Reactive Oxygen Species-Responsive Nanoparticles for the Treatment of Ischemic Stroke

Olivera Rajkovic, Charlotte Gourmel, Richard d’Arcy, Raymond Wong, Ivana Rajkovic, Nicola Tirelli, and Emmanuel Pinteaux*

Ischemic stroke is a leading cause of death and long-term disability worldwide, yet availability of current treatments is limited. The downstream events resulting from cerebral ischemia lead to an imbalance in the production of harmful reactive oxygen species (ROS) over endogenous antioxidant mechanisms. Thus, treatments that can reduce this imbalance can limit the extent of injury resulting from ischemic stroke. In this work, the potential of novel ROS-scavenging PEGylated polymeric nanoparticles (NPs) composed of poly(propylene sulfide) (PPS) (PPS-NPs) for ischemic stroke therapy is evaluated in vitro and in a mouse model of stroke. In vitro results show that PPS-NPs display remarkable anti-oxidant and anti-inflammatory properties, whilst exhibiting negligible cytotoxicity. NPs administered intravenously rapidly accumulate in ischemic brain regions as visualized through fluorescence imaging, reduced infarct volume, blood-brain barrier damage, neuronal loss, and neuroinflammation as determined by immunohistochemistry, and improved recovery of neurological function as determined through behavioral analyses. Crucially, the data show that the therapeutic time window for administration of PPS-NPs extends up to 3 h, a clinically relevant time window for stroke. The findings provide new strong evidence that these NPs may be an effective antioxidant therapy for ischemic stroke.

1. Introduction

Ischemic stroke is a leading cause of death and disability worldwide,[1] accounting for 1 out of every 20 deaths in the United States[2] and 11.8% of deaths worldwide.[3] Treatment options are limited to thrombolysis by intravenous (iv) administration of recombinant tissue plasminogen activator (t-PA) and endovascular thrombectomy to physically remove the blood clot.[4,5] These interventions are restricted to a narrow therapeutic window, have unwanted side-effects, and can only be given to a limited number of patients.[6,7] Thus, there is an urgent need for novel therapies that can limit the effects of stroke-induced brain injury. After cerebral ischemia and particularly reperfusion, the production of reactive oxygen species (ROS) is dramatically increased, leading to neuronal injury and death.[8] In addition, ROS are critical in initiating the post-ischemic inflammatory response which further exacerbates ischemic injury.[9] There is an abundance of experimental and clinical research supporting ROS as a promising therapeutic target for acute ischemic stroke.[10–15] However, low molecular weight antioxidants (for instance NXY-059) have historically not translated well in the treatment of stroke patients. On the other hand, several promising antioxidant nanoparticles (NPs) have been developed in recent years, with the perspective capacity of suppressing ROS-induced damage following ischemic stroke.[16–18] For example, Liu and colleagues have demonstrated that melanin NPs (MeNPs) scavenge multiple reactive oxygen and nitrogen species (RONS), suppress RONS-induced inflammation, and are neuroprotective in vitro.[19] They also showed in a rat model of ischemic stroke that MeNPs reduce brain superoxide levels and infarct volume. Carbon NPs, hydrophilic carbon clusters, conjugated to poly(ethylene glycol) (PEG) termed PEG-HCCs, administered during reperfusion after transient middle cerebral artery occlusion (MCAo) in acutely hyperglycemic rats, reduce infarct size, hemisphere swelling, hemorrhage score, and improve neurological functional outcome.[20] Exemplary work by Kabanov and co-workers on superoxide dismutase 1 (SOD1) containing nanozymes has shown that they are able to significantly reduce infarct volumes in both mice[21] and rat[22] models of ischemic stroke. In this work, we have synthesized and used new
Scheme 1. Top: PPS NPs are produced first by emulsifying the monomer (propylene sulfide, PS) in water with the help of a PEGylated surfactant (Pluronic F127; it is a triblock copolymer featuring two terminal PEG chains flanking an internal block of poly(propylene glycol), PPG). By the addition of an in situ deprotected initiator, PS is polymerized within the droplets; at the end of the process, PPS chains feature terminal thiols, which are used for the purpose of fluorescent labelling (in this case Cy5.5 maleimide) and cross-linking (pentaerythritol tetraacrylate, acting as a multiple Michael-type acceptor). The cross-linked nanoparticles retain Pluronic chains on their surface and their size is controlled on the basis of the initial monomer/surfactant ratio. Bottom: PPS-NPs are injected intravenously into a mouse model of stroke, where they accumulate in the ischemic brain via and EPR-like effect (note: the blood–brain barrier is severely compromised in stroke). ROS (signified as the yellow–red area) are significant potentiators of stroke morbidity and is sequestered by the antioxidant PPS-NPs in turn reducing stroke severity.

