The effect of lipid nanoparticle PEGylation on neuroinflammatory response in mouse brain

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A B S T R A C T

Nanocarrier-based drug delivery systems have attracted wide interest for the treatment of brain disease. However, neurotoxicity of nanoparticle has limited their therapeutic application. Here we demonstrated that lipid nanoparticles (LNs) accumulated in the brain parenchyma within 3 h of intravenous injection to mice and persisted for more than 24 weeks, coinciding with a dramatic activation of brain microglia. Morphological characteristic of microglial activation also observed in LNs-treated Cx3cr1GFP / lo / mice. In vivo study with two-photon confocal microscopy revealed abnormal Ca 2+ waves in microglia following LNs injection. The correlated activation of caspase-1, IL-1β and neurovascular damage following LNs injection was attenuated in P2X 7 / lo / mice. PEGylation of LNs reduced correlated nanoparticles aggregation. Moreover, PEGylation of LNs ameliorated the P2X 7 / caspase-1 / IL-1β signalling-dependent microglia activation and neurovascular damage. In conclusion, PEGylation of LNs is a promising biomaterial for brain-targeted therapy that inhibits P2X7-dependent neuroinflammatory response.

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1. Introduction

There is increasing evidence that nanomedicine will bring significant advances in the development of therapeutic modalities [1–3]. For instance, nanoparticles have been shown to represent promising drug delivery systems for brain disease treatment [4–6]. However, the host immune response as well as neurotoxicity of nanoparticle is an impediment to clinical translation. Till now, the in vivo neuroscience evidences regarding stability and safety of nanoparticles is still lacking [7–9].

Drug delivery to the brain using nanoparticles via crossing the blood–brain barrier (BBB) may provide a brain disease treatment strategy [10]. The enhanced retention in the brain capillaries, opening of tight junctions and endocytosis mechanisms have been reported to improve the BBB passage of nanoparticles [10,11]. However, inappropriate BBB permeability has been suggested to be a cause of nanoparticle toxicity in neurovascular brain components [11,12]. For example, exposure to TiO 2 nanoparticles leads to impairment of spatial recognition memory by disturbing mouse brain enzymes and neurotransmitter systems [11]. Lipid nanoparticles (LNs) are tiny colloidal carriers composed of a biodegradable lipid matrix that have advantages over other colloidal carriers for brain targeting [10,13,14]. Therefore, performing an in vivo biokinetic evaluation of the biological behaviour and brain toxicity of LNs is important [12,15,16], because lack of knowledge regarding how these particles affect normal cellular processes within the brain has limited their use.

Microglia are the primary immune sentinels of the brain and respond rapidly to brain risk factors and strictly controlled events [17,18]. Ferric oxide nanoparticle exposure triggers microglial activation, recruitment and phagocytosis [19]. Microglial activation...
may act as an alarm as well as defence system when exogenous nanomaterials invade, aggregate and are deposited in the brain. However, the question how microglia elicits neuroinflammatory responses during nanoparticle-mediated neurotoxic injury is a currently unexplored research problem.

The present study aimed to evaluate LNs as a brain drug delivery system by assessing their safety in vivo. The impact of the LNs on neurovascular components of brain and its potential molecular mechanisms were analysed in mice. This study will provide in vivo neuroscience evidences on development of appropriate nanocarriers for brain-targeted drug delivery systems that exhibit high efficacy and less neurotoxicity.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise noted.
2.2. Preparation of fluorescence labelled LNs and PEGylation of LNs

