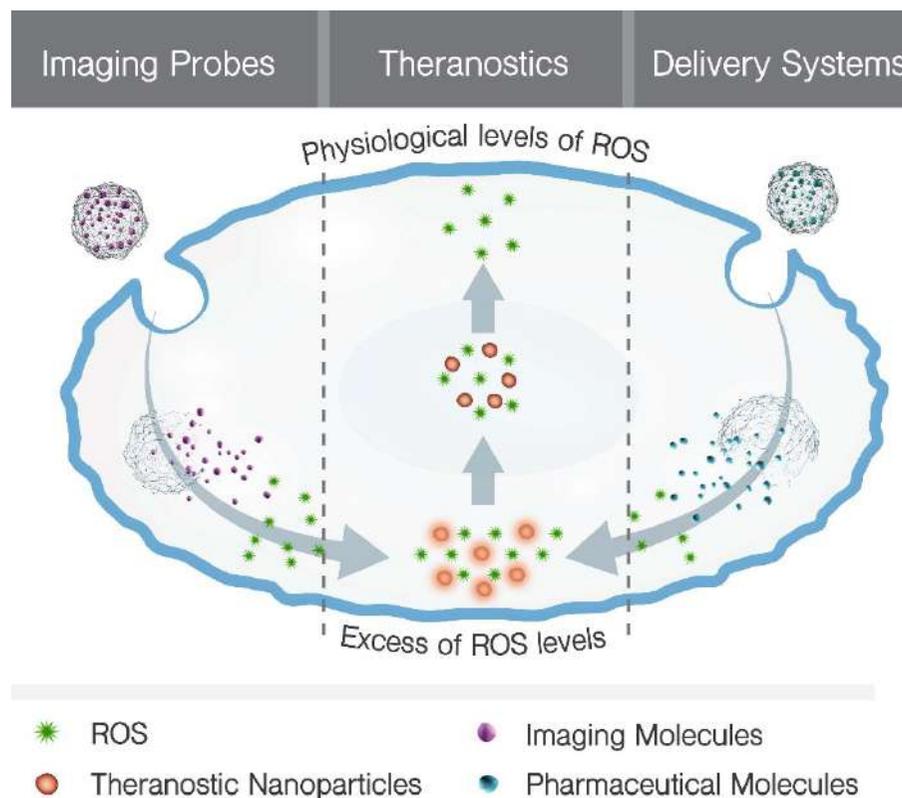
Details of the previously mentioned studies can be found in **Table 11**.

**Table 11.** Studies reporting on the diagnostic/theranostic abilities of inorganic nanoparticles in ischemic stroke.

Material	Therapeutic substance	Therapeutic ability/feature	Biological models	Targeting moiety	Mechanism of action	Ref.
SPIONs-loaded polymersomes internalized by MSCs	N/A	MRI	Male SD rats	N/A	T <sub>2</sub> contrast agent	[94a]
Commercial SPIONs	N/A	MPI	C57BL/6J mice	N/A	T <sub>2</sub> contrast agent	[94b]
Poly(ethyleneimine) & PDLLA diblock copolymer	SPIONs/NgR siRNA	Enhanced neuronal differentiation / Improved recovery / MRI	<i>In vitro</i> : primary NSCs <i>In vivo</i> : male SD rats	N/A	Promotion of NSCs' differentiation through NgR gene silencing	[95a]
PEG-coated BaHoF <sub>5</sub>	N/A	CTA and CTP	<i>In vitro</i> : BCECs / murine RAW264.7 <i>In vivo</i> : Kunming mice / male SD rats	N/A	Multimodal CT	[95b]
DPPC / cholesterol / DPPE-PEG2000	Citicoline	Fluorescence & PET imaging	Male Wistar rats	CD106 / IgG-1	Chemical exchange saturation transfer	[96a]
Cholesterol / PC / PE	Angiogenic peptides	Increased vascular density & angiogenesis / PET imaging	Male SD rats	N/A	Increased expression of angiotensin-2 and TGF-1b	[96b]
SPIONs/Silica/ Gold - MSCs	N/A	Reduction of the infarction / MRI & PAI	Male C57BL/6J mice	N/A	T <sub>2</sub> contrast agent Increased MSCs homing in the ischemic brain	[97]
SPIONs/Albumin/ ApoE	NGF / U0126	Neurite outgrowth / Reduction of the infarction / MRI	<i>In vitro</i> : PC12 cells <i>In vivo</i> : Male Wistar rats	ApoE	T <sub>2</sub> contrast agent Delivery of NGF and a MEK inhibitor	[98]
EPCs	RWJ 67657	Enhanced angiogenesis / MRI & NIRFI	Male wild type mice & male diabetic rats	N/A	Enhanced EPCs homing	[99a]
Platelets	N/A	Revascularization/ Blood flow restoration / Reduction of the infarction/ MRI	Male C57BL/6J mice	Integrins $\beta 1/\beta 3$ GPIIb-IX-V & GPVI	Remodeling of stroke lesion through the delivery of PNBs	[99b]