poly(propylene sulfide) (PPS) NPs, to reduce post-stroke inflammation and damage through scavenging ROS. The NP surface is coated with poly(ethylene glycol) (PEG) chains, from the surfactant used in their preparation (Pluronic F127); this composition was chosen to allow for biocompatibility and long circulation times in vivo; a graphic sketch of the nanoparticle synthetic method is shown in Scheme 1, and size distribution, cryo-EM and zeta potential are provided in Figure S3 and Table S1, Supporting Information. The core is made of chemically cross-linked PPS, which is an organic polymer with a high density of sulfur (II) atoms (sulfides, also known as thioethers); such groups are potent ROS scavengers, for example, methionine residues offer a well-known biological example of this activity.[23] Upon exposure to ROS, the sulfide groups can be converted to more polar and thus more hydrophilic groups (sulfoxides, sulfones). Typically, this increase in polarity/solubilization has been exploited as a ROS-responsive release mechanism of encapsulated payloads for targeted drug-release.[24,25] We have previously shown that PPS-NPs feature different oxidation-responsive mechanisms in response to the presence of different ROS.[26] In particular, exposure to hydrogen peroxide (H2O2) induces swelling and loss of the PEGylated surface layer without affecting the NPs (lack of) cytotoxicity. PPS-NPs have recently been used as versatile vaccine delivery systems,[27–29] and interestingly a very similar NP formulation, also consisting of a ROS-reactive thioether and cross-linked core, has been shown to reduce neuroinflammation and improve the therapeutic outcome in a mouse model of traumatic brain injury,[30] although the PPS-NPs tested in our study are less toxic at higher concentration, less hydrophilic (hence lower ROS solubility at early time points) and larger (therefore better suited
PPS-NPs decrease the release of pro-inflammatory cytokines in vitro. A–C) Murine primary mixed glial cells were primed with LPS (0.5 µg mL$^{-1}$; 24 h) then exposed to PPS-NPs (1 mg mL$^{-1}$) of varying sizes (50–150 nm) and LPS (0.5 µg mL$^{-1}$; 24 h). PPS-NPs decreased (A) IL-6, (B) TNF-α release and were not cytotoxic (C), as measured by ELISA (A and B) and LDH assay (C). D–F) BV2 microglial cells were primed with LPS (0.5 µg mL$^{-1}$; 24 h) then exposed to 50 nm PPS NPs (0.1–5 mg mL$^{-1}$) and LPS (0.5 µg mL$^{-1}$; 24 h). PPS-NP treatment decreased (D) IL-6 and (E) TNF-α release. (F) BV2 cells were incubated with PPS-NPs (0.5–5 mg mL$^{-1}$; 24 h) and cell viability was assessed using MTT assay. Statistical analyses were performed using one-way ANOVA followed by Tukey (A–C) or Dunnett (D–F) corrected post-hoc analysis (ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control (LPS; A–E); (untreated; F), ns $p > 0.05$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus 50 nm PPS (A–C). All data are presented as mean ± SEM ($n = 3–5$). UNT indicates untreated.

for a slower and prolonged scavenging effect). Despite their potential ROS scavenging abilities and negligible cytotoxicity,[25,26,31,32] PPS-NPs have not yet been tested in the context of ischemic stroke, although PPS-derived microspheres have recently shown promise in a mouse model of hind leg ischemia.[33] Here we show that PPS-NPs exhibit antioxidant properties for multiple types of ROS in vitro with negligible cytotoxicity and a remarkable therapeutic activity in an in vivo model of ischemic stroke.

2. Results and Discussion

2.1. PPS-NPs Decrease Proinflammatory Cytokine Release and Scavenge Multiple Types of ROS In Vitro

The anti-inflammatory effects of PPS-NPs were studied by enzyme-linked immunosorbent assay (ELISA) in primary cultures of mixed glial cells, and BV2 cells (an immortalized murine microglial cell line). First, as previously observed, we have confirmed the PPS-NPs negligible cytotoxicity; using two separate methods of cell viability assessment, lactate dehydrogenase (LDH) assay (measure of membrane integrity) (Figure 1C) and MTT assay (metabolic activity) (Figure 1F) and two different cell types (primary murine mixed glial and BV2 microglial cells) which showed excellent viability even up to concentrations as high as 5 mg mL$^{-1}$.

Cells were then subjected to a lipopolysaccharides (LPS) stimulation (0.5 µg mL$^{-1}$), for 24 h initially and then a further 24 h; this significantly increased the release of proinflammatory cytokines interleukin (IL)-6 (391-fold for primary cultures (Figure 1A); 57-fold for BV2 cells (Figure 1D)) and tumor necrosis factor (TNF)-α (24-fold for primary cultures (Figure 1B); 22-fold for BV2 cells (Figure 1E)) compared to untreated control, confirming LPS as inducers of inflammatory cytokine release. In primary cultures, treatment with PPS-NPs (1 mg mL$^{-1}$; 24 h) of different sizes (50, 75, 115 nm) significantly decreased IL-6 (78%, 47%, 53%, respectively; Figure 1A) and TNF-α levels (97%, 84%, 76%, respectively; Figure 1B) compared to LPS control, demonstrating their potent anti-inflammatory properties in a NP-size dependent fashion (smaller particles displaying a higher potency than larger particles). We hypothesize that the inversely proportional relationship between NP size and activity is due to the higher surface area of smaller NPs enabling a faster ROS scavenging (as also demonstrated in previous works).[26] As 50 nm NPs were found to have the strongest anti-inflammatory effects as well as negligible cytotoxicity, all further experiments were carried out using NPs of this size; however, it should be noted that in applications where a slower and more prolonged anti-inflammatory/anti-oxidant effect is desired (e.g., chronic inflammatory diseases) larger particles may be better suited.

In BV2 microglial cultures, a significant decrease in IL-6 levels compared to LPS control were also found to be concentration-dependent (0.5 (32%), 1 (36%), 2 (73%), 5 (96%) mg mL$^{-1}$ PPS-NPs for 24 h), suggesting the anti-inflammatory effect is also proportional to PPS-NP concentration (Figure 1D). Unlike IL-6 which required a PPS-NP concentration of ≈5 mg mL$^{-1}$, a NP
due to the otherwise inertness of the PPS-NPs, we are inclined to assume the potent (pharmacological) anti-inflammatory properties are mediated through their antioxidant nature. We therefore first evaluated in vitro their concentration-dependent ability to reduce the concentration of clinically relevant ROS (hydrogen peroxide (H$_2$O$_2$), superoxide, and nitric oxide (NO)) that are known to potentiate the severity of ischemic stroke.\cite{34} H$_2$O$_2$ scavenging activity of PPS-NPs was confirmed using ROS-Glo H$_2$O$_2$ Assay (Figure 2A) which utilizes a boronic-ester protected luciferin substrate. H$_2$O$_2$ treatment (15 µM; 24 h) significantly increased luminescence (ninefold) compared to untreated control whereas treatment with PPS-NPs for 24 h significantly decreased luminescence in a concentration-dependent manner (1 (33%), 2 (72%), 5 (85%) mg mL$^{-1}$ NPs). Superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C, a well-established method for measuring superoxide,\cite{35–38} was used here to investigate the ability of PPS-NPs to reduce extracellular superoxide (O$_2^-$) released by BV2 cells (Figure 2A (Middle)). Menadione, a known inducer of ROS was added at 100 µm for 1.5 h, and resulted in significantly increased O$_2^-$ levels (fivefold) compared to the untreated controls; however, co-treatment of menadione with PPS-NPs for 1.5 h significantly decreased O$_2^-$ levels in a concentration-dependent manner (1, 2, 4 mg mL$^{-1}$ of PPS-NPs leading to a 39%, 54%, and 69% reduction in O$_2^-$ levels respectively). Crucially, it should be noted that previous studies have clearly demonstrated that PPS-NPs/thioethers are unable
to scavenge $O_2^-$ [39] this would therefore indicate that superoxide release is inhibited upstream of its release rather than being directly scavenged by PPS-NPs, possibly through reducing the oxidative burden on the native cellular antioxidants (e.g., glutathione, thioredoxin, superoxide dismutase, catalase, etc).