Octadecylamine and rhodamine B isothiocyanate (RITC) chemical conjugate (ODA-RITC) were first synthesised for LNs labelling [20]. Briefly, 25.14 mg octadecylamine (Fluka chemie AG, Switzerland) and 50 mg rhodamine B isothiocyanate (Amresco, USA) were dissolved in 6 ml ethanol. The reaction was conducted at 50°C for 24 h protected from light. After the reaction was complete, the reaction mixture was cooled to room temperature, and 50 ml distilled water was added to precipitate the ODA-RITC. The precipitate was collected via filtration through a 0.45 μm filter (Millipore) and washed twice with 20 ml distilled water. The final product was lyophilised and stored in the dark for further use. RITC labelled LNs were then prepared using the solvent diffusion method in an aqueous system, as reported in a previous study by our group [20]. Briefly, 15 mg monostearin (Shanghai Chemical Reagent Co. Ltd., China), 15 mg medium chain triglyceride (Shanghai Chemical Reagent Co. Ltd., China) and 1.5 mg ODA-RITC were completely dissolved in 1 ml ethanol in 70°C water bath. The resultant organic solution was quickly dispersed in 9 ml volume of an aqueous phase containing 0.1 wt% polysorbate 80 (Shanghai Shenyu Pharmaceutical & Chemical Co. LTD, Shanghai, China) under mechanical agitation (DC-40, Hangzhou Engineering Instruments, China) at 400 rpm 5 min at room temperature to obtain RITC-

Fig. 2. Lipid nanoparticle accumulation induced microglia activation in mouse brain. (A) Temporal distribution of rhodamine-labelled LNs after injection. Fluorescent staining of rhodamine-labelled LNs (red) and NeuN (green) were performed 24 h after LNs injection in mice. Scale bar = 20 μm. (B) Rhodamine-labelled LNs (red) and GFAP (green) double staining was performed 24 h after LNs injection. Scale bar = 20 μm. (C) The immunohistochemical localisation of rhodamine-labelled LNs (red) and ED1 (green) was examined 24 h after LNs injection. Scale bar = 20 μm. The results obtained by quantitative analysis of ED1-positive staining in (C) are summarized in (D). The data are presented as box-and-whiskers plots. ***P < 0.001 versus control (n = 6 slices from 5 animals for each group). (E) Lipid nanoparticles initiated microglia-mediated phagocytosis in the mouse brain cortex. Representative immunostaining data demonstrating microglia-mediated phagocytosis in the mouse brain cortex. Scale bar = 10 μm. DAPI counterstaining indicates cell nuclei (blue). (F) Representative immunostaining data showing a majority of the LNs localized outside brain microvessels in the Tie2-GFP mouse cortex. White arrows indicate the Tie2-GFP-positive microvessels. Scale bar = 50 μm. (G) LNs did not affect brain microvessel density supported by representative Z-stack image of microvessels in cortical regions 168 h after LNs injection in Tie2-GFP mice. Scale bar = 50 μm. LNs, lipid nanoparticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
labelled LNs with a 3 mg/ml concentration. The polyethylene glycol (PEG) coating of LNs (PLNs) were prepared using our previously reported methods [21]. Briefly, 3 mg polyethylene glycol monostearate with an ethylene glycol polymerisation degree of 40 (Tokyo Kasei Kogyo Co., LTD., Japan) was used instead of 1.5 mg monostearin and 1.5 mg medium chain triglyceride. To prepare near infrared DiR-labelled LNs or PLNs, 10 μL (1.0 mg/ml) DiR dimethyl sulfoxide solution was used instead of 1.5 mg ODA-RITC.

2.3. Characterization of the size, morphology and zeta potential for lipid nanoparticles

The particle size and zeta potential for all of nanoparticles in dispersion were determined with a Zetasizer (3000HS, Malvern Instruments, UK) after the prepared dispersion performed 100-fold dilutions with distilled water. The particle sizes and surface morphologies of the nanoparticles were further observed via transmission electronic microscopy (TEM) (JEM-1230, JEOL, Japan). The samples were placed on copper grids and stained with 2% (w/v) phosphotungstic acid for viewing.

