Using a Förster-resonance energy transfer (FRET)-based detection system (FedEcs) to monitor nanoparticle cargo delivery to the brain

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Abstract

Nanotechnology holds great promise to improve delivery of therapeutics to the brain. Current experimental approaches are, however, hampered by the lack of tools to dynamically monitor cargo delivery *in vivo*. We developed highly fluorescent lipid nanodroplets (LNDs) that carry a Förster-resonance energy transfer (FRET)-based drug delivery detection system able to monitor cargo release (FedEcs) *in vivo*. We investigated the distribution, stability, and cargo release of FedEcs-LNDs in the healthy and ischemic mouse brain by intravital multiphoton microscopy. We dynamically observed that LNDs accumulated within cerebral microclots after ischemia, caused by magnetic nanoparticles (Nano-stroke), and released their cargo. Furthermore, the blood-brain barrier (BBB) became permeable at sites of microclots thereby allowing FedEcs-LNDs to cross the BBB and to deliver their cargo to the brain parenchyma. Consequently, FedEcs represents a novel tool to quantitatively investigate the nanocarriers biodistribution and cargo release using intravital microscopy and may thus tremendously ease their translational validation.

Introduction

Nanotechnology is currently revolutionizing the delivery of therapeutics, however, nanomedicines for the treatment of brain diseases are sparse. Among existing nanoscale platforms, lipid nanodroplets (LNDs) hold particular promise[1]. LNDs have a lipid membrane and a liquid core thereby mimicking low-dense lipoproteins. Their liquid core is an attractive reservoir for encapsulation of drugs and contrast agents[1] and their lipid shell is biocompatible and therefore regarded as safe for the application in patients (GRAS)[2-4]. LNDs are characterized by a long circulation time in blood, which make them attractive for targeting tissues[5]. In particular, previous studies showed that LNDs remained intact in the blood circulation for multiple hours after injection and accumulated in tumours in nearly intact form[5].

Despite these advantageous properties, investigating the biodistribution of LNDs and other NPs in the brain remains a fundamental challenge because current detection modalities are not well suited to detect whether NPs cross the blood-brain barrier (BBB), enter the brain parenchyma, and release their cargo. To overcome this issue, we developed highly fluorescent LNDs which are detectable *in vivo* and carry a Förster-resonance energy transfer (FRET)-based drug delivery detection system (FedEcs) which allows the visualization of cargo release *in vivo*. Using *in vivo* 2-photon microscopy, correlative light-electron microscopy, and a mouse model of cerebral ischemia based on magnetic nanoparticles (Nano-stroke), we observed that 30 nm FedEcs-LNDs accumulate in microthrombi forming in brain capillaries after a stroke, cross the BBB, and release their cargo at both locations.

Results

Development of highly fluorescent lipid nanodroplets (LNDs) carrying a Förster-resonance energy transfer (FRET)-based cargo delivery detection system (FedEcs)

We sought to design a highly fluorescent nanodroplets which harnesses FRET to dynamically assess the structural integrity of the particles and the release of their encapsulated cargo. To that end, we formulated LNDs from LabrafacWL and Cremophor ELP® by a spontaneous nanoemulsification[6] and loaded them with two fluorophores which undergo FRET. The fluorescent dye F888, which was specially designed for high loading into LNDs and high fluorescence intensity following 2-photon excitation[7], was selected as FRET donor and the Cy3 derivative DiI was selected as FRET acceptor. DiI was coupled to tetraphenylborate (TPB) to ensure efficient encapsulation inside LNDs with minimal dye leakage[8] (**Figure 1A; Suppl. Figure S1 A**). Upon degradation of the LNDs and/or cargo release, the distance between the donor and acceptor dyes increases thereby leading to the loss of FRET and a subsequent reduction of the ratio between donor and acceptor fluorescence intensity (**Figure 1B**)[5]. This property of the LNDs should now function as a FRET-based cargo delivery detection system (FedEcs). In contrast to previously reported FRET-LNDs operating in NIR region for whole mice imaging[5], here we propose FRET-LNDs specially designed for 2-photon intravital imaging in capillary