The Griess Reagent System was used to investigate NO formation by measuring its more stable breakdown product, nitrite ($NO_2^-$) [40] (Figure 2A (right)). LPS stimulation (1 µg mL$^{-1}$; 48 h) significantly increased (49-fold) $NO_2^-$ levels compared to untreated control, confirming LPS-induced $NO_2^-$ release by BV2 cells. Treatment with PPS-NPs for 48 h significantly decreased $NO_2^-$ levels in a concentration-dependent manner (0.5 (45%), 1 (68%), 2 (74%), 4 (90%) mg mL$^{-1}$ NPs). The particles are unlikely to directly scavenge NO, due to its very low oxidant activity, while its reduction may be more likely due to lower expression of induced nitric oxide synthase (iNOS), which is upregulated via NF-kappa B pathways, similarly as TNF-$\alpha$; [41] previous studies have also highlighted the ability of PPS to scavenge peroxynitrite, [42] a reactive oxidative product of NO and superoxide which in turn can also break down into nitrite or cause additional nitrosative inflammatory stresses. [43] In ischemic stroke, NO production through neuronal-NOS (nNOS) is likely a result of the membrane-permeable character of ROS scavenging activity due to their PEGylated nature, the PPS-NPs are small size (50 nm) and PEGylation, as studies have shown that PEGylated polymeric NPs between 10 and 100 nm in size are able to remain in the systemic circulation for hours. [47-49]

2.3. PPS-NPs Accumulate in Ischemic Brain after MCAo and are Cleared through the Liver and Spleen

Poor brain delivery is a major contributing factor to the continued failure of potential ischemic stroke treatments. NPs are promising candidates for overcoming the challenges of brain drug delivery, as it is well established that the blood–brain barrier (BBB) is highly disrupted after cerebral ischemia; [51-53] NPs offer the possibility to passively target it through an enhanced permeability and retention (EPR)-like effect similar to that observed in tumors. [54-56] The ability of PPS-NPs to accumulate in the damaged brain was evaluated in a mouse model of stroke (MCAo). Mice were intravenously administered Cy5.5-labelled PPS-NPs (100 mg kg$^{-1}$) 0.5 h after filament removal (reperfusion). Mice were sacrificed, organs extracted, and ex vivo fluorescence imaging of the ischemic brains was conducted 2 and 24 h after PPS-NP-injection. As shown in Figure 3B, quantification (Figure 3B2) and fluorescence images (Figure 3B3) showed that accumulation of PPS-NPs into the ischemic brain occurred rapidly (as early as 2 h post-injection); this is an important finding given that ROS levels begin to increase almost immediately after cerebral ischemia. [57-58] thus NPs need to be available for ROS sequestration acutely after stroke-onset to ensure therapeutic efficacy.

As shown in Figure 3B1,3, NP accumulation was localized to the ischemic area (left) and area of BBB-damage, suggesting that BBB breakdown allows NPs to leak into brain parenchyma and accumulate in the injured area due to an EPR-like effect and localize primarily in the core but also noticeably in the penumbra (Figure 3B1). In addition, distribution of PPS-NPs into peripheral organs was evaluated (Figure 3B4,5). PPS-NPs were observed mainly in the liver and spleen at 24 h (and to a lesser extent in the kidneys), suggesting clearance by these organs, as expected for polymeric NPs of this size. [59-61] Of note, the retention of anti-inflammatory PPS-NPs in liver and spleen for at least 24 h may also be in itself beneficial for stroke outcome, as previous studies have shown that targeting the peripheral inflammatory response, in which the liver and spleen play prominent roles, improves post-stroke recovery. [62-63]

Interestingly, the average radiant efficiency into the brain was significantly increased (fourfold) at 24 h compared to

