Noninvasive Imaging of Early Venous Thrombosis by 19F Magnetic Resonance Imaging With Targeted Perfluorocarbon Nanoemulsions

Sebastian Temme, PhD; Christoph Grapentin, PhG; Christine Quast, MD; Christoph Jacoby, PhD; Maria Grandoch, MD; Zhaoping Ding, MD; Christoph Owenier, BSc; Friederike Mayenfels, PhD; Jens W. Fischer, PhD; Rolf Schubert, PhD; Jürgen Schrader, MD*; Ulrich Flögel, PhD*

Background—Noninvasive detection of deep venous thrombi and subsequent pulmonary thromboembolism is a serious medical challenge, since both incidences are difficult to identify by conventional ultrasound techniques.

Methods and Results—Here, we report a novel technique for the sensitive and specific identification of developing thrombi using background-free 19F magnetic resonance imaging, together with α2-antiplasmin peptide (α2AP)-targeted perfluorocarbon nanoemulsions (PFCs) as contrast agent, which is cross-linked to fibrin by active factor XIII. Ligand functionality was ensured by mild coupling conditions using the sterol-based postinsertion technique. Developing thrombi with a diameter <0.8 mm could be visualized unequivocally in the murine inferior vena cava as hot spots in vivo by simultaneous acquisition of anatomic matching 1H and 19F magnetic resonance images at 9.4 T with both excellent signal-to-noise and contrast-to-noise ratios (71±22 and 17±5, respectively). Furthermore, α2AP-PFCs could be successfully applied for the diagnosis of experimentally induced pulmonary thromboembolism. In line with the reported half-life of factor XIIIa, application of α2AP-PFCs >60 minutes after thrombus induction no longer resulted in detectable 19F magnetic resonance imaging signals. Corresponding results were obtained in ex vivo generated human clots. Thus, α2AP-PFCs can visualize freshly developed thrombi that might still be susceptible to pharmacological intervention.

Conclusions—Our results demonstrate that 1H/19F magnetic resonance imaging, together with α2AP-PFCs, is a sensitive, noninvasive technique for the diagnosis of acute deep venous thrombi and pulmonary thromboemboli. Furthermore, ligand coupling by the sterol-based postinsertion technique represents a unique platform for the specific targeting of PFCs for in vivo 19F magnetic resonance imaging. (Circulation. 2015;131:1405-1414. DOI: 10.1161/CIRCULATIONAHA.114.010962.)

Key Words: fluorocarbons ■ magnetic resonance imaging ■ molecular imaging ■ pulmonary embolism ■ venous thrombosis

Thrombosis plays a crucial role in a variety of cardiovascular diseases such as myocardial infarction, deep venous thrombosis, and pulmonary embolism, which are major causes of morbidity and mortality. More recently, it has been shown that patients with tumor have up to an 7-fold increased risk of developing thromboembolic events1,2 caused by systemically increased proinflammatory conditions and the release of prothrombotic factors. Thus, visualization and specific identification of thrombi by imaging techniques address an important clinical problem.

Clinical Perspective on p 1414

Thrombi can be visualized noninvasively by ultrasound,3 computed tomography,4 or magnetic resonance (MR) imaging (MRI).5 MRI is free of ionizing radiation and has high spatial resolution in deep tissues that are not accessible by ultrasound. However, thrombus detection by conventional MRI...
with ¹H MR angiography or T1/T2-weighted ¹H MRI is difficult because small, nonocclusive thrombi have only a minor impact on blood flow and may not give rise to a clear signal in weighted images. To overcome this limitation, gadolinium-based probes raised against fibrin within the thrombus have been developed (EP-2104R).⁶⁻¹¹ Another specific marker of developing thrombi is factor XIIa (FXIIa), which cross-links α₂-antiplasmin with fibrin during the early phase of thrombus formation.¹²,¹³ Probes based on α₂-antiplasmin have been used for ex vivo or in vivo labeling of thrombi by near-infrared fluorescence, scintigraphy, or gadolinium-enhanced ¹H MRI.¹⁴⁻¹⁷