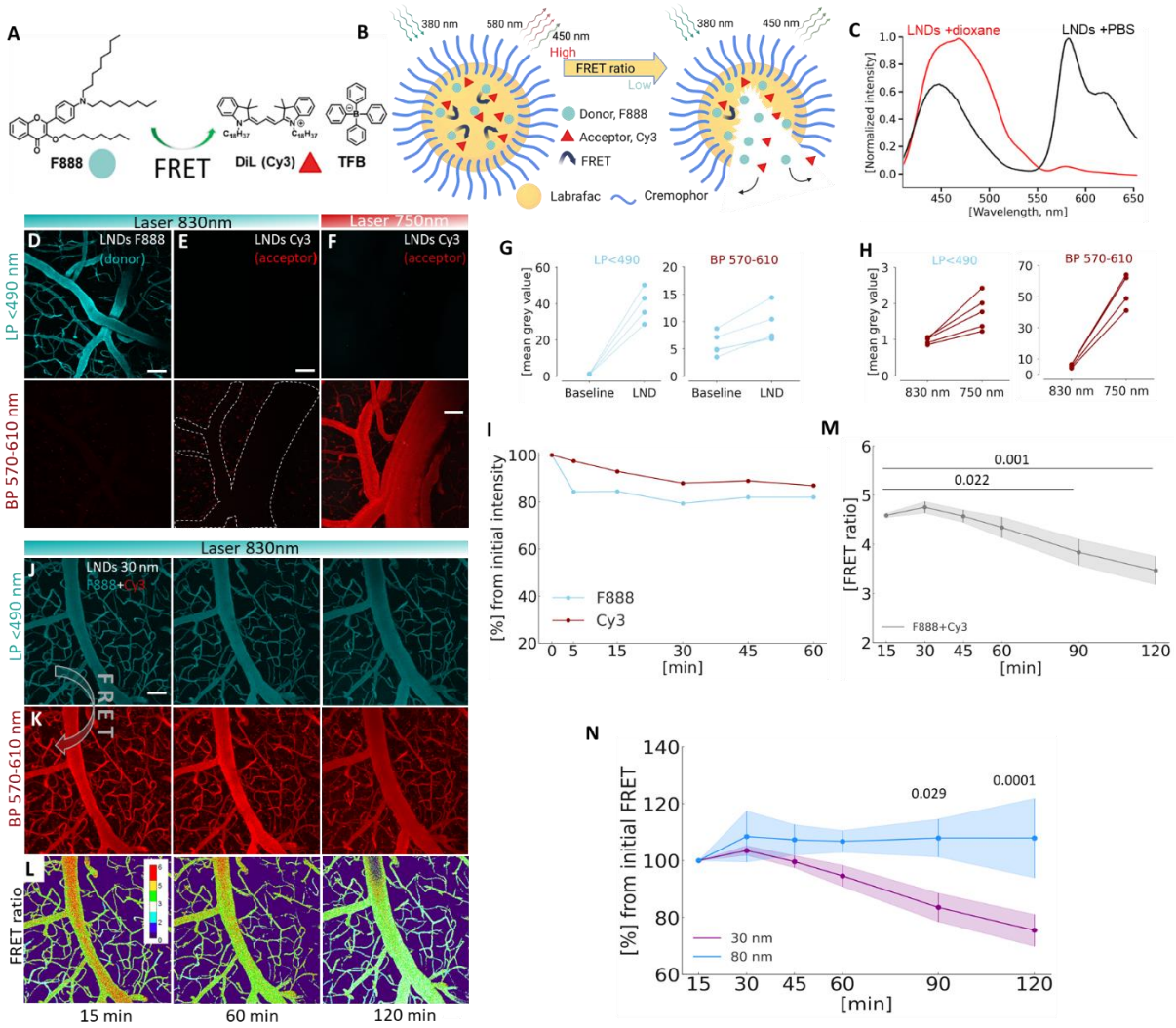


Figure 1. LNDs loaded with FRET system enable real-time *in vivo* quantitative fluorescent ratiometric imaging of nanocarrier (NC) integrity. A. Chemical structure of F888 and Dil (Cy3) coupled with a counterion tetraphenylborate, which causes FRET. Experiment design. **B.** Concept of FRET NCs that can shift their colour upon carrier decomposition, thus reducing the total FRET ratio. Schematic drawing of a LND consisting of an oil core (yellow; Labrafac WL), surfactant (blue; Cremaphore, Kolliphor® ELP), and the fluorescent FRET pair dyes F888 (blue shere) and Cy3 (red triangle) bound to a hydrophobic counter-ion. **C.** Fluorescence spectrum of FRET FRET-LNDs containing F888 and DiI-TPB (2 wt% loading each) diluted in PBS (back) and dioxane (red). **D-F.** Representative 2-photon images of mouse cortical vessels after injection of 30-nm LNDs loaded solely by F888 (donor) and Cy3 (acceptor). Maximum intensity projection (MIP) of 150 µm Z-stacks; laser power 4.5-13%; donor channel – LP<490 nm; acceptor channel – BP 570-610 nm; scale bar - 50 µm. **D.** Injected 30-nm LNDs loaded with F888 (donor), excitation laser wavelength 830 nm. Presence of bright fluorescent signal in donor channel. **E.** Injected 30-nm LNDs loaded with Cy3 (acceptor), excitation laser wavelength 830 nm. Absence of a signal in both the donor and acceptor channel. Dashed line outlines invisible vessel, which become visible only in 750 nm. **F.** Injected 30-nm LNDs loaded with Cy3 (acceptor), excitation laser wavelength switched to 750 nm. Presence of bright fluorescent signal in acceptor channel. **G.** Increase in fluorescent intensity after injection of donor-loaded LNDs (n=5 regions of interest in the vessels) in the donor (LP<490) and acceptor (BP 570-610 nm) channel. **H.** Shifting of the laser wavelength from 830 nm to 750 nm increases fluorescent intensity of the acceptor-loaded LNDs (n=5 regions of interest in the vessels) in both the donor and acceptor channel. **I.** Stability of LNDs loaded by F888 (donor) and Cy3 (acceptor) in the cerebral microcirculation of naïve mice during 1 hour (n=1). **J, K.** Representative 2-photon MIP images of mouse cortical vessels 15-120 min after injection of 30-nm FedEcs-LNDs loaded by FRET pair (F888+Cy3), excitation laser wavelength 830 nm. Due to the FRET effect, the acceptor become visible by BP 570-610 detector under a laser wavelength of 830 nm. **L.** By colour coding the ratiometric changes of the FRET ratio, it enables us to perform direct 3D real-time imaging of NC integrity in the blood

circulation. **M.** Quantification of time-depended LNDs disintegration in the mouse brain vasculature using FRET ratio. Data are presented as mean ± standard deviation (SD). One-way ANOVA was used, n=3. **N.** Comparative analysis of the degradation of circulated LNDs of two different sizes (30- and 80-nm) using FRET ratio. Data presented as mean ± SD. Two-way ANOVA was used, n=3.

resolution. LNDs with diameter 30- and 80-nm, containing either the donor, acceptor, or both dyes were formulated (**Suppl. Figure S1B**). At an excitation wavelength of 380 nm, LNDs of both sizes showed the fluorescent properties of the donor, *i.e.* fluorescence emission at 450 nm, as well as a second fluorescence peak at 580 nm, corresponding to the expected emission of the acceptor due to FRET (**Suppl. Figure S1 C,D**). The LNDs generated are composed 98% of lipids and 2% of their constitution is represented by the donor and acceptor fluorophores respectively. At this weight, FRET efficiency between the donor and acceptor reached 86% for 30 and 80 nm LNDs (**Suppl. Figure S1 E**). Dilution of FedEcs-LNDs into dioxane, which is expected to destroy LNDs, led to the loss of the FRET signal, so that the emission was dominated by the donor emission (**Figure 1C**). These data demonstrate that the currently developed FRET system can detect LND integrity/cargo delivery.

Using FedEcs to investigate the stability of LNDs in healthy blood vessels *in vivo*

To investigate the critical issue of whether FedEcs-LNDs are bright enough to be visualized *in vivo* and to validate their functionality, we injected FedEcs-LNDs systemically into mice and investigated the cerebral vasculature by *in vivo* 2-photon microscopy (2-PM). FedEcs-LNDs were bright enough to be visualized *in vivo* and LNDs containing either the donor (F888) or acceptor (Dil-TPB) behaved as expected, *i.e.* fluorescence was only observed when the particles were excited with the wavelength specific for the respective fluorophore (830 or 750 nm; **Figure 1D-H**). Longitudinal imaging confirmed these results suggesting that LNDs loaded with both fluorophores are stable *in vivo* (**Figure 1I**). LNDs loaded with FRET donors and acceptors (FedEcs-LNDs) emitted bright fluorescence in both channels (**Figure 1J, K**), suggesting that FRET occurs and can be dynamically assessed in vivo. Thereby, we can dynamically assess real-time particle integrity using the FRET ratio, quantifying the acceptor/donor ratio (**Figure 1L**). Longitudinal analysis of the FRET ratio of circulating 30-nm LNDs in healthy mice showed a slow decrease in the FRET signal, which reached 80% of the initial FRET ratio value over 120 min (**Figure 1M**). 80-nm LNDs had a substantially higher (p=0.0001) stability in the blood circulation since they remained intact for two hours (**Figure 1N**) most likely due to a higher oil to surfactant ratio. Thereby, we can dynamically assess real-time particle integrity using the FRET ratio, quantifying the acceptor to donor ratio. To our knowledge, this is the first report of a nanoparticle system able to produce a stable FRET signal *in vivo* suitable to intravital 2-PM.

Employing FedEcs to define the distribution of LNDs after ischemic stroke *in vivo*

To investigate how LNDs behave under pathological conditions, we induced focal cerebral ischemia by occluding the middle cerebral artery of a mouse with an endovascular filament for one hour and systemically injected highly fluorescent LNDs 30 min after reperfusion (**Suppl. Figure S2A-B**). We focused on 30 nm LNDs, because we recently showed that NPs of this size are small enough to cross the blood-brain barrier via transcytosis[9]. Animals were sacrificed 60 minutes after LND injection and we detected a wide spread accumulation of LNDs within the ischemic territory by histological analysis (**Suppl. Figure S2C**). Staining of blood vessels (lectin) and red blood cells (Ter119) indicated that LNDs were located within cerebral vessels and colocalized with trapped red blood cells indicative of thrombus formation. A high intensity area around LND-stained vessel occlusions suggested that LNDs may have extravasated into the brain parenchyma (**Suppl. Figure S2C, i-iii**).

We then aimed to investigate this process dynamically and with the highest possible resolution to decipher reveal the route/mechanism by which LNDs have crossed the blood brain barrier and whether the accumulation of LNDs in ischemic brain may have affected their ability to carry and deliver cargo. For this purpose, we developed a novel stroke model based on a technique previously used to induce focal cerebral ischemia in juvenile mice[10]. This particular model uses magnetic nanoparticles (MNP) to transiently occlude cortical blood vessels. In detail, animals received a systemic injection of MNP and subsequently a strong mini-magnet was placed on top of the cranial window implanted for *in vivo* imaging (**Suppl. Figure S3A**). The magnetic field trapped the 200 nm pegylated MNPs within the cerebral vasculature (**Suppl. Figure S3B i-ii**) thereby causing cerebral ischemia (Nano-Stroke). Reperfusion was induced by removing the magnet after one hour (**Suppl. Figure S3B iii-iv**). This model occluded cortical blood vessels up to a depth of 500 µm (**Suppl. Figure 3C-E**) and allowed immediate live 2-PM of reperfused cerebral vessels (**Suppl. Figure S3F**) followed by histological evaluation (**Suppl. Figure S3G-K**). Using the Nano-Stroke model and FedEcs-LNDs we investigated the biodistribution of systemically applied LNDs after experimental stroke by *in vivo* 2-PM (**Figure 2A**). First, we focused on LNDs with preserved integrity according to their FRET (acceptor) signal (**Figure 2B**). Post-reperfusion, we observed LND accumulation within non-flowing segments of cerebral vessels (**Figure 2C, yellow arrow**). Some LND accumulations **(Figure 2D, yellow arrow)** were accompanied by subsequent extravasation of LNDs into the brain parenchyma (**Figure 2D**, **80 min, red arrow**). Quantification showed that the number of LND accumulations 80 min after ischemic stroke was 8-fold higher than in sham operated mice **(Figure 2E;** $p<0.0001$**; Ext. data Fig. S4)** and that 90% of all observed micro-occlusions occurred in vessels less than 6 µm, *i.e*. brain capillaries (**Figure 2F**), implying that LNDs were trapped in microvascular-occlusions. Interestingly, 25% of all microthrombi observed after stroke were associated with extravasation of LNDs **(Figure 2G)**, while no extravasations were detected in sham operated animals. Most importantly, extravasation of LNDs were only observed at sites where micro-