2.4. In vivo nanoparticles imaging

The near-infrared DiR fluorescent probe (Molecular Probes Inc. OR, USA) was used to investigate the distribution of labelled unmodified LNs and PLNs through in vivo fluorescent imaging using nude mice as an animal model. The mice were injected with 0.2 ml of DiR-labelled unmodified or PLNs via the tail vein. The mice were then anesthetised through diethyl ether inhalation and placed in a chamber, and fluorescent images were obtained using the in vivo imaging system (Maestro, CRl, Inc.). Fluorescent images of treated mice were acquired with a near-infrared filter set (the excitation wavelength was 684–729 nm, and the emission filter was 745 nm long pass) using an exposure time of 500 ms. The software acquired multispectral image cubes in 10 nm steps within a spectral range of 740–950 nm. For ex vivo analysis, the mice were sacrificed 24 h post-injection, and their organs were excised. Fluorescent ex vivo images of the organs were obtained using the same imaging procedure as applied for the in vivo measurements. The real-time distribution of DiR within the organs was evaluated based on the signal-to-noise ratio.

2.5. Mouse strains

Homozygous P2RX7/− mice (B6.129P2-P2rx7tm1Gab/J), B6.Cg-Tg (Thy1-YFP)2Jrs/J and Tie2-GFP (STOCK Tg [Tie2GFP] 2875Sad/J, Bar Harbor, Me), were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Cx3cr1GFP− transgenic mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used to visualise ramified microglia. Additionally, adult male C57BL/6J wild-type mice were used in the present study. The rhodamine (control) and rhodamine-labelled LNs or PLNs (30 μg per g body weight) was intravenous administered in mice. All mice were housed under a 12-h light/dark cycle and had access to food and water ad libitum. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the care and use of laboratory animals and were approved by The Committees for Animal Experiments at Zhejiang University in China.

2.6. Immunohistochemical labelling, image acquisition and analysis

Animals were anesthetised and trancardially perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). For immunohistochemical labelling, 35 μm-thick coronal brain sections were cut anterior or posterior to the bregma using a vibrating microslicer (VT1000S; Leica, Wetzlar, Germany). The brain sections were then incubated at room temperature with 0.01% Triton-X-100 in PBS for 30 min, followed by incubation in 3% bovine serum albumin (BSA) in PBS for 1 h. To perform immunohistochemical labelling, slices from the experimental animal brain sections were incubated with antibodies against ED1 and NeuN (Chemicon, Temecula, CA, USA) or GFAP (Sigma Chemical, St Louis, MO) overnight at 4°C. The brain sections were placed on VectaShield (Vector Labs) on glass slides. Immunofluorescence was visualised using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). Tie2-GFP mice were employed to analyse the capillary density in the brain cortex, with or without LNs injection. Morphological parameters of Cx3cr1GFP+ transgenic mice defined to characterize the nanoparticles-induced microglia morphology change: Filaments total length (μm), Mean diameter (μm3) and Soma volume (μm3). The relative fluorescence intensity of rhodamine-labelled nanoparticles and ED1 staining in cortex was quantified by using the software package MATLAB R2011b.

![Fig. 3. Characteristics of PEGylated lipid Nanoparticles. Particle size (A), zeta potential (B) and transmission electronic microscopy (TEM) image (C) of PEGylated LNs. (D) In vivo fluorescence images of nude mice 15 min after injection with near-infrared DiR-labelled LNs (left) and PEGylated LNs (right). (E) Optical imaging of tissues in nude mice 24 h after LNs injection (above) and PEGylated LNs injection (below). LNs, lipid nanoparticles; PLNs, PEGylated lipid nanoparticles.](image-url)
The 3D filled plots were processed by using ImageJ v1.45 with the accompanied 'Interactive 3D Surface Plot' plug-in. The statistical and plotting software package Graphpad Prism v5.0 (GraphPad Software, Inc., USA) was used to perform one-way ANOVA and t-test.