Recently, ¹⁹F MRI has emerged as a promising novel technique for molecular imaging. For this, emulsified, biologically inert perfluorocarbons (perfluorocarbon nanoemulsions [PFCs]) are used as a contrast agent to follow the fate of ex vivo or in vivo PFC-labeled cells.¹⁵⁻²⁰ Because ¹⁹F is physiologically found in biological tissue in only trace amounts, the resulting fluorine signal displays an excellent degree of specificity. Merging of ¹⁹F images with corresponding ¹H data sets enables the exact anatomic localization of the ¹⁹F signal. Because ¹⁹F MRI generates a positive contrast as a “hot spot,” it is especially useful for heterogeneous tissue in which ¹H-based contrast is challenged by susceptibility artifacts or sparse proton density, complicating the interpretation of effects obtained by other contrast agents. Until now, ¹⁹F MRI has been used predominantly for immune cell tracking in a variety of clinically relevant inflammation models.²¹⁻³¹ This approach is based on the rapid uptake of intravenously injected PFCs by circulating monocytes that subsequently migrate into the inflamed area, resulting in a local accumulation of ¹⁹F-loaded immune cells.

In the present study, we report a novel procedure for the generation of targeted PFCs that makes use of a sterol-based postinsertion technique (SPIT) to generate α₂-antiplasmin-labeled PFCs (α₂AP-PFCs). SPIT allows modification of preformed PFCs under mild conditions that maintain the functionality of labile ligands. Using α₂AP-PFCs, we were able to detect the formation of developing deep venous thrombi and pulmonary embolism with ¹H/¹⁹F MRI in vivo with a high specificity and sensitivity.

Methods
An expanded Methods section can be found in the online-only Data Supplement.

Perfluorocarbon Nanoemulsions
PFCs were prepared as previously reported (see the online-only Data Supplement for more details).²²,²³

Sterol-Based Postinsertion Technique
Generation of the Cholesterol-PEG−Maleimide Anchor
An equimolar mixture of maleimide-PEG₁₀₀₀−NH₂ (Jenkem Technology, Plano, TX), cholesterol chloroformate (Sigma Aldrich, Seelze, Germany), and the activator triethylamine (Carl Roth, Karlsruhe, Germany) in water-free methylene chloride was prepared. The mixture was stirred for 24 hours under exclusion of light in a nitrogen atmosphere. The resulting cholesterol-PEG₁₀₀₀−maleimide was purified by chromatography with the use of a Sephadex LH-20 column and validated by ¹H nuclear magnetic resonance spectroscopy. Aliquots were stored at −80°C under argon.

Coupling of α₂AP to the Cholesterol-PEG Anchor
To generate PFCs for site-specific targeting of thrombi, we used a 14-amino acid peptide derived from α₂AP that is known to be cross-linked to fibrin at the glutamine Q3 by FXIIa.²⁵⁻²⁷ As control, Q3 was converted to alanine (Q3A), leading to a low-affinity substrate for FXIIa.²⁷ Both peptides were further functionalized with a cysteine residue at amino acid position 13 (Figure I in the online-only Data Supplement) for coupling to the cholesterol-PEG anchor. For immunofluorescence studies, carboxyfluorescein was linked via an additional lysine at the ε-terminal tryptophan (W14).

All peptides (Genaxxon, Ulm, Germany) were dissolved in sterile phosphate buffer (10 mmol/L phosphate isotonized with glycercol, pH 7.4) to 5 mg/mL. The peptides were added to the cholesterol-PEG₁₀₀₀−maleimide anchor, and the mixture was shaken at 17°C for 20 hours at 700 rpm. The cholesterol anchor was used in 10-fold excess compared with the ligand, thus allowing a quantitative coupling of peptides. During the incubation period, the thiol group of the cysteine residue and the maleimide group form a stable thioether bond.²⁰ Free maleimide groups were subsequently deactivated by the addition of mercaptoethanolamine. This way, a mixture of cholesterol-PEG₁₀₀₀−peptide and deactivated cholesterol-PEG₁₀₀₀ (approximate ratio of 1:10) was obtained and used in the following insertion step.

Postinsertion
Preformed PFCs were incubated with the obtained mixture on a rotary shaker at 17°C for 1 hour. As illustrated in Figure 1A, this leads to the spontaneous insertion of the cholesterol moiety into the phospholipid layer of the PFCs (molar ratio of phospholipid to cholesterol derivative, 20:1). PEGylated but nontargeted PFCs were formed by incubation with nonmodified cholesterol-PEG₁₀₀₀ only.