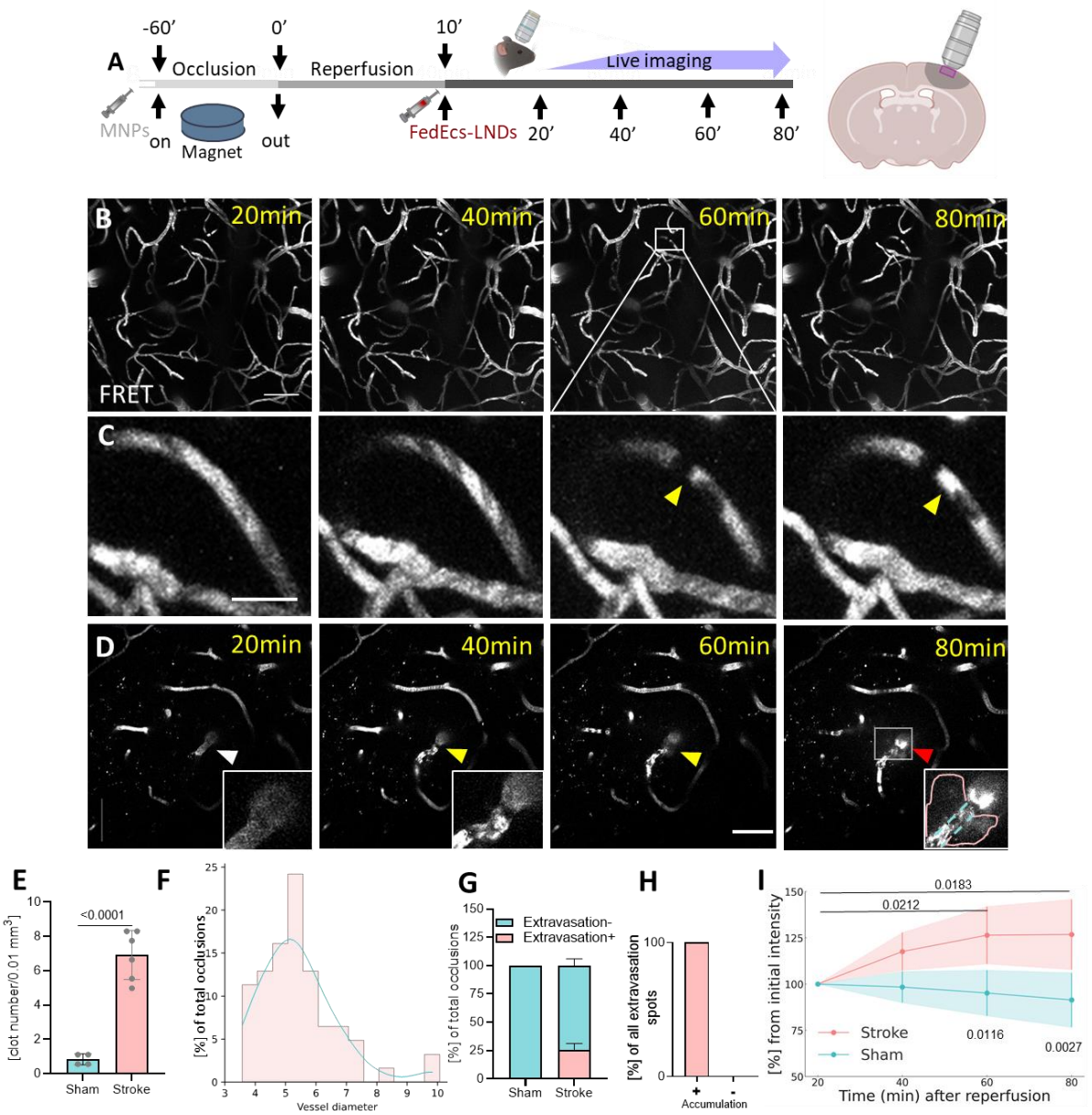
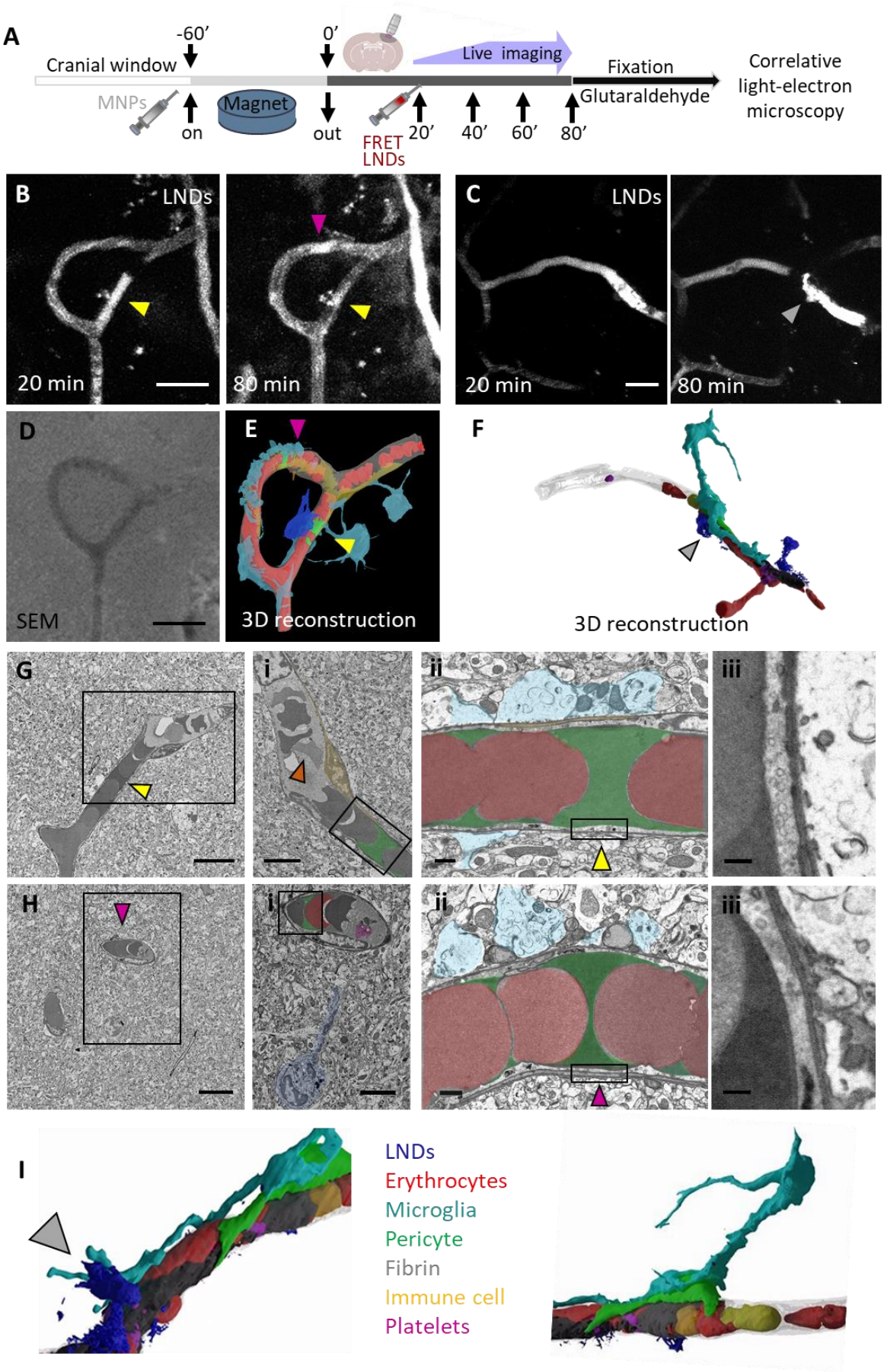