2.7. Two-photon calcium fluorescence imaging

In vivo fluorescent dye loading and Ca\(^{2+}\) imaging were performed through a small open cranial window by using C57Bl/6J wild-type mice [22]. The membrane-permeant Ca\(^{2+}\) indicator Oregon Green 488 BAPTA-1 AM (OGB-1 AM) was used as a quantitative measure of the steady-state intracellular Ca\(^{2+}\) concentration [22].

OGB-1 AM (0.8 mM, Invitrogen) was dissolved in DMSO with 20% pluronic acid (F-127, Sigma) and mixed in artificial CSF containing sulforhodamine 101 (SR101, 200 μM, Invitrogen). A standard patch-clamp pipette was filled with OGB-1 AM and SR101 solution and inserted into the cortex at a depth of 200–250 μm below the pial surface. OGB-1 AM and SR101 were pressure ejected from the pipette for 60–120 s. Finally, a cover slip was fixed to the skull above the cranial window. Calcium fluorescence imaging was started 1 h after successful dye loading to allow for proper staining. Calcium fluorescence was imaged in cortical cells with a two-photon Olympus microscope (Fluoview FV1000) using a Mai Tai DeepSee tunable ultrashort laser (800 nm, Spectra Physics) [22,23]. The excitation light was focused using a 25× water-immersion objective lens (1.05 numerical apertures).

Fig. 4. Effect of PEGylated lipid nanoparticles on brain microglia activation. (A) The immunohistochemical localisation of rhodamine-labelled LNs (red) and ED1 (green) was examined 24 h after the injection of mice with LNs and PEGylated LNs. Scale bar = 20 μm. (B) Representative immunostaining demonstrating that PEGylated LNs have less of an effect on microglial activation compared with unmodified LNs injected into Cx3cr1\(^{GFP}\) transgenic mice. Scale bar = 40 μm (C–E) Quantitative analysis of morphological changes in microglia 24 h after the injection with LNs and PEGylated LNs. (C) The Filaments total length (μm), (D) Mean diameter (μm) and (E) Soma volume (μm\(^3\)) were measured in Cx3cr1\(^{GFP}\)- transgenic mice (\(n = 5\)). **P < 0.001 versus controls; *P < 0.05, ***P < 0.001 versus LNs. LNs, lipid nanoparticles; PLNs, PEGylated lipid nanoparticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.8. Western blotting

Proteins were extracted, and samples containing equivalent amounts of protein were loaded onto 10–15% gels for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were blotted using the following primary antibodies: spectrin, MMP-9, Phospho-GluR1 (Ser831) (Millipore, Billerica, MA, USA); P2X5, P2X4, II-1β, caspase-1 and TIMP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HtrA2 (R&D Systems, Wiesbaden, Germany); Occludin and Claudin-5 (Invitrogen, San Diego, CA); Phospho-Thr286-CaMKII [24], Phospho-Ser603-Synapsin1 (Thermo scientific) and β-actin (Sigma Chemical, St. Louis, MO). Immunoreactive proteins were visualised using an enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK).

2.9. Statistical analysis

The data are presented as the mean ± S.E.M. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey’s test for multigroup comparisons and the unpaired Student’s t-test for two group comparison. P < 0.05 indicated statistically significant differences.

3. Results

3.1. Biodistribution of LNs in mouse brain

The most challenging aspect of research in the field of nanomedicine is the difficulty of performing in vivo nanoparticle measurements, tracing and observation in brain [25]. To investigate the brain distribution of LNs, rhodamine-labelled LNs and THY1-YFP transgenic mice were used (Supplementary Fig. S1). The overall cortical brain LNs distribution pattern is shown in Fig. 1A. LNs preferentially localised to cortical pyramidal layers II/III and IV/V (Fig. 1A). THY1-YFP-positive dendritic processes extended into the hippocampus and showed a high immunoreactivity, whereas LNs preferentially distributed to CA1 and CA3 pyramidal cell layers (Supplementary Fig. S2). Stress causes axonal/dendritic brain morphology changes, especially in the hippocampus, which is particularly vulnerable to stress because of its high glucocorticoid receptor density [26]. The accumulation of rhodamine-labelled LNs can be observed 3 h after injection and further increased in brain in a time dependent way (Fig. 1B). As shown in Fig. 1B and C, a significant elevation in rhodamine positivity was observed 3 h after LNs injection, peaked at 24 h and remained increased for even 168 h.