Characterization of PFCs
The resulting emulsions were characterized by photon correlation spectroscopy on a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) to determine the hydrodynamic diameter, the polydispersity index, and the ζ potential. Compared with nonmodified PFCs, we observed a slight increase in size (diameter: nontargeted PFCs, 149±15 nm; α₂AP-PFCs, 165±13 nm), a similar size distribution (polydispersity index: nontargeted PFCs, 0.14±0.01; α₂AP-PFCs, 0.16±0.05), but a less negative ζ potential (nontargeted PFCs, −37.2±4 mV; α₂AP-PFCs, −11.7±7 mV) for the targeted or PEGylated PFCs. This indicates the successful incorporation of chol-PEG−α₂AP into the PFC nanoparticles. ¹⁹F MRI measurements confirmed that all nanoemulsions exhibited the same fluorine content (Figure IIB in the online-only Data Supplement).

Animal Experiments
Animal experiments were in accordance with institutional guidelines on animal care. Male mice (C57BL/6; body weight, 25–30 g; age, 8–10 weeks) used in this study were bred at the central animal facility of the Heinrich Heine University (Düsseldorf, Germany). They were fed a standard chow diet and received tap water ad libitum.

Induction of Venous Thrombi and Pulmonary Thromboembolism
During surgery, mice were kept under anesthesia with 1.5% isoflu- rane. Buprenorphin was injected for analgesia. A median laparotomy was performed, and the inferior vena cava was exposed at the anatomic level of both kidneys. Subsequently, a filter paper (1x4 mm²) soaked with 10% FeCl₃ was placed on top of the vessel and incubated for 8 minutes. To ensure the location of the FeCl₃-soaked filter paper on top of the vessel surface, 2 stretches of parafilm were placed on both sides of the vessel. After removal of the filter paper, the vessel was washed with 0.9% NaCl to remove residual
FeCl₃, PFCs (3 mmol/kg body weight) were injected into the tail vein 5 minutes before thrombus induction or 5, 15, 30, 60, or 90 minutes after thrombus induction. Subsequently, MRI scans were performed at 2, 8, or 24 hours after surgery. To induce pulmonary thromboembolism, a mixture of human thrombin (Sigma-Aldrich, Seelze, Germany) and 2AP-PFCs (or unmodified PFCs/Q3A-PFCs as control) was injected that resulted in an 80% survival rate. 1H/19F MRI measurements were performed 24 hours later.

MRI Studies
Experiments were performed with a vertical 9.4-T Bruker AVANCE III Wide Bore nuclear magnetic resonance spectrometer (Bruker, Rheinstetten, Germany) operating at frequencies of 400.21 MHz for 1H and 376.54 MHz for 19F measurements using microimaging units as described previously.22–25,27,32 Mice were anaesthetized with 1.5% isoflurane and were kept at 37°C during the measurements. Data were acquired with the use of a 25-mm birdcage resonator tunable to 1H and 19F. After acquisition of the morphological 1H images, the resonator was tuned to 19F, and anatomically matching 19F images were recorded (see the online-only Data Supplement for a more detailed description of MRI setup, acquisition parameters, and quantification procedures). An overview over all imaging parameters used for 1H/19F MRI is given in Table I in the online-only Data Supplement.

In Vitro Thrombus Studies
Human blood was obtained by venous puncture and collected on ice. Blood (100 μL) was transferred to a round-bottom 96-well plate and incubated at 37°C for 15 minutes. Next, 25 μL PFCs (nontargeted PFCs or α2AP-PFCs) were added to each well, and the plate was further incubated for 90 minutes at 37°C under constant motion. The blood clots were extensively washed with cold PBS and subjected to 1H/19F MRI. Details about PFC uptake studies by murine blood are given in the expanded Method section of the online-only Data Supplement.

Cytotoxicity Assay
Murine splenocytes were obtained from C56BL/6 mice, incubated with the different PFCs, and analyzed as described in the expanded Method section of the online-only Data Supplement.