Figure 2. Spontaneous capillary microclot formation following Nano-stroke opens the blood-brain barrier for FedEcs-LNDs. A. Experiment design as described at **Suppl. Fig. S3A** with additional longitudinal intravital imaging every 20 minutes during 1 hour. Schematic representation of the field of view during intravital microscopy. Grey zone – the area under magnet with 60-min-occluded vessels. Magenta – imaging area (depth is ~150 µm). **B.** A representative maximum intensity projection (MIP) of Z-stack of longitudinal 2-photon intravital imaging of cortical area after Nano-stroke model in mouse injected with FedEcs lipid nanodroplets (LNDs). Scale bar – 50 µm. **C.** Zoomed from 4B, representative images of spontaneous microvascular clot formation with subsequently accumulated LNDs (yellow arrow). Scale bar – 10 µm. **D.** Representative longitudinal real-time images of spontaneous microvascular clot formation followed by extravasation of LNDs into the brain parenchyma (yellow arrow). Scale bar - 50 µm. **E.** Analysis of numbers of microvascular clots at 80 min post-reperfusion compared to sham animals. Data are presented as mean ± standard deviation (SD). Unpaired t-test. Stroke: N=79 clots (6 mice); Sham – 4 mice. **F.** Size-distribution of the vessels with microclots. Data are presented as proportion of vessel diameter where microclots were detected. N=62 clots (6 mice). **G.** Analysis of association between microclot (accumulation) and blood-brain barrier dysfunction (extravasation). N=20. **H.** Proportion of microvascular clots associated with extravasation of the LNDs. Data represented as mean ± SD. N=4 (sham) and n=6 (nano-stroke). **I.** Longitudinal analysis of the accumulation of FedEcs LNDs in the ischemic brain area. Data represented as mean ± SD. Two-way ANOVA was used, n=4 (sham) and n=6 (nano-stroke).

occlusions formed **(Figure 2H)**, suggesting that micro-occlusions compromised the permeability of the adjacent capillary wall and were critically involved in the opening of the BBB. Longitudinal *in vivo* imaging of LNDs allowed us to detect that particles gradually accumulated within the ischemic brain parenchyma (**Figure 2I**). Altogether, LNDs do not only accumulate within the vessel lumen after ischemic stroke, but also extravasate into the ischemic brain parenchyma. Thus, our data visualize for the first time that LNDs enter the ischemic brain and may thus have the potential to carry drugs into ischemic tissue.

Since MNPs are photoluminescent under 2-photon excitation, visible at light microscope as well as electron-dense, they can be used as fiducial markers for correlative light-electron microscopy (CLEM). Capitalizing on this feature we monitored LNDs accumulations and extravasation *in vivo* and subsequently relocated these "hot-spots" in serial electron microscopy based on the retained MNPs inside the occluded blood vessel **(Suppl. Figure S5; Methods**). This allowed us to match images obtained by 2-PM *in vivo* with the highest resolution images obtained by scanning electron microscopy (SEM). For this purpose, we induced MNPs-mediated cerebral ischemia in mice for one hour, injected FedEcs-LNDs 20 min after reperfusion, and imaged cerebral microvessels by *in vivo* 2-PM **(Figure 3A)**. We identified three micro-occlusions by *in vivo* microscopy: two without extravasation appeared in different time points – 20 min and 80 min after reperfusion **(Figure 3B, yellow and magenta arrows)** and one with extravasations **(Figure 3C, grey arrow)** and re-identified them by SEM **(Figure 3D; Movie S01)**. After reconstructing both vessel segments in 3D (**Figure 3E, F**; **Movie S02, 03**), we identified the cellular composition and the microstructure of the occluded vessels (**Figure 3E-I**). Microglial and pericytes surrounded the occluded vessels, while erythrocytes, platelets and LNDs co-localized within the occlusions (**Figure 3E-F**). When further zooming in into the ultrastructure of the microvascular occlusions, we found fibrin nets (orange arrow) in between red blood cells, further suggesting that the observed occlusions were microthrombi (**Figure 3G and H**). Collectively, these findings show that stroke induces the formation of microclots within the cerebral microcirculation and that LNDs trapped within these occlusions independent of their time formation.

Using CLEM, we precisely investigated the microstructure of brain endothelial cells (BECs) in the proximity of microclots (**Figure 3Gii-iii & Hii-iii**). Interestingly, microclots which formed early after reperfusion according to *in vivo* imaging had affected erythrocytes morphology, while those formed later had erythrocytes with the normal biconcave shape (**Figure 3Gii&Hii**). The BECs in the vicinity of older clots had a much higher number of caveolae (23 caveolae/µm; **Figure 3Giii**) than BECs next to fresh microclots (2.8 caveolae/µm; **Figure 3Hii,iii**), implicating that a persisting contact between BECs and clots may induce the formation of caveolae, the prerequisite for the subsequent extravasation of LNDs into the brain parenchyma. This is supported by the observation that LNDs indeed extravasate next to microclots (**Figure 3I, grey arrow**). Thus, our electron microscopy data show that stroke-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Fibrin
Immune cell
Platelets

Figure 3. Correlative light-electron microscopy revealed ultrastructural changes in sites of accumulation and extravasation of FedEcs-LNDs. A. Experiment design as described in **Suppl. Fig. S3A** with longitudinal intravital imaging during 60 minutes followed by fixation, embedding and serial sectioning for correlated volume electron microscopy (EM) by automated serial sections to tape (ATUM; **Suppl. Fig. S5**). **B.** Representative 2-PM images of post-stroke mouse brain capillaries containing "old" (20 min post-reperfusion; yellow arrow) and "fresh" (80 min; magenta arrow) spontaneous microvascular clots. Scale bar 20 µm. **C.** Representative example of a microvascular clot (20 min) transitioning towards LNDs extravasation (80 min; grey arrow) into the brain parenchyma ("leaky" microclot). Scale bar 20 µm. **D.** Summed slices of a **s**canning electron microscopy (SEM) stack of the relocated region from the 2-PM experiment **(Suppl. Fig.S5)**. Scale bar 20 µm. **E.** Rendered three-dimensional reconstruction of the volume SEM data thereof. Red (erythrocytes); yellow (pericytes); green (LNDs); sky blue (astrocytes); dark blue (microglia); magenta (platelet). **F.** Rendered three-dimensional reconstruction of volume electron microscopy of the relocated region from **Fig. 3C**. Red – erythrocytes; dark blue – LNDs; cyan – microglia; grey – fibrin; green – pericyte; magenta – platelets; yellow – leukocytes; transparent grey – BEC. See in Movie S03. **G.-H.** Single, segmented high-resolution SEM images of the "old" (G; yellow arrow) and "fresh" (H; magenta arrow) microclots from 3B. Scale bar 10 µm. **G,i.** Zoomed area from G. containing an "old" microclot with accumulated LNDs (green) and a pericyte constricting the capillary (yellow). Orange arrow – dysmorphic erythrocytes trapped into fibrine. **G,ii.** Zoomed area from E,i containing an occluded capillary with accumulated LNDs (green) between erythrocytes (red). Sky blue – astrocytic endfeet. Yellow arrow – relocated capillary area with accumulated LNDs showing a higher than in normal capillaries fluorescent intensity in 2-PM. Scale bar – 1 µm. **G,iii.** Zoomed brain endothelium cell (BEC) from J,ii. containing a large amount of vesicular-like structures. Scale bar – 200 nm. **H,i.** Zoomed area from H. containing a "fresh" microclot with accumulated LNDs (green) between erythrocytes (red) and microglia cell (dark blue) with its processes directed towards the platelet cells (magenta) trapped into fibrine. **H,ii.** Zoomed area from H,i containing an occluded capillary with accumulated LNDs (green) between erythrocytes (red). Sky blue – astrocyte endfeet. Magenta arrow – relocated capillary area with accumulated LNDs having higher fluorescent intensity in 2-PM. Scale bar – 1 µm. **F,iii.** Zoomed BEC from F,ii. with low level of vesicular-like structures. Scale bar – 200 nm. **H,iii.** Zoomed BEC from H,ii. with high level of vesicular-like structures. Scale bar – 200 nm. **I.** Zoomed F. demonstrating that extravasation occurs from the place where microthrombi were formed. FedEcs LNDs – dark blue; Erythrocytes – red; Microglia – cyan; Pericyte – green; Fibrin – grey; Immune cell – yellow; Platelets – magenta. Grey arrow – place of extravasation relocated from **Fig. 3C**.

induced persisting microclots within the cerebral microcirculation progressively promote an environment highly suited for the increased prevalence of endothelial transcytosis through caveolae, which may be associated with BBB opening and may therefore allow LNDs to enter the brain parenchyma.