2.2. In Vivo Pharmacokinetics of PPS-NPs

To evaluate whether PPS-NPs may be characterized by a prolonged half-life (i.e., “stealth” character, which is typical of PEGylated nanomaterials), mice were intravenously administered Cy5.5-labelled PPS-NPs (100 mg kg$^{-1}$). Blood samples were taken at specific time points, and the fluorescence intensity in whole blood was quantified. PPS-NPs were no longer detected in the blood after 24 h, and appear to show evidence of an early phase of accelerated clearance (within the first hour after injection) followed by a period of considerably slower decrease in concentration (Figure 3A). This may stem from the large dose used, which is only permitted by the exceedingly low toxicity of the particles: indeed, their high blood concentration may trigger physico-chemical processes leading to some rapid capture in the liver. Considering only this second part of the elimination kinetics, our finding is similar to the 6 h blood half-life recorded in a previous study of 40 nm PPS-NPs into Sprague Dawley rats. [46] This rather long circulation half-life can be attributed to the combination of small size (50 nm) and PEGylation, as studies have shown that PEGylated polymeric NPs between 10 and 100 nm in size are able to remain in the systemic circulation for hours days. [47-49]
Figure 3. A) In vivo pharmacokinetics of PPS-NPs. The time dependency of PPS-NPs concentration would suggest a rapid (minutes) phase of elimination, followed by a slower phase with a half-life in the region of 9.5 h. B) PPS-NPs accumulate in ischemic brain after intravenous injection. B1) Localization of PPS-NPs in the brain after 24 h. B2) Quantification of early (2 h) and delayed (24 h) brain accumulation. B3) Representative ex vivo fluorescence images of NP accumulation into the ischemic area (left). B4) Accumulation of NPs into peripheral organs post-stroke. B5) Quantification of fluorescence images show NPs mainly in the liver and spleen. Statistical analyses were performed using unpaired student’s t-test (*p < 0.05), and two-way ANOVA followed by Tukey corrected post-hoc analysis (ns p > 0.05, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ 2 h versus 24 h within organ). $ represents significance versus liver (2 h), # versus liver (24 h). All data are presented as mean ± SEM ($n$ = 3 or 8). PPS-NPs accumulate and are retained in the ischemic brain for at least 7 days after delayed delivery of NPs 3 h post-MCAo. C1) Quantification of NP brain accumulation 7 days post-MCAo ($n$ = 12). Unpaired student’s t-test (**$p < 0.001$). C2) Representative ex vivo fluorescence image. All data are presented as mean ± SEM.
2 h, suggesting that NPs continue to i) accumulate and ii) be retained for at least 24 h, demonstrating their potential for extended therapeutic efficacy. Furthermore, this confirms that the majority of particles in the brain at 24 h accumulated after 2.5 h from induction of stroke. A more clinically relevant time point for therapy-administration is often considered to be 3 h after induction of stroke.[64] Mice were therefore injected with either vehicle (saline) or Cy5.5-labelled PPS-NP 3 h after onset of reperfusion and sacrificed 7 days later, enabling a longer-term analysis of mouse behavior, NP retention, neuroinflammation, and neuroprotection. Remarkably, ex vivo fluorescence images and their quantification revealed the significant presence of PPS-NPs in the brain ischemic area (left) for at least 7 days, further demonstrating their potential for extended therapeutic efficacy (Figure 3C). Additionally, these data confirm that the accumulation and retention of particles in the brain even after a clinically relevant delay in treatment (3 h) and suggest only a minor difference between injection at 30 min or 3 h post-reperfusion.

2.4. PPS-NP Treatment Reduces Microglial Activation, Neutrophil Infiltration, and Vascular Activation in the Brain 24 h after Cerebral Ischemia

Studies have shown that ischemia-induced excess ROS formation leads to neuroinflammation,[65,66] which is characterized by a rapid activation of resident microglial cells,[67] enhanced expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1)[68] which in turn leads to brain infiltration by neutrophils.[69] Therefore, we investigated whether administration of PPS-NPs affects neuroinflammation 24 h (Figure 4A)
and 7 days (Figure 4B) after experimental stroke. Following the same experimental protocol, we found at 24 h a significant (271-fold) increase in the number of activated microglia (per mm$^2$) in the ipsilateral hemisphere compared to the contralateral hemisphere in vehicle treated mice, confirming MCAo-induced microglial activation. Importantly, PPS-NP-treated mice exhibited a significant (28%) reduction in the number of activated microglia in their ipsilateral hemisphere compared to vehicle treated mice. Similar results were found for % activated microglia (29% reduction Figure 4A3) and mean microglial activation score (23% reduction, Figure 4A4). These results were mirrored in the data at 7 days (injected 3 h post-reperfusion) which displayed a 55% reduction in the number of activated microglia in the ipsilateral hemisphere of the PPS-NP-treated mice with respect to the vehicle control.

Intercellular Adhesion Molecule 1 (ICAM-1) expression in cerebral endothelial cells is stimulated via inflammatory mediators such as IL-1β and TNF-α,[70] and allows for the (firm)
binding of circulating leukocytes such as neutrophils, ultimately permitting their extravasation through the BBB. \[71,72\] In ischemic stroke, once neutrophils and other polymorphonuclear cells have crossed the BBB, they are able to release ROS, myeloperoxidase (catalyzing the formation of highly damaging ROS, hypochlorite) \[73–75\] and proteases (in particularly MMP9), which together potentiate inflammation and degrades extracellular matrix, further destabilizing the BBB and causing significant damage to the surrounding brain tissues. \[34,71\] Indeed, therapies to reduce neutrophil/leukocyte accumulation after stroke, such as ICAM-1 blockades have proven effective therapies for reducing the severity of ischemic stroke. \[76\] In this study, ICAM-1 was therefore quantified via immunohistochemistry which, as expected revealed a significant (fivefold) increase in expression in the ipsilateral hemisphere compared to the contralateral hemisphere in vehicle treated mice, confirming MCAo enhanced ICAM-1 expression (Figure 5A). Notably, PPS-NP-treated mice exhibited a significant (47%) reduction in ICAM-1 expression in their ipsilateral hemisphere compared to ipsilateral hemisphere of vehicle-treated mice. Correspondingly, the number of neutrophils (per mm²) in the ipsilateral hemisphere compared to the contralateral hemisphere was also significantly increased, in vehicle-treated mice (Core; 43-fold, Penumbra; 93-fold) confirming MCAo-induced neutrophil infiltration (Figure 5B). Strikingly, PPS-NP-treated mice exhibited a significant reduction in neutrophil infiltration in the ipsilateral hemisphere compared to the contralateral hemisphere (Core; 43%, Penumbra; 62%) confirming MCAo-induced neutrophil infiltration (Figure 5B). Moreover, we found the PPS-NPs were able to reduce stroke-induced neuronal loss in the ipsilateral lobe from 69% (in the vehicle) to only 41% at 24 h post-stroke (Figure 7D). This corresponds to 48% less neurons in vehicle treated mice with respect to the PPS-NP-treated mice. At 7 days after stroke, we found there was still a 17% reduction in the number of remaining neurons in the ipsilateral hemisphere compared to the contralateral hemisphere in vehicle treated mice; however, there was no significant difference observed in PPS-NP-treated mice, suggesting that they did not exhibit significant