Flow Cytometry
Mice were killed by cervical dislocation, and thrombi were excised and digested in streptokinase/plasmin (150U/2 U/mL) for 30 minutes at 37°C under constant shaking. To generate a single-cell suspension, thrombi were passed through a 70-μm cell strainer (BD Biosciences, Heidelberg, Germany), washed with fluorescence-activated cell sorter buffer, and stained for 30 minutes with fluorochrome-coupled antibodies as described in the online-only Data Supplement.

Histology and Fluorescence Microscopy
Excised thrombi and lungs were fixed in 4% paraformaldehyde or embedded in Tissue-Tek (Weckert Labortechnik, Kitzingen, Germany) and frozen at −20°C. Sections of 8 or 14 μm were cut and processed for immunohistochemical staining as described previously (see the online-only Data Supplement for more details).

Statistics
All data were evaluated for normal distribution with the Shapiro-Wilk test and are given as mean±SD. Statistical difference was assessed
Results
Visualization of Deep Venous Thrombi by \(^{1}H/^{19}F\) MRI In Vivo

To generate PFCs for the targeting of developing thrombi, we used a 14–amino acid peptide derived from \(\alpha_{2}\) AP (Figure I in the online-only Data Supplement), which is cross-linked by FXIIIa to fibrin at the glutamine Q3 during the early phase of the thrombus development.\(^{15-17}\) As control, we applied an FXIIIa low-affinity peptide in which glutamine was replaced by alanine in position 3 (Q3A).\(^{15,16}\) Both peptides were coupled to cholesterol-PEG-maleimide and inserted into preformed PFCs by SPIT (Figure 1A).

To explore the suitability of \(\alpha_{2}\)AP-PFCs for early thrombus detection, we induced nonocclusive thrombi in the inferior vena cava and injected \(\alpha_{2}\)AP-PFCs or Q3A-PFCs intravenously 5 minutes before thrombus induction. After 2 hours, \(^{1}H/^{19}F\) MRIs were acquired in the proximity of the thrombus induction site. As shown in Figure 1B, animals that received \(\alpha_{2}\)AP-PFCs display a strong background-free \(^{19}F\) signal at the site of the newly formed thrombus that can be dimly recognized in \(^{1}H\) MRI at the ventral side of the vena cava as a dark gray structure (arrows in Figure 1B, bottom). Although \(\alpha_{2}\)AP-PFCs clearly delineated the thrombus, no signal was found in the area surrounding the surgery (Figure 1B, top), indicating the specificity of labeling. \(^{19}F\) signals were found to be substantially reduced when PFCs with Q3A–control peptide were used (Figure 1B, bottom). Quantification of all data revealed a strongly enhanced \(^{19}F\) signal for \(\alpha_{2}\)AP-PFCs compared with control Q3A-PFCs (P<0.05; Figure 1B, right). Note that the mean diameter of the thrombi was <1 mm (0.74±0.16 mm; n=8) and that mere angiographic MR \(^{1}H\) scans did not permit us to precisely detect the location of the thrombus because only minor alterations in the blood flow were observed (Figure IIIA in the online-only Data Supplement). Although thrombi could be detected in high-resolution spin-echo MRIs, the calculated contrast-to-noise ratio for \(^{1}H\) and \(^{19}F\) signals between thrombus and the adjacent vessel lumen, muscle, and connective

![Figure 2. Time course of the \(^{19}F\) signal in thrombi and blood. A, Sections of in vivo \(^{1}H/^{19}F\) magnetic resonance imaging (MRI) scans of the same animal 2, 8, and 24 hours after thrombus induction. B, Kinetics of the total \(^{19}F\) MR signal in venous thrombi (arbitrary units [a.u., red circles] and \(^{19}F\) signal in blood (signal-to-noise ratio [SNR], blue circles) after \(\alpha_{2}\)-antiplasmin peptide (\(\alpha_{2}\)AP)–perfluorocarbon nanoemulsion (PFC) injection determined by in vivo \(^{19}F\) MRI. C, Time course of the blood signal (signal-to-noise ratio) after injection of PEGylated and unmodified PFCs determined by \(^{19}F\) MRI (left). Area under the curve calculated from the blood \(^{19}F\) signal over time (\(^{19}F\) signal·h) for \(\alpha_{2}\)AP-PFCs, PEG-PFCs, and PFCs (right). B and C, Data represent mean±SD of n=3 experiments.](http://circ.ahajournals.org/doi/fig/10.1161/CIRCULATIONAHA.114.011785)
tissue clearly revealed a strongly enhanced specificity of the \(^{19}\text{F}\) hot spots with contrast-to-noise ratios increased at least 3-fold compared with \(^1\text{H}\) images (Figure IIIB in the online-only Data Supplement).