3.2. The glial responses after LNs injection in mice

LNs are mainly composed of biodegradable lipids and present high in vivo tolerability [27]. It was reported that intravenously injected LNs are rapidly cleared from systemic circulation by opsonisation [28]. However, we are concerned about LNs neurotoxicity because we observed abundant LNs brain aggregation (Fig. 1). To identify whether LNs injection induced morphological changes in different cell types, we conducted immunostaining for cell-specific markers to examine cellular responses after LNs injection. First, no changes were observed in neuronal morphology or numbers based on NeuN immunostaining (Fig. 2A). Consistent
with a previous study [29], we demonstrated that these nanoparticles appear to localise intracellularly within a specific neuronal cytosolic compartment and that they were absent from the cell nucleus (Fig. 2A). In addition, the LNs did not induce astrocyte activation until at least 24 h after injection, as assessed by changes in the number and morphology of GFAP-expressing astrocytes (Fig. 2B).

We subsequently addressed the impact of the LNs on microglial activation. ED1 immunoreactivity significantly increased in LNs-injected mouse brains compared with control animal brains, indicating that LNs injection induces microglial activation. Indeed, we observed longitudinal changes in microglial activation in response to LNs, which was apparent as early as 3 h after injection and led to significant morphological changes 24 h after LNs injection (Fig. 2C and D). Moreover, 168 h after LNs injection, microglial activation was characterised by phagocytosis process of microglia (Fig. 2E).

The neurovascular network of the adult brain is relatively stable, and vascular endothelial cells exist in a differentiated, quiescent state and rarely proliferate. Brain injury reportedly induces angiogenesis or brain repair with increased microvessel density [30,31]. However, little is known about the pathophysiological changes that occur in microvessels after LNs injection. In the present study, we used Tie2-GFP mice to obtain Tie2+ microvessels after LNs injection. The collected data demonstrated that a majority of LNs localised within the brain parenchyma, and only a few remained within the microvasculature (Fig. 2F). Furthermore, capillary diameter analysis revealed no differences between control or LNs treatments in Tie2-GFP mouse cortex for at least 168 h after LNs injection (Fig. 2G), suggesting that brain microvascular remodelling is not involved in the present context.

3.3. Effect of LNs PEGylation on neuroinflammatory responses

Modification of the surfaces of delivery vehicles to be used as drug carriers with PEG has shown potential for improving the stability and in vivo performance of various drug and gene vectors [32,33]. The rhodamine-labelled PLNs prepared by the present method were 27.80 ± 4.31 nm in diameter and had a -12.1 ± 1.6 mV zeta potential (Fig. 3A–C and Supplementary Table S1). There were no obvious changes in particle size after LNs PEGylation; however, the absolute zeta potential value was decreased compared with unmodified LNs (Fig. 3A–C and Supplementary Table S1). Ex vivo fluorescence images of tissues demonstrated that the liver was the major organ responsible for the clearance of PEGylated and unmodified LNs (Fig. 3D, E). Notably, we demonstrated that the LNs distribution in the brain was significantly enhanced by PEGylation (Fig. 3E), whereas the absolute surface charge values of the PEG-modified nanoparticles were lower than those of the unmodified LNs (Supplementary Table S1).

We further examined the effect of PEGylated LNs on brain inflammatory responses compared with unmodified LNs. As shown in Fig. 4A, less ED1-positive staining was observed in mice after injection with PEGylated LNs compared with unmodified LNs-treated mice (Fig. 4A), indicating that the PEGylated LNs did not significantly induce microglial activation in the mouse brain.