2.5. Intravenous Administration of PPS-NPs Decreases Acute Brain Damage after MCAo

To investigate whether treatment with PPS-NPs influences acute neuronal death after stroke, brain damage was assessed in vehicle and PPS-NP-treated mice (injected with 100 mg kg⁻¹ Cy5.5-NPs) at 24 h after acute ischemic brain injury. Infarct volume (Figure 7A) and BBB damage (Figure 7B) were both significantly reduced (24% and 38% respectively) in PPS-NP-treated compared to vehicle treated mice. Moreover, we found the PPS-NPs were able to reduce stroke-induced neuronal loss in the ipsilateral hemisphere compared to the contralateral hemisphere in vehicle treated mice; however, there was no significant difference observed in PPS-NP-treated mice, suggesting that they did not exhibit significant
Treatment with PPS-NPs decreases brain damage acutely after middle cerebral artery occlusion (MCAo). 24 h after MCAo, a significant decrease is observed in infarct size and blood–brain barrier (BBB) damage between vehicle and PPS-NP-treated mice, as measured by A) Nissl (cresyl violet) staining and B) immunoglobulin G (IgG) infiltration. C) Improvement in the 28-point neuroscore at day 3 after MCAo. Intravenous injection of PPS-NPs is neuroprotective 24 h after MCAo. (D1 and E1) Immunohistochemistry of NeuN (red) positive (+) neurons in ipsilateral or contralateral hemispheres of core region in vehicle or PPS-NP-treated mice as labelled. Scale bar 50 µm. (D2 and E2) Area of NeuN+ neurons in the ipsilateral and contralateral hemispheres of the core region of vehicle and NP treated mice was quantified using Image J software. The area of NeuN+ neurons in the ipsilateral hemisphere was calculated as a percentage of the area of NeuN+ neurons in the corresponding contralateral hemisphere for vehicle and PPS-NP-treated mice. Statistical analyses were performed using (D and E) repeated measures two-way ANOVA followed by Sidak corrected post-hoc analysis (ns p > 0.05, **p ≤ 0.01), and (C) Mann–Whitney test performed per day (***p ≤ 0.05). All data expressed as mean ± SEM (n = 6–7 for A, B, and E, or 10–12 for C and E).

or permanent neuron loss (Figure 7E). Together, these findings suggest that treatment with PPS-NPs reduces acute BBB disruption and neuronal loss after stroke, which is consistent with previous studies showing that antioxidant NPs are neuroprotective and reduce BBB dysfunction after acute ischemic stroke.\cite{18,20,78}

2.6. Delayed Delivery of PPS-NPs is Neuroprotective and Improves Recovery of Neurological Function after MCAo

As shown in Figure 7C, PPS-NP-treated mice displayed a significantly lower (33%) 28-point neuroscore at day 3 after MCAo compared to vehicle treated mice, indicating a significantly
enhanced recovery of neurological function even at this early time point. Collectively, these results indicate that, in terms of neuroprotection and recovery of neurological function, the therapeutic time window for administration of PPS-NPs indeed extends at least up to 3 h. Notably, particles were still present in the brain at 7 days post-stroke, highlighting their potential as a longer term therapy.

3. Conclusion

We have evaluated the therapeutic potential of oxidation-responsive PPS-NPs to limit the ROS-mediated cascade of inflammatory and degenerative events occurring after acute ischemic stroke. In vitro assays show that PPS-NPs are potent antioxidant and effective anti-inflammatory agents, whilst exhibiting negligible cytotoxicity. In our study, we have used LPS to study the ROS-scavenging properties of PPS-NPs on inflammation since groundbreaking study found that LPS stimulation in immune cells drives ROS production leading to a pro-inflammatory state.[79] Intravenously injected PPS-NPs had a favorable blood circulation, taking 24 h to fully clear allowing for significant accumulation in the brain. NP injections acutely, (0.5 h) after onset of reperfusion, are able to quickly accumulate in ischemic brain and reduce infarct volume, BBB damage, and neuronal loss, as well as neuroinflammation, early (24 h) after ischemic brain injury. Crucially, we show that the therapeutic time window for administration of PPS-NPs extends at least up to 3 h, a clinically relevant time window for stroke, with reduced neuroinflammation and neuronal loss 7 days after ischemia; in addition, PPS-NP treatment significantly improved recovery of neurological function. Remarkably, the PPS-NPs are retained in the brain for at least 7 days, indicating their potential for an extended therapeutic action. We propose that our observations strongly support the further development and testing of PPS-NPs as an antioxidant therapy for ischemic stroke.

4. Experimental Section

Materials: All materials were supplied by Sigma-Aldrich unless otherwise specified. Mouse DuoSet ELISA kits and ICAM-1 antibodies were purchased from R&D Systems (UK), Griess Reagent System, ROS-Glo H2O2 Assay, and CytoTox 96 Non-Radioactive Cytotoxicity Assay were purchased from Promega (UK). Prolong mounting agent, white 96-well plates, HBSS, Pierce BCA Protein Assay Kit, CellROX Deep Red Reagent, and all secondary antibodies were purchased from ThermoFisher (UK). Fetal bovine serum; Gibco (UK). Isopentane and DPX mounting medium; Fisher Scientific (UK). Biotinylated anti-mouse IgG antibody and Vectastain Elite ABC HRP Kit; Vector Laboratories (UK). Isosulfane; AbbVie Ltd (UK). EMLA; Astrazeneca (UK). 6-0 monofilament; Doccol (Sharon, MA, USA). Normal donkey serum; Jackson Laboratories (Bar Harbor, ME, USA). Heat blanket; Harvard Apparatus (Edenbridge, Kent, UK). Laser Doppler monitor; Oxford Optronix (Abingdon, UK). IVIS Lumina II system; Caliper Life Sciences (USA). Freezing sled microtome; Bright (Cambridgeshire, UK). Microplate reader; Synergy HT BioTek (UK). FlexStation 3; Molecular Devices (USA) used for cellROX/pharmacokinetic experiments.