To test whether the \(^{19}\text{F}\) signal increases over time, we determined the time course of the \(^{19}\text{F}\) signal 2, 8, and 24 hours after thrombus induction. From the representative magnifications of merged \(^1\text{H}/^{19}\text{F}\) data sets (Figure 2A) and the quantitative analysis of all experiments (Figure 2B), it can be seen that there was no further PFC accumulation within the thrombus after 2 hours (Figure 2B, red circles). As expected, delayed application of the \(\alpha^2\text{AP}\)-PFCs after thrombus induction resulted in a continuous decline in the \(^{19}\text{F}\) signal (Figure IVA in the online-only Data Supplement). In line with the reported half-life of FXIIIa activity of 20 to 30 minutes,15–17 some \(^{19}\text{F}\) signal could still be observed when \(\alpha^2\text{AP}\)-PFCs were injected 60 minutes after thrombus induction, whereas after 90 minutes, PFC deposition was not detected any longer. In separate in vivo experiments with untreated animals, we determined the \(^{19}\text{F}\) signal in the blood by MRI (\(^{19}\text{F}\) FLASH) and found that the blood half-life of peptide-targeted PFCs was \(\approx2\) hours (Figure 2B, blue circles). A similar half-life was found for PEGylated PFCs without peptide (Figure 2C, left), indicating that the PEGylation but not the peptide determines the kinetics within the circulation (Figure 2C, right). Interestingly, unmodified non-PEGylated PFCs remained in the bloodstream for a much longer time, with a half-life of \(\approx20\) hours (Figure 2C).

To further corroborate the location of the \(^{19}\text{F}\) signal within the thrombus, excised tissue samples were analyzed ex vivo by histology and by high-resolution \(^1\text{H}/^{19}\text{F}\) MRI. Hematoxylin and eosin and Sirius Red staining confirmed thrombus formation, with the presence of trapped erythrocytes as a hallmark of deep venous thrombi (Figure 3A). Ex vivo MRI identified the thrombus as structure with inhomogeneous contrast surrounded by the dark vessel lumen and the embedding agarose (dark gray, Figure 3B; left, longitudinal sections; right, axial sections). Detected \(^{19}\text{F}\) signal was clearly restricted to the thrombus and exhibited a patchy pattern distributed over the entire thrombus. This result was confirmed by histology through the use of PFCs with carboxyfluorescein-labeled \(\alpha^2\text{AP}\)-peptide, which also showed a patchy distribution of the fluorescence signal within the thrombus (Figure 3C, top). No signal could be observed in control thrombi from animals that received Q3A-carboxyfluorescein-PFCs (Figure 3C, bottom).

**Specificity of \(\alpha^2\text{AP}\)-PFCs for Developing Thrombi**

Trapping of PFC-loaded monocytes or PFCs themselves might have contributed to the observed \(^{19}\text{F}\) signal in the thrombus. Therefore, we explored whether unmodified PFCs or PEGylated PFCs without peptide enrich in venous thrombi. As shown in Figure 4A, intravenous administration of PFCs or PEG-PFCs before thrombus induction did not lead to the deposition of any \(^{19}\text{F}\) signal within thrombi (white arrows, Figure 4A). Flow cytometry on the cellular composition of thrombi revealed a small amount of CD45^+CD11b^+ immune cells (Figure 4B, left and middle) that consist predominantly of neutrophils and a negligible number of monocytes (Figure 4B, right). Interestingly, unmodified PFCs, which are known to be avidly taken up by macrophages,22,23 gave rise to a strong \(^{19}\text{F}\) signal in the inflamed area of surgery (Figure 4A, top, yellow arrows). In contrast, PEGylated PFCs did not accumulate in inflamed areas (Figure 4A, bottom). This phenomenon is most likely attributable to an impaired uptake of PEGylated PFCs by monocytes under these conditions. This assumption was confirmed by separate experiments, where the uptake of PEGylated PFCs by blood monocytes and RAW macrophages was found to be strongly reduced compared with neat
PFCs (Figure 4C). Thus, neither PFC-loaded immune cells nor passive PFC accumulation contributed substantially to the $\alpha_2$AP-PFC–derived $^{19}$F signal in FeCl$_3$-induced deep venous thrombi.