We next sought to determine whether LNs triggered adverse effects on normal cellular processes within neurovascular units, such as neuronal or microvascular damage or detrimental biochemical responses. First, the 150 kDa spectrin breakdown product was examined by immunoblotting, which is indicative of the degree of neurovascular damage following LNs injection. A significant increase in the level of the spectrin breakdown product was observed 3 h after LNs injection and was observed for at least 168 h (Fig. 5A). LNs induced an alteration of BBB restrictive characteristics and a complimentary response of Oculclin absent expression in mouse cortex (Fig. 5A), which are critical prerequisites for brain physiology [35]. Matrix metalloproteinase-9 (MMP-9) is upregulated in focal cerebral ischaemia and involved in BBB breakdown during disease processes [36]. Here, we confirmed that LNs injection induced significant MMP-9 activation in the mouse cortex (Fig. 5A), offering further evidence that LNs have deleterious effects on BBB integrity. In contrast, our data demonstrated that PEGylated LNs have less detrimental effects on neurovascular units (Fig. 5A). Nanoparticles can induce enhanced lipid peroxidation and inflammatory reactions in rat brains, which could be a major toxicological paradigm for engineered nanoparticles [37,38]. Mitochondria are likely to be sensitive to nanoparticle cytotoxicity [39]; therefore, we further verified the subcellular high temperature requirement protein A2 (HtrA2) distribution following LNs or PEGylated LNs injection. The continued releasing of HtrA2 from the mitochondrial intermembrane space into the cytosol is a sensitive indicator of mitochondrial dysfunction [40,41]. Notably, cytosolic HtrA2 was elevated in the LNs group, whereas the HtrA2 content in the mitochondrial fractions was significantly decreased (Fig. 5B). Notably, there was no significant shift of endogenous HtrA2 from the mitochondria to the cytosol after the administration of PEGylated LNs. Thus, unmodified LNs induced significant lipid peroxidation and oxidative stress in the brain. Moreover, 24 weeks after LNs injection, microglial activation...
was characterised by transformation from a highly ramified to an amoeboid morphology (Fig. 5C, Supplementary Video 4). Importantly, PEGylation relieves LN-induced morphological changes in the microglia (Fig. 5C) and neurovascular damage (Fig. 5D) until 24 weeks in mouse brain after intravenous injection.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2013.07.009.

3.5. Molecular mechanisms of LN-induced neuroinflammatory response

Although several prior studies have revealed that rapid microglial activation plays a crucial role in mediating brain responses [18,42], we know little about how microglia are activated during LN-mediated neurovascular injury. Disturbance of ionotropic P2X7 signals may be relevant to risk factor-induced brain inflammation, as suggested by recent observations of microglial activation, movement and paracrine signalling in brain injury animal models [18,43,44]. Therefore, we next ascertained whether the observed LN injection-induced microglial activation occurred in conjunction with P2X7 receptor-dependent signalling. Notably, we observed a dramatic and continuous increase in P2X7 immunoreactivity 3 h and 24 h after LN injection, which remained elevated for at least 168 h (Fig. 6A). To further define how P2X7 modulates these events, we next addressed whether P2X7 activation induces downstream signalling and releases proinflammatory mediators. Consistent with the overproduction and release of the mature, bioactive form of IL-1β following LN injection, we detected a significant elevation of caspase-1 expression, indicating a P2X7/caspase-1/IL-1β inflammatory signalling response (Fig. 6A). As shown in Fig. 6A, in contrast to the significant elevation of P2X7, caspase-1 and IL-1β protein levels observed 168 h after the injection of unmodified LNs, no significant inflammatory responses were observed following PEGylated LN administration. Thus, in contrast to unmodified LNs, PEGylated LNs appear to have fewer effects on microglial activation and present reduced detrimental effects on the neurovascular unit.