To investigate this process on the functional level *in vivo*, mice received a systemic injection of LNDs and were then subjected to 60 min Nano-stroke. The cerebral microcirculation was imaged by *in vivo* 2-PM before and after ischemia **(Suppl. Figure 6)**. Images obtained immediately after reperfusion showed multiple microclots co-localising with LND extravasation **(Suppl. Figure 6, yellow arrows)**, supporting our histological and EM findings that microclots serve as an entry point for LNDs into the brain parenchyma. Occluding vessels by MNP we could open BBB.

Monitoring cargo delivery of LNDs in the brain using FedEcs

Entry of LNDs into the brain parenchyma is, however, not equivalent with cargo delivery. Therefore, we used the newly developed FedEcs functionality of our highly fluorescent LNDs to monitor whether LNDs decompose after being trapped in microclots and transmigrate into the brain

parenchyma. For this purpose, we measured the FRET ratio in flowing versus non-flowing segments of the microvasculature after cerebral ischemia over-time by quantitative *in vivo* FRET imaging **(Figure 4A)**. We identified stable FRET ratios of LNDs circulating in plasma (**Figure 4B**, yellow colour), while the

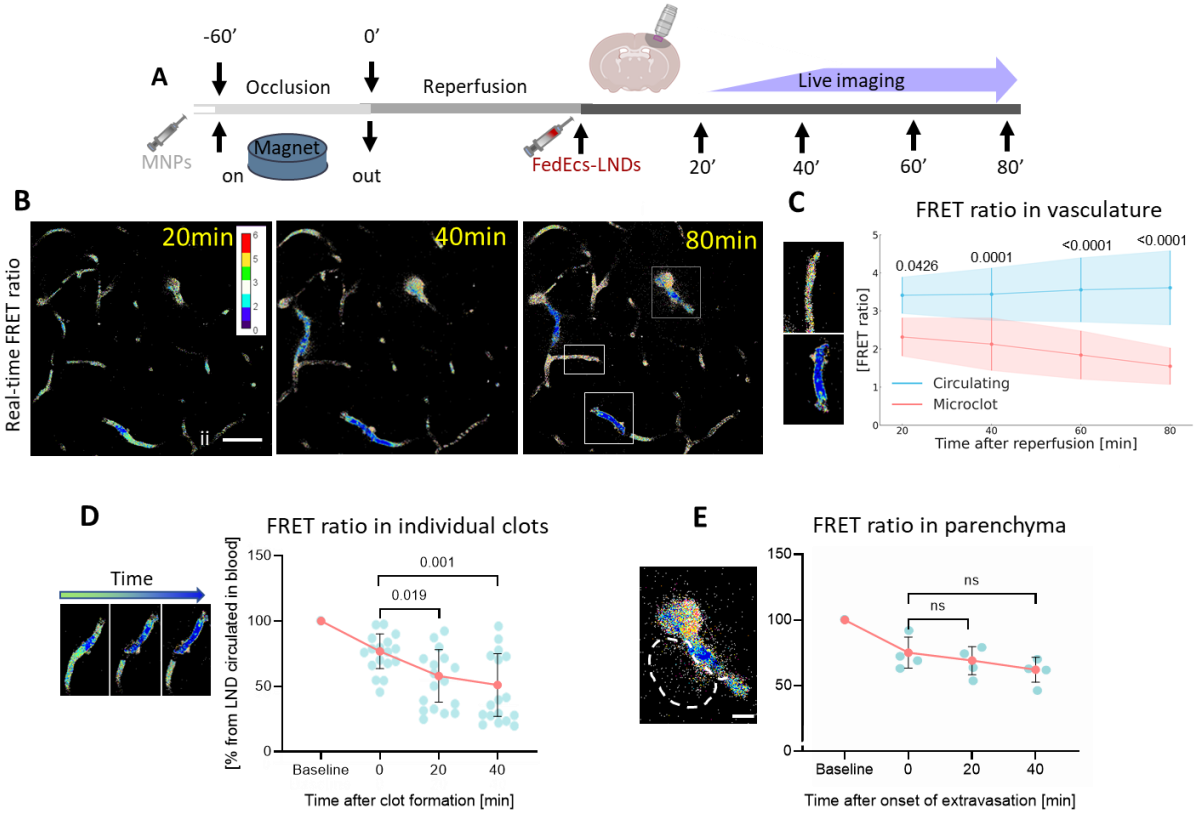

Figure 4. FRET ratio reveals higher decomposition of FedEcs-LNDs in microclots, but not in brain parenchyma as well as delivery of cargo into the brain parenchyma. A. Experiment desing. **B.** Representative longitudinal real-time images of visualisation of FRET ratio related to decomposition of FedEcs-LNDs in vasculature of ischemic brain area. Scale bar - 50 µm. **C.** Longitudinal analysis FRET ratio related to decomposition of FedEcs-LNDs in vasculature of ischemic brain area during 80 minutes after recanalization. Data are presented as mean ± SD. Two-way ANOVA was used, circulated n=16-17 ROIs; microclots n=6-13; 5 mice. **D.** Time-dependent analysis of relative FRET changes of LNDs located inside individual clot. **E.** Time-dependent analysis of relative FRET changes of extravasated into brain parenchyma LNDs. Data are presented as single points and mean ± SD. One-way ANOVA was used, n= 17 clots and n=4 extravasations, respectively. Scale bar – 10 µm. Yellow arrow – clot; dashed area – extravasated FedEcs-LNDs emitting FRET.

FRET ratio of LNDs trapped within microclots was significantly reduced (**Figure 4B**, blue colour). The loss of FRET is caused by a loss of interaction between the two fluorophores undergoing FRET and is therefore a quantitative measure of particle decomposition. Hence, our data indicate that LNDs are stable in plasma, but start decomposing once being trapped in microclots. This observation is further supported by the analysis of individual microclots over time (**Figure 4C**). Once trapped in a microclot, the FRET ratio of LNDs starts declining and reaches about 50% of its baseline value after 40 min. Interestingly, a very similar trend to decay of FRET ratios can also be observed when focusing the analysis on LNDs extravasated into the brain parenchyma (**Figure 4C**). FRET signal at parenchyma means that intact LNDs entered brain via microthrombi. Additionally, these LNDs show a constant

decay of their FRET ratio suggesting that LNDs release their cargo, in our case the fluorophores undergoing FRET, after entering the brain parenchyma.

In summary, using highly fluorescent LNDs and FedEcs, a novel *in vivo* real-time cargo release detection system, we assessed the biodistribution of nanoparticles-based drug carriers *in vivo* and found that lipid nanodroplets accumulate inside microthrombi, cross the BBB, and enter the brain parenchyma while releasing their cargo. Thus, FedEcs represents a novel and useful tool to investigate the therapeutic potential of nanoparticle-based drug delivery systems and to directly visualize cargo delivery *in vivo* with a high spatial and temporal resolution.

Discussion

Targeted drug delivery using nanotechnology has two conflicting aims: nanoparticles must reliably enclose their cargo and transport it to the target tissue, but release it there effectively[11]. Both aims are hard to achieve and even more challenging to investigate experimentally due to insufficient technologies for particle tracking and cargo release in living organisms. In the current study we developed highly fluorescent LNDs with a built-in cargo release function based on FRET and used these nanoparticles (NPs) to investigate the biodistribution of LNDs and cargo release in a novel, magnetic NPs-based model of ischemic stroke in mice. Using *ex vivo* and *in vivo* multiphoton fluorescent microscopy, we demonstrate that LNDs accumulate and thereby reliably label microthrombus formation within the cerebral microcirculation after ischemic stroke. Microthrombi open the blood-brain barrier (BBB) thereby enabling LNDs to extravasate into the brain parenchyma. *In vivo* FRET imaging suggests that LNDs release their cargo within microthrombi and, at a lower rate, also in the brain parenchyma. These data explain the cellular and subcellular mechanisms responsible for the retention of liposomes in ischemic brain published earlier[12-14], define the exact site of particle retention, and suggest LNDs as potential drug carriers for the delivery of therapeutics to the brain under conditions where microthrombi are present, *i.e.* ischemic stroke or traumatic brain injury[9].