\*ApoE: Apolipoprotein E, CTA: Computed tomography angiography, CTP: Computed tomography perfusion, DPPC: Dipalmitoyl phosphatidyl choline, DPPE: 1,2-Bis(diphenylphosphine) ethane, EPCs: Endothelial progenitor cells, GPIIb-IX-V: Receptor complex, GPVI: Receptor complex, IgG: Immunoglobulin G, MEK: Mitogen-activated protein kinase, MPI: Magnetic particle imaging, MRI: Magnetic resonance imaging, MSCs: Mesenchymal stem cells, NGF: Nerve growth factor, NIRFI: Near-infrared fluorescence

imaging, PAI: Photoacoustic imaging, PC: Phosphatidyl choline, PE: Phosphatidyl ethanolamine, PEG: Polyethylene glycol, PET: Positron emission tomography, PNBs: platelet-derived bio-nanobubbles, RWJ 67657: Selective inhibitor, SPIONs: Super paramagnetic iron oxide nanoparticles, TGF-1 $\beta$ : Tumor growth factor 1 $\beta$ .



**Figure 5. The overproduced ROS after ischemic stroke can be used as a stimulus in order to design smart biomaterial-based therapeutics. Based on this, ROS-responsive nanostructures for imaging, therapy, or their combination (theranostic) can be fabricated. On the left, ROS-responsive imaging probes increase their fluorescence after their release from the ROS-sensitive nanostructure, and their subsequent contact with the overproduced ROS. On the right, a similar ROS-sensitive nanostructure releases its therapeutic cargo due to the overproduced ROS. In the center, the released imaging probes and pharmaceutical molecules act synergistically, providing detection (increased fluorescence) and therapy (ROS scavenging), leading finally to physiological ROS levels.**

## 7. Conclusion and perspectives

As with the majority of CNS diseases, an ischemic stroke results in functional and structural disorders that may subsequently lead to cognitive, motor, and speech problems. Although a high number of studies for the treatment of ischemic stroke have been presented within the most recent years, there remains an immense need for the development of smart nanotherapeutics that will replace the current drug therapies. Tissue plasminogen activator and mechanical thrombectomy that currently constitutes the only two FDA-approved treatment options,<sup>[2]</sup> fail to treat the post-ischemic stroke side-effects, allowing for the high percentages of morbidity worldwide. On the other hand, although the first generation of nanotherapeutics based on nanoparticles, hydrogels, and/or other therapeutic nanostructures have demonstrated encouraging results, their therapeutic effect is inhibited by numerous factors, including the inability of most of these structures to cross the BBB.<sup>[100]</sup> Also, toxicity and immunogenicity, low encapsulation efficiencies, and non-controlled biodistribution represent a series of significant obstacles that need to be overcome when aiming to cure this specific disease. Along with the limitations above, the poor pharmacokinetics and the slow diffusion of these DDS into the brain parenchyma increase the difficulties of controlled and targeted delivery of nanotherapeutics.<sup>[100]</sup>

Additional consideration should also be given to the use of invasive techniques as well as to the time window in which each treatment can be applied. Although tPA results in the dissolution of the clot and subsequent blood reperfusion, the narrow administration time window (4-6 hours) limits its therapeutic efficacy rendering it unsuitable for many patients. To overcome this time limitation, mechanical thrombectomy can be applied,<sup>[101]</sup> but once again, not all patients can undergo an invasive operation of this kind. As stated, tPA can dissolve the clot, but it is unable to treat pathophysiological causes that lead to the post-ischemic stroke effects such as overproduction of reactive oxygen and nitrogen species, overproduction of matrix metalloproteinases, neuronal cell death, damaged BBB and others.