To further evaluate intracellular LN neurotoxicity, we extended our work by determining whether changes in calcium homeostasis are related to LN injection, as basal [Ca^{2+}]{i} increases are central to the regulation of the executive functions of activated microglia [45]. To address these possibilities, two-photon fluorescent lifetime imaging microscopy was used to measure the dynamic changes in calcium homeostasis immediately after LN injection in living mice (Fig. 6B, Supplementary Video 5). We used OGB-1 AM, a functional calcium indicator, to test the LNs-induced functional changes in neurons and astrocytes [22]. In saline-treated mice, the calcium waves remained stable throughout the observation period (Fig. 6B). It has been hypothesised that a glial calcium wave propagates between neighbouring cells, which is a major factor mediating intercellular communication between neurons and glial cells [45].

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To provide genetic evidence supporting the role of P2X7 in LN-mediated neurotoxicity, we used P2X7−/− mice. Less caspase-1 and IL-1β induction in the brain was observed 168 h after LN injection in P2X7−/− mice compared with WT (wild-type) mice (Fig. 6C). Our data demonstrated that P2X4 protein levels were not perturbed after LN injection and indicated that P2X4 was not essential for microglial activation (Fig. 6C). In addition, no significant MMP-9 activation of tight junction protein changes was observed in P2X7−/− mice 168 h after LN injection compared with wild-type controls, indicating that animals lacking P2X7 signalling were resistant to LN-mediated neurovascular injury (Fig. 6D). We also examined potential changes in the expression levels of phospho-CaMKII-Thr286 (the active form of CaMKIIz) following LN injection in cortical brain extracts. Decreases in CaMKIIz (Thr286), Synapsin1 (Ser603) and GluR1 (Ser831) phosphorylation were observed in WT mice following LN injection, whereas the levels remained relatively stable in P2X7−/− mice (Supplementary Fig. S3). These data, coupled with our microglia-specific immunohistochemical data, indicate that P2X7/caspase-1/IL-1β intracellular signalling in microglia is correlated with the severity of LN-mediated neurotoxicity (Fig. 6F).

4. Discussion

Development of appropriate nanocarriers for brain-targeted drug delivery systems that exhibit less neurotoxicity may provide therapeutic modalities for brain diseases. In this study, we demonstrated an unexpected neurotoxicity of LNs in mice brain, which accumulated in the brain parenchyma as soon as 3 h after intravenous injection were observed at least until 168 h post-injection. Moreover, LN aggregation triggers microglial-mediated neuroinflammation and neurovascular injury in the mouse brain via P2X7/caspase-1/IL-1β-dependent mechanisms. Importantly, PEGylation of LNs reduced brain aggregation of nanoparticles and microglia-mediated neuroinflammatory responses; accordingly, PEGylation of LNs attenuated neurovascular damage compared with uncoated LNs in the mouse brain. Overall, these data suggest that PEGylated LNs have great promise for the development of applications to treat human brain disease.

Microglial cell activation is a rapidly occurring cellular response to exogenous stimulation [17,18,42]. Here, we found that LN aggregation triggers microglial-mediated neuroinflammation in the mouse brain, although the LNs used in these experiments are generally considered to be non-toxic [46]. In light of the proposed roles for nanoparticle aggregation [47], we were concerned about nanoparticles agglomeration and aggregation after LNs injection in the brain. This biomolecular layer adsorbs to nanoparticle surfaces immediately upon contact with a biological environment [47].

The basal [Ca^{2+}]{i} increases are central to the regulation of the executive functions of activated microglia [45]. Interestingly, our time-lapse imaging revealed that intravenous LN injection induced rapid calcium waves in mouse astrocytes and neurons. Here, we suggest that local neuron-astrocyte networks are rapidly perturbed and subsequently contribute to LN injection-mediated microglial activation. This theory is supported by more recent evidence demonstrating that neuron-astrocyte calcium waves might be capable of triggering microglial responses [48].