Cell Cultures and Animals: Primary cultures of mixed glial cells were prepared from the brain of 0–5-day-old (C57BL/6) mice (Envigo, UK). Mouse neonates were sacrificed via cervical dislocation and the heads were cut into 2 mL pre-warmed mixed glial culture media (MGCM) (DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL−1 penicillin and 100 µg mL−1 streptomycin). Midline incisions were made through the skin and skull, the brain isolated, and the cerebral and olfactory bulbs discarded. The remaining tissue was homogenized in 10 mL of MGCM. The cell suspension was centrifuged at 800 x g for 7 min at room temperature, the supernatant removed, and the pellet resuspended in 10 mL of fresh MGCM. This cell suspension was plated onto 24-well plates and maintained for 5 days. Prior to each treatment, the supernatant was removed and fresh MGCM added. Cells were then grown for 48 h before the first media change, then media was changed every 3–4 days. Cells were grown in 24-well plates at 37 °C, 90% humidity, and 5% CO2 until 70–80% confluent. Cells were detached with 0.5% trypsin-EDTA, counted, and seeded at the appropriate density for each experiment. For in vitro experiments, all incubations were carried out at 37 °C, 90% humidity, and 5% CO2 unless otherwise specified. Mice (male, C57BL/6, Charles River UK) were maintained under standard laboratory conditions: ambient temperatures of 21 °C (±2 °C), humidity of 60–70%, 12 h light cycle, ad libitum access to water and standard rodent chow. All surgeries were performed with the surgeon concealed to the treatment, and all behavioral and histological analyses were performed by a blinded observer. Treatments were randomly allocated. All animal experiments adhered to the ARRIVE[80] and IMPROVE[81] guidelines, and were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986, EU Directive 2010/63/EU for animal experiments and approved by the Home Office and the local Animal Ethical Review Group, University of Manchester.

Mixed Cial Cell Cytokine Release Experiments: On day 13 in vitro, cells were treated for 24 h with LPS (0.5 µg mL−1) in fresh DMEM. The following day, cells were incubated for an additional 24 h with LPS (0.5 µg mL−1) and PPS-NPs (1 mg mL−1) of varying sizes (50–150 nm). Control groups consisted of untreated cells, and LPS stimulated cells with no nanoparticle treatment. Culture supernatants were collected and centrifuged at 13 000 rpm for 5 min and immediately analyzed for cell death (see “Lactate dehydrogenase assay”). Remaining supernatants were analyzed for the presence of cytokines IL-6 and TNF-α (see “Enzyme-linked immunosorbent assay” (ELISA)).

BV2 Cell Cytokine Release Experiments: BV2 cells were seeded at 1.4 × 105 cells per well in 24-well plates and were incubated overnight. The following day, cells were treated for 24 h with LPS (0.5 µg mL−1) in fresh RPMI-1640 before an additional 24 h incubation with LPS (0.5 µg mL−1) and PPS-NPs (50 nm; 0.1, 0.5, 1, 2, and 5 mg mL−1). Control groups consisted of untreated cells, and LPS stimulated cells with no nanoparticle treatment. Culture supernatants were collected, centrifuged at 13 000 rpm for 5 min, and analyzed for the presence of IL-6 and TNF-α by ELISA.

Cell Viability Measurements: MTT assay was used to test the effect of PPS-NPs on cell viability. BV2 cells were seeded at 1 × 104 cells per well in 96-well plates in RPMI-1640 medium and were incubated overnight. The following day, cells were exposed to PPS-NPs (50 nm; 0.5, 1, 2, 4, and 5 mg mL−1) for 24 h. After 24 h incubation, medium was removed, cells were washed twice with PBS and 100 µL of fresh RPMI-1640 (serum free and without phenol red and glutamine) was added to each well. To each well, 10 µL of the AB Solution (MTT) was added and incubated at 37 °C for 4 h. At the end of the incubation period, all but 25 µL of medium was removed from each well and 50 µL of DMSO was added to each well and mixed thoroughly before incubation at 37 °C for 10 min. Within an hour, each well was mixed again, and the absorbance was measured on a microplate reader at 540 nm. To assess cell survival, optical densities were normalized to 100% cell viability control (cells with no nanoparticle treatment).

Hydrogen Peroxide (H2O2) Scavenging In Vivo: Cell free H2O2 scavenging activity of PPS-NPs was analyzed in vitro using ROS-Glo H2O2 Assay according to manufacturer’s instructions. Briefly, treatments (80 µL) were added to white 96-well plates and incubated for 18 h. H2O2 Substrate Solution (20 µL) was added to each well and plates were returned to the incubator for final 6 h of treatment. Next, 100 µL ROS-Glo Detection Solution was added to each well and incubated at room temperature for 20 min. Relative luminescence units were recorded using a microplate reader.
reader. Treatments were as follows: control groups consisted of Milli-Q purified water, or a combination of Milli-Q water and H$_2$O$_2$ (final concentration 15 µM). NP treatments (50 nm; final concentration 1, 3, and 5 mg mL$^{-1}$) were made up in Milli-Q water with H$_2$O$_2$ (final concentration 15 µM).

**Superoxide Scavenging In Vitro**: BV2 cells were seeded at 1 x 10$^5$ cells per well in 24-well plates and were incubated overnight. The following day, cells were washed once with PBS before treatment with menadione (100 µM) and PPS-NPs (0.5, 1, 2, and 4 mg mL$^{-1}$) in HBSS (no phenol red and without calcium and magnesium). Control groups consisted of untreated cells, and menadione (100 µM) stimulated cells with no nanoparticle treatment. Reference samples consisted of cells exposed to a combination of menadione (100 µM) and SOD (40 µg mL$^{-1}$). Cytochrome c (80 µM) and catalase (20 µg mL$^{-1}$) were immediately added into each well and plates were incubated for 90 min. Culture supernatants were collected, centrifuged at 13 000 rpm for 5 min at 4 °C, and transferred into transparent 96-well plates to measure the absorbance on a microplate reader at 550 nm. The reduction of SOD-inhibitable ferricytochrome c was calculated from the change in absorbance at 550 nm against the reference samples, using an extinction coefficient of cytochrome c of 21 nM$^{-1}$ cm$^{-1}$, to give extracellular superoxide production (nmol).