Detection of Pulmonary Thromboembolism

Venous thrombi are strongly prone to cause pulmonary thromboembolism. We therefore explored whether $^{19}$F MRI is also suitable for the detection of thrombi accumulating in the lung. To this end, we injected human thrombin, known to elicit pulmonary thrombosis, in combination with $\alpha_2$AP-PFCs. In vivo $^{1}$H/$^{19}$F MR analyses of the thorax revealed strong $^{19}$F signals within the lung after thrombin and $\alpha_2$-antiplasmin peptide ($\alpha_2$AP)–perfluorocarbon nanoemulsion (PFC) injection. The white line within the axial image (left) indicates the location of the corresponding coronal slice shown on the right. LV indicates left ventricle; PV, pulmonary vessels; RV, right ventricle; and SC, spinal cord.

Figure 5. $^{19}$F magnetic resonance imaging (MRI) of pulmonary embolism. A, Combined in vivo $^{1}$H/$^{19}$F MRI of the mouse thorax showing strong $^{19}$F signals within the lung after thrombin and $\alpha_2$-antiplasmin peptide ($\alpha_2$AP)–perfluorocarbon nanoemulsion (PFC) injection. The white line within the axial image (left) indicates the location of the corresponding coronal slice shown on the right. LV indicates left ventricle; PV, pulmonary vessels; RV, right ventricle; and SC, spinal cord. B, Ex vivo postmortem high-resolution $^{1}$H/$^{19}$F MRI of paraformaldehyde-fixed and agarose-embedded lung tissue indicating accumulation of $^{19}$F signal next to the right pulmonary branch. The location of the $^{19}$F signal (red) was further validated by 3-dimensional reconstruction of the data sets (right; Movie I in the online-only Data Supplement).
in the lung periphery (Figure 5B, right, and Movie I in the online-only Data Supplement).

**Labeling of Human Thrombi With α2AP-PFCs**

To test whether α2AP bound to PFCs can also label human thrombi, human thrombi generated in vitro were treated with untargeted PFCs or α2AP-PFCs during the early phase of thrombus formation. As shown in Figure 6A, we observed a 10-fold-increased 19F signal for α2AP-PFCs, indicating the specific incorporation of α2AP-PFCs. Of note, when α2AP-PFCs were applied after thrombus induction, similar time courses for the decline of the 19F signal were obtained for human thrombi generated ex vivo and thrombi generated in vivo in the mouse (Figure IV in the online-only Data Supplement). Moreover, we found that the uptake of PEGylated PFCs compared with neat PFCs is likewise impaired for human THP-1 monocytes (Figure 6B).

**Discussion**

In the present study, we report a novel technique for the noninvasive detection of thrombi and pulmonary embolism that is based on 19F MRI combined with α2AP-targeted PFCs. With the use of SPIT, targeting ligands were attached to preformed PFCs under mild conditions that maintained their integrity. This approach proved to be suitable for the detection of developing thrombi with a diameter of <1 mm in a reasonable scan time (30 minutes) and with high sensitivity (signal-to-noise ratio, 70). Specificity is further provided by the rapid clearance of α2AP-PFCs from the bloodstream by liver and spleen. In addition, uptake of α2AP-PFCs by blood monocytes is strongly impaired compared with unmodified PFCs, resulting in the absence of PFC accumulation in inflamed areas and therefore in a strongly reduced background signal.