To investigate the biodistribution of systemically applied NPs in the brain after stroke, we had to develop and improve several novel technologies. After observing retention of LNDs inside microclots in the ischemic brain by confocal microscopy, we used *in vivo* deep brain 2-photon imaging to understand the dynamics and the mechanism of this process. For this purpose, we developed a new MNPs-based stroke model, Nano-Stroke, which allowed us to image exactly the same cerebral vessels before and after reperfusion. The Nano Stroke model is based on the recently published SIMPLE stroke model performed in pups[10]. We adapted the SIMPLE model to adult animals by implanting a chronic cranial window, thus allowing us to visualize cerebral vessels in the ischemic brain longitudinally and with high resolution. Combining the Nano-Stroke model with highly fluorescent, super-bright FedEcs-LNDs and intravital 2-PM, we were able to demonstrate for the first time *in vivo* and in real-time that

following reperfusion systemically injected LNDs accumulate inside microthrombi and extravasate there, a so far missed phenomenon due to the use of low-fluorescence NPs.

Accumulation of LNDs inside microclots after cerebral ischemia may have a large medical potential. The standard treatment for ischemic stroke is recanalizing large cerebral arteries either by dissolving or removing the occluding thrombus by systemic administration of recombinant tissue plasminogen activator or by mechanical thrombectomy, respectively[15]. However, in many cases recanalization of large cerebral vessels does not result in reperfusion of the cerebral microcirculation and restoration of cerebral blood flow and may thus allow additional tissue damage to occur[16]. In previous attempts to dissolve microthrombi after stroke researchers attached the main thrombus-attaching protein (Cys-Arg-Glu-Lys-Ala) CREKA on the surface of nanocarrier[17-19], however, due to the lack of appropriate imaging techniques it remained elusive whether these particles delivered their cargo into microclots. In the current study, we did not use any active targeting strategy, still our LNDs ended up in large numbers within microclots. This finding is per se interesting, since future approaches to target microthrombi may include specific targeting as well, but it still remains unclear how such high numbers of NPs get trapped in microthrombi. One possible explanation could be that NPs get trapped in forming microthrombi, *i.e.* while the clot forms, however, the large number of NPs located between all components of the clot does not seem to support such a scenario. The more likely explanation is that it takes some time until clots form and fully occlude a cerebral microvessel, as suggested by previous investigations by us and others[20-22]. During this period, which may last for minutes or even hours, vessels are not patent for cells but for plasma and since NPs travel with plasma, they enter the clot and get trapped in between accumulated fibrin polymers and stalled red and white blood cells. This “filtering” mechanism fully explains the large number of LNDs we found in microclots and the specificity with which LNDs accumulate in these structures. This concept is well in line with previous studies which showed neuroprotective effects with nano-scale compounds after ischemic stroke[23, 24], but did not elaborate on the mechanisms of particle distribution and cargo delivery.

In the current study we chose LNDs as nanocarriers because they are regarded to be non-toxic and biodegradable and they have a relatively long half-life of up to 8h in mammalian blood[5]. To monitor cargo release, we equipped LNDs with a FRET-based cargo release detection system, FedEcs. FRET systems have been widely used in nanoscience for more than a decade[25], however, the novelty of our current approach was to use a FRET pair compatible with 2-photon excitation for intravital imaging of cargo release. This approach resulted in LNDs with such a high FRET signal that single LNDs could be visualized by 2-PM in mouse blood and brain *in vivo*. In comparison to recent, more traditional approaches to image drug delivery to the brain using fluorescent NPs[12, 26, 27], FedEcs has the large advantage of being able to visualize the actual moment when LNDs decompose and release their cargo, even in tiny amounts of crossed BBB LNDs. Thus, FedEcs are able to clearly distinguish between

artefactual cargo delivery to the brain, that is when dyes leaked from circulating nanocarriers cross the BBB, from factual cargo delivery, when carrier cross the BBB and release their cargo thereafter. Thus, FedEcs will allow researchers to monitor the release of cargo from individual NPs with high spatial and temporal resolution in all organs accessible to *in vivo* microscopy thereby avoiding all ambiguities caused by previous methods.

Conclusion

In conclusion, we successfully developed highly fluorescent lipid nanodroplets carrying a FRET-based cargo delivery system (FedEcs). FedEcs-LNDs are bright enough to be visualized individually in blood and brain of living mice by 2-photon microscopy. Using FedEcs-LNDs we were able to quantify the stability of LNDs in blood and demonstrate the preferential accumulation of LNDs in microvascular clots, extravasation of intact LNDs across the blood-brain barrier, and cargo delivery to the brain parenchyma following cerebral ischemia produced in controlled manner by magnetic nanoparticles. These findings highlight FedEcs as a reliable and potent dynamic indicator of cargo delivery and release and provide a future testing ground for the efficacy and delivery of a wide-range of targeted nanoscale therapy in future.

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Authors contribution

I.K. initiated and co-fund the study, led scientific aspects of the project, designed, performed and analysed most of experiments, wrote the first draft of the manuscript. N.A. formulated and optimised FRET-LNDs for 2-photon imaging. M.S. coordinated and performed CLEM experiment, helped write the manuscript. A.W. performed and designed *ex vivo* part of Nano-stroke experiment and its analyses, helped write the manuscript. V.B.T. formulated FRET-LNDs and performed their analyses. U.M. performed fMCAo stroke experiment. J.S. performed Nano-stroke and fMCAo experiments and analysis. T.M. led EM part of the project, coordinated CLEM experiments and analysis. SF designed, coordinated, performed Nano-stroke and CLEM experiments. A.K. led FRET-LNDs aspect

of the project, provided infrastructure, supervised and cowrote final draft of the manuscript. N.P. led main animal experimental aspects of the project, co-fund the study, provided infrastructure, supervised, cowrote the final manuscript. All authors critically reviewed, contributed to and approved the final manuscript.

Material and Methods

1. Formulation of LNDs

Dye loaded nanoemulsions were obtained by spontaneous nanoemulsification. Briefly, the dyes R18-TPB (prepared as described before)[28] was dissolved in LabrafacWL® at 2% by weight. In case of FRET-LNDs, F888 (FRET donor, prepared as described before[5, 7]) and DiI-TPB (FRET acceptor, prepared as described before[8]) Then, 60 mg of the oil with the dye were mixed with 40 mg Cremophor ELP® (also called Kolliphor ELP®) and homogenized under magnetic stirring at 40 °C for 10 min. The LNDs were obtained by the addition of ultrapure (Milli-Q) water (230 mg) under rapid magnetic stirring. Size distributions were determined by dynamic light scattering using a Malvern Zetasizer ZSP.

2. Animals

Male 6-8 weeks old, 18-22 g, C57Bl6/J mice from Charles River Laboratories (Sulzfeld, Bavaria, Germany) were used. Mice were group-housed under pathogen-free conditions and bred in the animal housing facility of the Institute of Stroke and Dementia Research (Muenchen, Germany), with food and water provided ad libitum (21 ± 1°C, at 12/12 hour light/dark cycle). Animal husbandry, health screens, and hygiene management checks were performed in accordance with Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations[29]. All experiments were carried out in compliance with the National Guidelines for Animal Protection of Germany with the approval of the regional Animal care committee of the Government of Upper Bavaria, and were overseen by a veterinarian. The data were collected in accordance with the ARRIVE guidelines[30].

3. Stroke fMCAo

Ischemic stroke animal model via filament middle-cerebral artery occlusion (fMCAo) was performed as previously described[31]. Briefly, buprenorphine (0.1 mg/kg) injected animals, 30 min later were anesthetized with 1.8–2% isoflurane in 50% O_2 in air by face mask under the control of rectal temperature (37°C±0.1°C) with a feedback-controlled heating pad. Regional cerebral blood flow (rCBF) over the territory of the middle cerebral artery (MCA) was monitored via glued onto parietal skull probe using laser Doppler fluxmetry (Perimed, Stockholm, Sweden). A silicone-coated monofilament (Doccol Corporation, Sharon, MA, USA) was introduced into the left common carotid artery and advanced toward the Circle of Willis until a drop of rCBF more than 80% of baseline indicated occlusion of the MCA. Thereafter, animals were allowed to wake up. After 60 min, animals were re-anesthetized and the filament was removed to allow reperfusion. Immediately, femoral artery catheter was inserted and 30 min post-reperfusion LNDs or MNPs were injected in volumes 4 or 6 µL/g per animal, respectively.