**Nitric Oxide (Measured as Nitrite) In Vitro Assay**: BV2 cells were seeded at 2.5 x 10$^5$ cells per well in 96-well plates in RPMI-1640 medium and were incubated overnight. Media was replaced with phenol-red free DMEM supplemented with high glucose and 5% FBS and cells were left to acclimatize in the incubator for 4 h. Cells were then treated for 48 h in RPMI (1 µg mL$^{-1}$) and PPS-NPs (50 nm; 0.5, 1, 2, and 4 mg mL$^{-1}$). Control groups consisted of untreated cells, and LPS stimulated cells with no nanoparticle treatment. After 48 h of stimulation, culture supernatants were collected and centrifuged at 13 000 rpm for 5 min. Supernatants were transferred into 96-well plates and tested with the Griess reagent according to supplier’s recommendations, measuring absorbance at 550 nm. Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite in culture medium. In addition, protein content in each well was evaluated. Briefly, cells were washed three times with PBS and 0.5% Triton X-100, with protease inhibitor, was added for 10 min at 37 °C to lyse cells. The lysates protein content was measured using Pierce BCA Protein Assay Kit according to manufacturer’s instructions.

**Immunocytochemistry with CellROX Deep Red Reagent for Detecting Oxidative Stress**: BV2 cells seeded at 3 x 10$^4$ cells per glass coverslip in 12-well plates and were incubated overnight. The following day, cells were incubated with or without PPS-NPs (50 nm; 1 µg mL$^{-1}$) in fresh RPMI-1640 for 24 h. For the final hour of incubation, cells were treated with or without menadione (100 µM) at 37 °C. Cells were then stained with CellROX Deep Red (5 µM) by adding the probe to the medium and incubating cells at 37 °C for 30 min. Medium was removed and cells were washed three times with PBS. Cells were then fixed with formaldehyde (4%) for 20 min before washing three times with PBS. Coverslips were left to air dry in the dark overnight before mounting onto slides with ProLong Diamond Antifade Mountant with DAPI. Mounting medium was left to dry overnight. Images were collected on a Zeiss Axiosim.DG upright microscope using a 40x 0.7 Plan-neofluar objective and captured using a Coolscope HQ2 camera (Photometrics) through Micromanager Software v1.4.23. Specific band pass filter sets for DAPI and Cy5 were used to prevent bleed through from one channel to the next. Images were then processed using Image J software.

**Oxidative Stress Quantification in BV2 Cells using CellROX Deep Red Reagent**: BV2 cells were seeded at 5 x 10$^4$ cells per well in black 96-well plates, in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and were incubated overnight. The following day, cells were incubated with or without PPS-NPs (50 nm; 1 µg mL$^{-1}$) for 24 h. For the final hour of incubation, cells were treated with or without menadione (100 µM) at 37 °C. Cells were then stained with CellROX Deep Red Reagent (5 µM) by adding the probe to the medium and incubating cells at 37 °C for 30 min. Medium was removed and cells were washed two times with PBS. 100 µL of fresh PBS was added to each well and fluorescence intensity in wells was measured in a microplate reader with an excitation of 644 nm and an emission of 665 nm. The values derived from the no-cells/no-dye PBS controls were subtracted from each point, in order to correct for background fluorescence.

**Enzyme-Linked Immunosorbent Assay (ELISA)**: The levels of IL-6 and TNF-α were quantified by ELISA using mouse DuoSet kits following the manufacturer’s instructions. For each assay, samples were diluted as measured, and protein levels were calculated according to a four-parameter logistic (4-PL) curve fit.

**Lactate Dehydrogenase Assay**: Cell death of primary mixed glial cells after LPS and PPS-NP treatments was analyzed using a lactate dehydrogenase (LDH) assay kit according to the manufacturer’s instructions. In brief, to assess cell death, supernatants were collected, LDH was measured, and optical densities were normalized to 100% cell death control. An increase in LDH measurements was interpreted as an increase in cell death.

**Pharmacokinetic Study**: Mice (6 weeks old, 22 ± 3 g) were injected via the tail vein with Cy5.5-labeled PPS-NPs (50 nm; 100 mg kg$^{-1}$). At pre-established time points (5 min, 1, 3, 6, 12, and 24 h), blood samples were taken from the tail vein by heparin coated capillary tube into a 1.5 mL heparinized tube. Whole blood was diluted in PBS, transferred to black 96-well plates and fluorescence intensity of Cy5.5-NPs was measured in a microplate reader (excitation: 673 nm, emission: 707 nm). Mice injected with saline (0.9%) were used for background controls, and values shown are background-subtracted averages of three replicates. The samples were quantified (µg mL$^{-1}$) against a standard curve prepared by adding defined amounts of Cy5.5-NPs to solutions of whole blood diluted in PBS. The blood circulation half-life was determined by Exponential Two phase decay analysis, carried out in GraphPad Prism (7.0).