Disturbance of ionotropic P2X7 signals may be relevant to risk factor-induced brain inflammation [18,43,44]. Correlated with the overproduction of caspase-1 and release of the bioactive form of IL-1β following LN injection, we detected a dramatic and continuous increase in P2X7 immunoreactivity 3 h and 24 h after LN injection, indicating a P2X7/caspase-1/IL-1β inflammatory signalling response. P2X7−/− mice exhibited markedly reduced neuro-inflammatory responses following LN injection. In addition, no significant tight junction protein and cognition-related protein changes were observed in P2X7−/− mice 168 h after LN injection compared with wild-type controls, indicating that animals lacking P2X7 signalling reduced vulnerability to LN-induced neurovascular damage.

A complete understanding of the mechanisms of interaction between nanoparticles and target cells that may lead to changes in the local brain environment is required [49,50]. Our present data
demonstrated that LNs induced complex cellular and molecular events in the brain, including early disturbances in calcium waves, oxidative stress, tight-junction protein degradation and subsequently neuronal damage, during which microglial activation participates in the LNs-mediated inflammatory response. Our data describe previously unknown mechanisms of LNs-induced neurotoxicity at the acute and subacute stages of the LNs-induced aberrant P2X7/caspase-1/IL-1β signalling associated with LNs-induced inflammatory response. Improvements in our understanding of these P2X7-dependent inflammatory mechanisms could lead to therapeutic strategies aimed at preventing or minimising LNs-induced neurotoxicity.

Based on our observation, the agglomeration and aggregation of LNs would be sufficient to induce inflammatory responses, therefore indicating that they are not suitable for application as an in vivo drug carrier. Hence, the optimisation of nanoparticle characteristics should balance brain entry and potential neurotoxicity. PEG surface modification resulted in a prolonged circulation time due to inhibiting nonspecific protein adsorption, opsonisation and phagocytosis by the mononuclear phagocytic system [51,52]. By using the biochemical and OX42+1/CD11b+ transgenic approach, our present study shown that PEGylated LNs have fewer effects on microglial activation and present reduced detrimental effects on the neurovascular unit. Thus, PEGylated LNs effectively improve BBB transport rates, possibly by reducing non-specific adhesion to cytoskeletal elements, thus leading to lower levels of brain agglomeration and aggregation [20].

With respect to the reduced inflammatory responses in the brain caused by PEG-modified nanoparticles, there are several mechanisms that could explain the biocompatibility of PEGylated LNs and why they do not disrupt major cellular functions of neurovascular components. PEG-modified nanoparticles and unmodified LNs exhibit similar brain distribution profiles, but LNs tended to aggregate in the brain, despite showing stable physicochemical properties in vitro (Supplementary Table S1). The reduced brain aggregation of PEGylated LNs may be due to their hydrophilicity, surface energy or protein affinity [30]. Here, we demonstrated that the absolute value of the PEG-modified nanoparticle surface charge was lower than that of the unmodified LNs (Supplementary Table S1). Indeed, PEG polymers reportedly modify the nanoparticle surface to create a steric barrier, hence impeding protein adsorption and decreasing complement system activation as well as macrophage uptake [53].

5. Conclusion

Our data describe previously unknown mechanisms of LN-induced neuroinflammatory response and aberrant P2X7/caspase-1/IL-1β signalling associated with LN-induced microglial activation and neurovascular injury. In this study, we demonstrated the benefits of modification of the LNs surface with PEG over microglia-mediated inflammatory responses and neurovascular damage. Thus, we suggest that the PEG-modified LNs represent a promising material for the preparation of a potential drug delivery system for brain-targeted disease therapy due to their enhanced biocompatibility compared with unmodified LNs. Because efficient BBB penetration and brain distribution are important prerequisites for brain targeting, our findings are significant for the optimisation of nanomedicines to effectively enter the brain and relieve potential neurotoxicity.

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Appendix A. Supplementary data

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