4. Ex vivo: confocal (fMCAo, nano-stroke), 2-photon

Animals were injected via femoral catheter by 50 µl of DyLight 649 Labeled Lycopersicon Esculentum (Tomato) Lectin (Vector Laboratories, Burlingame, CA, US) and 5 min later were transcardially perfused with 4% PFA in deep anesthesia. Free floating coronal 80 µm sections were prepared as previously described[32]. The sections were blocked and simultaneously stained with the primary antibody in buffer (1% bovine serum albumin, 0.1% gelatin from cold water fish skin, 0.5% Triton X-100 in 0.01 M PBS, pH 7.2–7.4) for 72h at 4°C. The following primary antibodies were used: iba-1 (rabbit, Wako, #019-19741, 1:200), NeuN (guinea pig, Synaptic Systems, #266 004, 1:100), GFAP-Cy3 (mouse, Sigma Aldrich, #2905, 1:200). After incubation sections were washed in PBS and incubated with the following secondary antibodies: anti-rabbit coupled to Alexa-fluor 488 (goat anti-rabbit, Thermo Fisher Scientific, 1:100), anti-rabbit coupled to Alexa-fluor 594 (goat anti-rabbit, Thermo Fisher Scientific, #A-11012, 1:200), anti-guinea pig coupled to Alexa-fluor 647 (goat anti-guinea pig, Thermo Fisher Scientific, #A-21450, 1:200) in secondary antibody buffer (0.05% Tween 20 in 0.01 M PBS, pH 7.2–7.4). Nuclei were stained with 4',6-Diamidin-2-phenylindol (DAPI, Invitrogen, #D1306) 1:10,000 or DRAQ5 (ThermoFisher, #65-0880-92) 1:1000 in 0.01 M PBS. Imaging was performed using confocal microscopy (ZEISS LSM 900, Carl Zeiss Microscopy GmbH, Jena Germany). For NeuN and iba-1 quantification, 40x magnification was used (objective: EC Plan-Neofluar 40x/1.30 Oil DIC M27) with an

image matrix of 512x512 pixel, a pixel scaling of 0.4 x 0.4 µm and a depth of 8 bit. Three different ROIs in the lesion from superficial to deeper levels were chosen and collected in 15 µm z-stacks with a slice distance of 1 µm. Homotypical areas were imaged on the contralateral hemisphere and used as controls. MAP2 intensity was then measured and normalized to contralateral.

4.1 Analysis of microglia coverage. Microglia coverage was manually assessed in a maximum intensity projection of iba-1 stained sections. ROIs were chosen in the center of the lesion at 250 µm depth from the cortex surface at -1mm from bregma. Homotypical ROIs were chosen on the contralesional hemisphere. 3 ROIs per section per hemisphere were chosen. For microglia coverage, manual cell counting was used. To assess microglia morphology, Sholl and fractal analysis were performed to indicate ramification, cell range and circularity using a modified protocol from Young and Morrison[33]. Z-stack images were converted to a maximum intensity projection and microglia cells were individually cut out using the polygon selection tool in ImageJ. Only cells which were entirely contained within the z-stack were selected. Images were then thresholded and binarized as well as resized to 600 x 600 pixels, keeping the original scale. Any speckles and debris around the cell were removed using the paintbrush tool. Sholl analysis was performed using ImageJ [34]. Centered on the soma, concentric circles with an increasing radius of 1 µm were drawn, the number of intersections measured at each radius.

After converting binary images to outlines, fractal analysis was performed using the FracLac plugin for ImageJ [35]. As described previously [33], the total number of pixels present in the cell image of either the filled or outlined binary image were calculated and later transformed to µm2 (pixel area = 0,208 µm2). Cell circularity was calculated as

$$\text{Circularity} = 4*\pi*\text{Area}/\text{Perimeter}^2.$$

Statistical analysis

All data is given as mean ± standard deviation (SD) if not indicated otherwise. For comparison between groups, Student t-test was used for normally distributed data and Mann-Whitney Rank Sum test for non-normally distributed data according to the result of Kolmogorov-Smirnov normality test. Measurements over time were tested between groups using One-way or Two-way ANOVA with Repeated Measurements, followed by Turkey's multiple comparisons test for normally and Sidak's multiple comparisons test for non-normally distributed data as post hoc test. Calculations were performed with Sigmaplot version 14.0 (Systat Software GmbH, Erkrath, Germany) and GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, California USA).

5. Cranial window preparation.

Before use, surgical tools were sterilized in a glass-bead sterilizer (FST, Heidelberg, Germany). Mice were anesthetized by an intra-peritoneal injection of medetomidine (0.5 mg/kg), fentanyl (0.05 mg/kg), and midazolam (5 mg/kg) (MMF) (140/10 mg/kg body weight, WDT, Bayer, Leverkusen, Germany). Subsequently, mice were placed and head-fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). Throughout the experiment, body temperature was monitored and maintained by a rectal probe attached to a feedback-controlled heating pad (Harvard Apparatus, Holliston, MA, USA). Eyes were protected from drying by applying eye ointment (Bepanthen, Bayer, Leverkusen, Germany). The scalp was washed with swabs soaked with 70 % ethanol. A flap of skin covering the cranium was excised using small scissors. The periosteum was scraped away with a 15-size scalpel (Swann Morton, Owlerton Green Sheffield, UK). The prospective craniotomy location above the primary somatosensory cortex (AP: −0.9 mm and ML: +3.0 mm relative to bregma) was marked with a biopsy punch (diameter 3 mm, Integra LifeSciences, Princeton, New Jersey, USA). The exposed skull around the area of interest was covered with a thin layer of dental acrylic (iBond Self Etch, Hereaus Kulzer, Hanau, Germany) and hardened with a LED polymerization lamp (Demi Plus, Kerr, Orange, CA, USA). A dental drill (Schick Technikmaster C1, Pluradent Frankfurt am Main, Germany) was used to thin the skull around the marked area. After applying a drop of sterile phosphate buffered saline (DPBS, Gibco, Life Technologies, Carlsbad, CA, USA) on the craniotomy the detached circular

bone flap was removed using forceps (S&T Vessel Dilating Forceps - Angled 45°, FST, Heidelberg, Germany). Subsequently, SuperGrip forceps were used to remove carefully the dura mater (S&T Forceps - SuperGrip Tips, FST, Heidelberg, Germany), and the brain was rinsed with saline. A circular coverslip (3 mm diameter, thickness #0, VWR International, Radnor, PA, USA) was placed onto the craniotomy and glued to the skull with histoacrylic adhesive (Aesculap AG, Tuttlingen, Germany), applied via dental microbrushes (0.5 mm tip diameter, Microbrush International, Grafton, WI, USA). The exposed skull was covered with dental acrylic (Tetric Evoflow A1 Fill, Ivoclar Vivadent, Schaan, Liechtenstein) and a custom-made head-post was attached parallel to the window for head-fixing mice in subsequent imaging sessions. After surgery, the anesthesia was antagonized with a combination of Atipamezol (2.5 mg/kg), Flumazenil (0.5 mg/kg) and Naloxon (1.2 mg/kg) i.p.. Finally, mice received a subcutaneous dose of the analgesic Carprophen (7.5 mg/kg body weight, Rimadyl, Pfizer, New York, NY, USA) and were allowed to recover from surgery in a heating chamber.

6. Intravital 2-photon

2-photon images were scanned as previously described[36]. Briefly, the anesthetized mouse was placed under an upright Zeiss LSM710 microscope equipped with a fs-laser (Mai Tai DeepSee, Spectra-Physics, Stahnsdorf, Germany), a 20x water immersion objective (W Plan-Apochromat 20x/1.0 NA, Zeiss) and a motorized stage. Ti:Sa laser (Chameleon Vision II) from 917 Coherent (Glasgow, Scotland) with an excitation wavelength of 830 nm and power of 5-22 % was used for detection. GAASP detector with an SP < 485 nm filter with master gain 600 for the donor channel and LP > 570 nm for the LNDs acceptor or FRET channel with master gain 600 were used. Images were taken as z-stacks (150 µm, 1024 x 1024, 8 bit, objective: W Plan-Apochromat 20x/1.0 DIC D=0.17 M27 75mm).