Edging forward, the development of non-invasive nanotherapeutics able to treat specific pathophysiological causes of the post-stroke effects and, with an improved administration time window, holds the promise of more effective treatments for ischemia, with the prospect of a better quality of life for stroke survivors. To this end, biomaterial-based <sup>[4a]</sup> and cell-based <sup>[4c]</sup> therapies, as well as a combination of both, have been reported. Besides this, the use of nanostructures that can act both as therapeutics and diagnostics further enhances the therapeutic outcome, since real-time monitoring of the delivered nanostructures provides additional information concerning their effect on the ischemic tissue. Based on non-invasive techniques like MRI, MPI, PAI, CTA, CTP, and PET and the use of theranostic nanostructures significant improvement on the treatment of a variety of CNS diseases including ischemic stroke can be achieved.

From a biomaterial point of view, nanostructures that respond to external and/or internal stimuli hold great promise as next-generation nanotherapeutics. Guidance through an external magnetic field of nanostructures incorporating SPIONs <sup>[92a]</sup> or nanoceria particles that can act as re-chargeable ROS scavengers <sup>[102]</sup> are two of the most promising developments presented. Additionally, magnetothermal stimulation of neurons <sup>[103]</sup> or controlled release using wireless actuators or actuators that respond to electrical signals <sup>[104]</sup> can also be rendered as suitable nanotherapeutics for the ischemic brain.

Another course of action that can be followed to enhance the therapeutic efficacy of the desired DDS is the proper functionalization of the current biomaterial-based DDS. This may utilize targeting moieties that specifically target the BBB and/or the infarcted area. In view of this, several peptides have been proposed in the literature that targets the endothelial cells of the BBB (*e.g.*, angiopep-2), <sup>[86]</sup> the infarcted tissue (*e.g.*, stroke homing peptide), <sup>[88]</sup> or cell-penetrating peptides <sup>[44d]</sup> that have the ability to enhance the uptake of the bearing nanostructures.

Conversely, from a cell-based point of view, the use of mesenchymal stem cells, progenitor cells, and more recently, the use of exosomes and cell-mimetic particles hold promise. These may represent an alternative form of therapy not only for cerebral ischemia but also for the majority of the CNS disorders.<sup>[105]</sup> Even though significant progress has been made thus far, we are not yet able to clinically use the systems mentioned above due to the limitations that each system imposes (*e.g.*, ethical issues, scale-up).

An additional reason for the unsuitability of the current nanotherapeutics to be clinically translated is the lack of proper model systems. A good model system should provide a better understanding of the biological mechanisms that underlie the pathophysiology of the disease. In parallel, it should allow a thorough investigation of the effect of the nanotherapeutics on these mechanisms.<sup>[106]</sup> Models that are based on the middle cerebral artery occlusion and do not mimic the pathophysiological heterogeneity (*e.g.*, duration, severity, extension) of different stroke types should not be considered relevant and should be modified. Furthermore, healthy rodents or other used animals that do not present comorbidities, as occur in several stroke patients, consist of one more limitation that leads to failure during the clinical trials and should be avoided. The development of experimental models mimicking the conditions of acute ischemia needs to be carefully considered since the use of the proper model will allow a further step towards the clinical translation of the presented nanotherapeutics.