**Cerebral Ischemia Induced by Transient Middle Cerebral Artery occlusion (MCAo)**: Induction of anesthesia was achieved by inhalation of 4% isoflurane (30% oxygen and 70% nitrous oxide gas) and was maintained at 1.75% isoflurane. During surgery, a rectal probe was used to assess body temperature, which was kept at 37 ± 0.5 °C with a heat blanket. A laser Doppler monitor was used to confirm a drop in CBF in the middle cerebral artery (MCA) region following occlusion and subsequent recovery of CBF. Focal cerebral ischemia was achieved by MCAo using a protocol described previously as follows: prior to incision, topical anesthetic (EMLA, 5% prilocaine and lidocaine) was administered on to the areas of skin requiring incision. A laser-Doppler probe located 6 mm lateral and 2 mm posterior from bregma was fixed on to the skull with vetbond. An incision was made into the middle front section of the neck, and the right common carotid artery was exposed and ligated. The internal carotid artery received temporary ligation. An incision in to the common carotid artery was made, followed by the insertion and advancement of a 6-0 monofilament through the internal carotid artery. After 17 min of occlusion (a short occlusion time was used to reduce mortality) the filament was removed allowing reperfusion. The neck wound was sutured and saline (0.5 mL, for rehydration) and buprenorphine (0.05 mg kg$^{-1}$, providing general analgesia) were administered subcutaneously (sc). Animals were allowed to recover in an incubator (26 °C), and then held in ventilated cages placed on a heat pad (24 °C) under standard laboratory conditions with ad libitum access to mashed food and water for 24 h. Finally, cages were returned to their home rack and kept under normal housing conditions. Mice were 8–10 weeks old at the time of surgery (25 ± 5 g).

**PPS-NPs Administration post-MCAo and Tissue Processing**: 30 min or 3 h after filament removal, mice were injected via the tail vein with Cy5.5-labeled PPS-NPs (50 nm; 100 mg kg$^{-1}$) or vehicle (0.9% saline). 2 h, 24 h, or 7 days after injection, animals were anesthetized with 4% isoflurane and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains and peripheral organs (lungs, heart, liver, spleen, and kidneys) were then dissected out, post-fixed in 4% PFA for 24 h, and imaged on an IVIS Lumina II system. Brains were subsequently transferred to 30% sucrose for a further 24 h. Brains were then frozen in isopentane maintained at −30 °C with dry ice, and stored at −80 °C. For immunohistochemistry and Cresyl violet staining, brains were sectioned into 30 µm sections on a freezing sledge microtome. Sections were stored in cryoprotectant solution (30% ethylene glycol, 20% glycerol, 0.66 % sodium phosphate dibasic dehydrate, 0.079 % sodium dihydrogen orthophosphate 1-hydrate, in distilled water (dH$_2$O)) at −20 °C.
Ex Vivo Whole Organ Imaging with IVIS: Collected organs were imaged on an IVIS Lumina II system, following the C57B16 fluorescence protocol. Quantification of fluorescence intensity was performed using the region of interest tool in Living Image software 3.2 (Caliper Life Sciences, USA), expressed as average radiant efficiency \( \text{p}[\mu\text{W/cm}^2]/\text{sr} \). Organs from vehicle treated mice were used to correct for background fluorescence. Images were analyzed with fluorescent efficiency scales, where the value of each pixel represents the fractional ratio of fluorescent emitted photons per incident excitation photon.

Infarct Volume Analysis: Lesion volumes were measured using Cresyl violet staining, as previously described. For each brain, infarcts were incubated with avidin–biotin–peroxidase complex (1:750) for 1 h. Sections triton for 1 h) was blocked, and sections incubated in biotinylated anti-GFAP were rinsed and mounted on glass slides, and dried in the dark at room temperature for 24 h. Slides were then cover slipped with DPX mounting medium and imaged once dry using an Olympus BX51 upright microscope with Cool snap ES camera (Photometrics, UK) for image capture. The total IgG volume for the brain was determined by measuring IgG on individual sections and integrating them to determine total IgG volume, as described earlier for lesion volume.

Immunohistochemistry: Free-floating brain sections were washed three times with phosphate buffered saline (PBS). Endogenous peroxidase activity (0.3% \( \text{H_2O}_2 \) in \( \text{dH}_2\text{O} \) for 10 min) was blocked, and sections were then washed. Non-specific staining (5% normal donkey serum in PB, 0.3% triton for 1 h) was blocked, and sections incubated in biotinylated antimouse IgG antibody (1:500) for 2 h. Sections were then washed and incubated with avidin–biotin–peroxidase complex (1:750) for 1 h. Sections were washed and detected by colorimetry using a diaminobenzidine (DAB) solution (0.05% DAB, 0.005% \( \text{H}_2\text{O}_2 \) in \( \text{dH}_2\text{O} \)). Sections were washed, mounted onto glass slides, and dried in the dark at room temperature for 24 h. Slides were then cover slipped with DPX mounting medium and imaged once dry using an Olympus BX51 upright microscope with Cool snap ES camera (Photometrics, UK) for image capture. The total IgG volume for the brain was determined by measuring IgG on individual sections and integrating them to determine total IgG volume, as described earlier for lesion volume.

Microscopy and Image Analysis: High power field images were collected on an Olympus BX51 upright microscope using a 20x objective (10x for neutrophils) and captured using Cool snap ES camera (photometrics, USA) through MetaVue software ( Molecular Devices, USA). Images were then analyzed with Image J software (National Institutes of Health, USA). For each animal, immunohistochemistry micrographs were analyzed on coronal sections of the same co-ordinates (approximately bregma level 0.84 mm, according to mouse brain atlas, www.mouse.brain-map.org), which represented the middle of the infarcted territory from anterior–posterior perspective. Both ipsilateral and contralateral hemispheres in penumbra and core regions were measured 

28-point Neuroscore Test: Animals were assessed for neurological deficits 1 day before MCAo surgery (baseline), and on days 1, 3, and 7 post-MCAo surgery. Animals were scored 1–4 (increasing value of severity) for seven behaviors as follows: body symmetry (open bench top), gait (open bench top), climbing (gripping surface, 45° angle), circling behavior (open bench top), front limb symmetry (mouse suspended by its tail), compulsory circling (front limbs on bench, rear suspended by tail), whisker response (light touch from behind). The sum of the scores for the seven behaviors was used as the overall score of the motor deficit per mouse. The maximum score is 28, indicating the most severe level of neurological deficit.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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