7. FRET in vivo

There were three types of LNDs which were used at FRET in vivo experiment: loaded with donor (F888), loaded with acceptor (Cy3) and with both donor and acceptor (FRET). All solutions were injected in volume 7.5 µl/kg body weight into the mice with implanted cranial windows. Mouse under MMF anesthesia were placed on the 2-PM stage. The LNDs were excited at 830 nm (and 740 nm for LNDs loaded with acceptor) and the emission was collected with SP < 485 nm (donor channel) and LP > 570 (acceptor/FRET channel) filters by non-descanned detectors (photomultiplier tube GaAsP, Zeiss). Three-dimensional z-stacks of 150 µm depth with 1 µm axial resolution and 1024×1024 pixels per image frame (0.6 µm/pixel) were acquired in multiphoton mode of the microscope over a time course of 120 min. The intensity was calculated at each channel as a total intensity of maximum intensity projection image using Fiji software. The FRET ratio was calculated using formula: FRETratio=FRET intensity/Donor intensity. Ratiometric analysis of LNDs integrity was performed in blood circulation 30-nm vs. 80-nm. Statistical analysis was performed using two-way ANOVA with Tukey correction for multiple comparison (n=3 for each group) with GraphPad® Prism (GraphPad Software, San Diego, CA, USA).

8. Nano-stroke, a transient mini-stroke mouse model

Stroke induction started one day after cranial window implantation. Throughout the experimental procedure, mice were anaesthetized with an intra-peritoneal injection of medetomidine (0.5 mg/kg), fentanyl (0.05 mg/kg), and midazolam (5 mg/kg) (MMF) (140/10 mg/kg) and placed on a heating pad to keep body temperature at 37 °C (Harvard Apparatus, Holliston, MA, USA). The head-holder of the mouse was fixed to a custom-made headpost and a neodymium-iron-boron (NdFeB) magnet was placed on the cranial window overlying the distal branches middle cerebral artery (MCA; Fig. S1B, i, ii). NdFeB magnets were custom-made (Zhenli Co. Ltd, Jiaozuo, Henan, China) with the following specifications: Cylindrical NdFeB magnets, grade N52, 1 mm long, 1.5 mm in diameter, magnetized along the cylindrical axis. Before injection, all solutions with nanoparticles were mixed thoroughly using a vortex shaker (VTX-3000L, LMS). Magnetic nanoparticles, Nanomag®-D (Micromod, Rostok, Germany), diameter 180 nm, PEG 2000 coated, were injected slowly at a volume of 6 µl/g body weight via a femoral artery catheter. Since iron oxide is a magnetite and each particle acts as an individual magnetic domain[37], we expected to block the vessels via interaction between a magnet and blood-

circulated MNPs. Safety, biocompatibility, and physical parameters of these MNPs were described previously[10]. Subsequently, injected MNPs (Fig. S1B, ii) into the blood stream were trapped in the magnetic field applied to the cerebral cortex, thereby occluding the underlying vessels as evidenced by the black colour filling the vessel lumen (Fig. S1B, iii). Reperfusion was then induced by removing the magnet. Ten minutes thereafter, the blood flow in the distal branches of middle cerebral artery (black arrow) as well as in most pial veins was restored (Fig. S1B, iv). Vessel occlusion was monitored and recorded under a stereo microscope M80 (Leica, Wetzlar, Germany), equipped with a video camera DFC 290 HP (Leica). After 1 hour the magnet was removed to induce reperfusion and a suspension of ultrabright FRET LNDs (7.5 µl/kg body weight) or Hypoxyprobe (60 mg/kg, NPI Inc., Edmonds, WA, USA) was injected. Systemic injection of fluorescent LNDs (Fig. S1A, i) was performed through same catheter used for MNPs. The platform used for performing the nano-stroke featured a custom heating pad and head-holder, which were compatible with the 2-PM stage. This allowed for easy relocation of the mouse to the microscope, enabling immediate intravital imaging of the reperfused area. Subsequently, mice were transferred under LSM 7 MP microscope (Zeiss, Oberkochen, Germany). LNDs were excited with 830 nm wavelength, laser power 5-11%, filters, as was described above, were SP < 485 nm and LP > 570 nm for donor and FRET respectively. Three-dimensional z-stacks of 150 µm depth with 1 µm axial resolution and 1024×1024 pixels per image frame (0.6 µm/pixel) were acquired in multiphoton mode of the microscope every 20 min over a time course of 60 min. Throughout the imaging session laser power was kept below 50 mW to avoid phototoxicity. Sham-operated animals received MNPs 20 min before imaging without magnet application. The microthrombi were identified visually as occluded vessels (lower-to-dark fluorescent intensity) or accumulated LNDs (higher fluorescent intensity). The total number of

9. Correlated light and ATUM Volume Scanning Electron Microscopy (CLEM).

In order to provide exact relocation of ROI from 2-PM to EM, the custom-made head post for head-fixation was used (Figure S05, A, white arrow), as described[38]. This enabled to relocate the imaging orientation from 2-PM to EM. Immediately after 2-photon imaging, mice were transcardially perfused with a mixture of 4% formaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences, EMS) in 0.1 M sodium cacodylate buffer, pH 7.4. After 1 h perfusion mice were decapitated and the right parietal bone with the ipsilateral window was removed. Subsequently, the mouse head was attached to the stage of a vibratome (VT1000S, Leica Biosystems) via the head post to cut the brain tissue with thickness 80 µm parallel to the imaging plane (Figure S05A). This way, a single brain slice containing all cortical layers and the complete somato-sensory cortex was obtained. The cortical brain slice was incubated in the same fixative overnight at 4 °C, washed with PBS at the next day, cut at 50 µm thickness on a vibratome and subsequently stored in 0.1 M sodium cacodylate buffer at 4°C until the start of the postembedding. Coarse lateral relocation of the cortical region of interest was guided by magnetic nanoparticles inspected under a standard binocular (Kern) and dissected at roughly 2x2 mm. A standard rOTO *en bloc* staining protocol[39] was applied including postfixation in 2% osmium tetroxide (EMS), 1.5% potassium ferricyanide (Sigma) in 0.1 M sodium cacodylate (Science Services) buffer (pH 7.4). Staining was enhanced by reaction with 1% thiocarbohydrazide (Sigma) for 45 min at 40°C. The tissue was washed and incubated in 2% aqueous osmium tetroxide, washed and further contrasted by overnight incubation in 1% aqueous uranyl acetate at 4°C and for 2h at 50°C. Samples were dehydrated in an ascending ethanol series and infiltrated with epon-araldite resin (LX112, LADD).

In order to preserve the same imaging orientation for EM from the 2-photon experiment, the block was trimmed parallel to the cortical surface. The advantage of MNPs is to be seen label-free in light, fluorescent and electron microscopies[37]. Thereby, the correlation strategy relied on electron-dense magnetic particles and further anatomical landmarks like vascular patterns (Figure S05, B) and regions of MNP leftovers in the reperfused area, far outside of the ROI. The area of interest was exposed by trimming using TRIM90 (Diatome) on the ATUMtome (Powertome, RMC). In total, 1500 serial sections were collected starting from the pial surface with a 35° ultra-maxi diamond knife (Diatome) at a nominal cutting thickness and resulting axial resolution of 100 nm and collected on freshly plasma-treated (custom-built, based on Pelco easiGlow, adopted from M. Terasaki, U. Connecticut, CT), carbon

nanotube (CNT) tape (Science Services). CNT tape stripes were assembled onto adhesive carbon tape (Science Services) attached to 4-inch silicon wafers (Siegert Wafer) and grounded by adhesive carbon tape strips (Science Services). EM micrographs were acquired on a Crossbeam Gemini 340 SEM (Zeiss) with a four-quadrant backscatter detector at 8 kV. In ATLAS5 Array Tomography (Fibics), wafer overview images were generated (2000 nm/pixel). In order to relocate the region imaged by light microscopy we acquired a data set covering 10 wafers (~200 sections, covering 20 µm depth of tissue each) at a resolution of 0.2x0.2x4 µm. This coarse EM map was aligned (TrakEM2)[40] and different cell types segmented (VAST)[41]. Building on this cellular map regions of interest surrounded but devoid of magnetic particle accumulations were selected for high resolution imaging at 20x20x100 nm voxel size. High resolution stacks were aligned, segmented and rendered (Blender)[42]. Accordingly, the first step was to identify the 2-PM field-of-view on a macro photograph of the cranial window (Figure S05, C, white arrows). This ROI was identified in low resolution EM using MNPs as fiducials and vascular patterns as endogenous landmarks (Figure S05, C, black arrows). Registration of the 2-PM and the low resolution SEM image volumes allowed us to target time-lapse features of interest like microthrombi formation or extravasation events (Figure S05, D, blue arrows) and acquire high-resolution volume SEM data thereof.

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