The failure of the currently-developed therapeutics to reach the market is attributable to several factors, one of which is the therapeutic time window. Although in clinical trials, long-time windows are used, during the preclinical studies, there is a time limitation that may affect the therapeutic outcome. In addition to this, during preclinical studies, the result is mostly judged from the reduction of the infarct size, while during the clinical phase, clinical and functional endpoints are assessed. Furthermore, the use of combinational treatments (*e.g.*, tPA + neuroprotectants) should also be taken into consideration since the combination of different treatments may not result in the desired therapeutic outcome.

In our opinion, one of the most significant drawbacks that leads to limited clinical translation of the majority of the developed nanotherapeutics is the lack of proper design during preclinical evaluations. For example, there are several studies in which strategies of randomization or blinding to reduce the risk of bias are not reported. The result can thus be a non-objective assessment. <sup>[107]</sup>

To improve clinical therapeutic outcomes, drastic changes need to be made. For example, new animal models, that are not based on healthy animals, but takes also into consideration the variety of comorbidities that real-life patients present, needs to be established Furthermore, following the guidelines for preclinical trials, as they were set by the Stroke Therapy Academic Industry Roundtable (STAIR), <sup>[108]</sup> will further improve the viability of the developed therapeutics in the clinical trials.

Another essential aspect is the type of drugs that are currently developed. Among the 430 drugs that were evaluated for the treatment of ischemic stroke from 1995-2015, only 19 succeeded in reaching the market, and of these, 11 of them were anti-thrombotic with a mere 8 for the prevention of stroke. <sup>[109]</sup> It is thus apparent that researchers should focus more on the development of neuroprotectants that increase the therapeutic time window and that protect the brain from the severe damage that is caused during reperfusion. Regeneration of the lost neurons should also be a significant concern. This would improve functional recovery and the number of reduced disability-adjusted life years (DALY).

Eventually, the combination of biomaterial- and cell-based therapies into autonomous stimuli-responsive hybrid nanosystems (*e.g.*, hydrogels incorporating mesenchymal stem cells and/or SPIONs/CeO<sub>2</sub> nanoparticles) will prove beneficial, allowing for completely radical treatment of cerebral ischemia. Moreover, the study of these nanosystems using proper experimental models that accurately mimic the pathophysiology of ischemic stroke will enable a faster transition from a preliminary laboratory study to a clinical trial.

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## Biographies



**Christos Tapeinos** research interests are focused on the design of smart bioengineering systems responding to various physical, chemical, and biological stimuli. He elaborated on a wide variety of biomaterial-based structures based on synthetic and natural polymers. Apart from the synthesis and the characterization of these nanostructures, he is also studying their biological interactions in living cells and tissues. Currently, Dr. Tapeinos works as an Experienced Researcher funded by an individual Marie-Curie Fellowship at the Smart Bio-Interfaces Research Line at the Italian Institute of Technology, where he focuses his research on the fabrication of biomimetic and neuroprotective nanocapsules for the treatment of post-ischemic stroke effects.



**Abhay Pandit** is an Established Professor of Biomaterials at the National University of Ireland, Galway. He is the Scientific Director of the Centre for Research in Medical Devices (CÚRAM), a multi-disciplinary academic-industry-clinician translational research center funded by Science Foundation Ireland. Prof. Pandit's research integrates material science and biological paradigms in developing solutions for chronic diseases. Prof. Pandit was elected to the American Institute of Medical and Biological Engineering (AIMBE) College of Fellows in recognition of his outstanding contributions to establishing a national research center to treat global chronic diseases. He has also been an elected member of the Council for both the Tissue Engineering and

Regenerative Medicine International Society and the European Society for Biomaterials.



### **Gianni Ciofani**

Gianni Ciofani is an Associate Professor at the Polytechnic University of Torino, Department of Mechanical and Aerospace Engineering (Torino, Italy) and Senior Researcher at the Italian Institute of Technology, where he coordinates the Smart Bio-Interfaces Research Line (Pontedera, Pisa, Italy). His research is mainly focused on smart nanomaterials for nanomedicine, bio/non-bio interactions, and biology in altered gravity conditions. In 2016 and 2018 he was awarded, respectively, a European Research Council (ERC) Starting Grant and an ERC Proof of Concept Grant.

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