Ultrasound is the gold standard for the diagnosis of deep venous thrombi of legs but is not suitable for structures located more deeply (eg, lung). 1H MRI has a high spatial resolution, but the application of targeted contrast agents based on iron oxide may be difficult, particularly in lung tissue, which appears black in conventional MR scans. MR angiography can indicate the presence of thrombi, but the detection of small thrombi with only little impact on the blood flow is challenging. Consistent with this notion, we found no clear evidence for thrombus formation by 1H-based MR angiography in the present study (Figure IIIA in the online-only Data Supplement). Unambiguous anatomic identification of thrombosis by high-contrast 1H spin-echo images, however, requires prior knowledge of the localization and a quite high resolution of the 1H MRI. Similar considerations apply for the gadolinium-based contrast agent (EP-2104R) that has successfully been used for thrombus detection in a variety of preclinical animal studies and in humans.5–11 In contrast, 19F MRI, because of the lack of 19F in the body, provides highly specific hot spots, and their anatomic location can be easily determined by merging with anatomic 1H reference scans. This enabled us to precisely detect α2AP-PFC-labeled thrombi in the vena cava with high specificity and excellent signal-to-noise and contrast-to-noise ratios because of the low level of tissue background. Quantification of the 19F signal within the thrombus revealed that 150 nmol 19F nuclei per voxel (0.16 mm3) resulted in a signal-to-noise ratio of ~70 (Figure VI in the online-only Data Supplement). This translates to a local PFC concentration of ~40 mmol/L, which is substantially higher than reported for EP-2104R (<0.25 mmol/L).9,35–39 However, there are several future options to optimize our present approach: Enhanced sensitivity can be obtained by increased voxel size or by improvements in hardware and imaging sequences. Zhong et al39 recently achieved an 8-fold decrease in scan time by implementing compressed sensing for 19F MRI, which could alternatively be used for lowering the PFC doses. On the other hand, given the high signal-to-noise ratio observed in this study, the applied dose of the contrast agent could be reduced several-fold in either case.

Generation of targeted PFCs with labile ligands is generally hampered by the manufacturing process, which usually requires high pressure to obtain stable nanoemulsions with a narrow size distribution.10 High-pressure homogenization causes substantial shear forces and cavitation; the latter generates local heat of up to 104 K by implosion of dispersant...
gas bubbles resulting from low static pressure. Obviously, such conditions are disadvantageous for the handling of sensitive ligands such as peptides, antibodies, or antibody fragments. This might explain the low signal-to-noise ratios of recent approaches for in vivo $^{19}$F MRI using targeted PFCs in a tumor model and after lung ischemia. SPIT, used here to modify preformed PFCs, overcomes the problem of possible ligand destruction when linked before high-pressure homogenization. Insertion of the targeting ligand by SPIT can be performed at room temperature with gentle agitation. Furthermore, SPIT-targeted PFCs can be produced in very small quantities down to $\leq$100 $\mu$L, which cannot be handled by conventional methods because homogenizers for such small volumes are not available. Importantly, this technique makes use of cholesterol-PEG, which is well tolerated by organism and cells (Figure IIC in the online-only Data Supplement). In addition, SPIT is not restricted to peptides and antibodies but also works with different reactive groups and ligands such as single-chain monoclonal antibodies or small proteins.

So far, $^{19}$F MRI has been widely used for immune cell tracking because of the efficient uptake of unmodified PFCs by blood monocytes after intravenous injection. Tracking of blood cells without phagocytic properties (such as T cells) or distinct cell types (dendritic cells, monocyte or macrophage subsets) previously required ex vivo labeling with PFCs and reimplantation. However, attachment of specific ligands for distinct surface epitopes to PFCs by the postinsertion technique should enable the direct in vivo labeling of target cells after intravenous injection in future studies.

MRI offers excellent spatial resolution, and the sensitivity of $\alpha_2$AP-PFCs should be suitable to detect newly formed small thrombi that only partially affect the blood flow in thromboembolic lungs or deep veins in a clinically relevant situation. Specific assessment of early thrombus formation versus organized thrombi will improve the selection of patients who benefit from fibrinolytic therapy and will help to adequately adjust doses in lysis schemata, thereby reducing serious side effects such as bleeding in situations when chronic thrombi are resistant to fibrinolytic therapy. In vivo imaging of FXIIIa activity via $\alpha_2$AP-PFCs could further aid in monitoring of imminent thrombus formation after implantation of such devices as pacemakers, valves, or scaffolds and in identifying latent thrombus generation in structures characterized by low blood flow velocities and prone to stasis such as the left atrial appendage. Besides coupling $\alpha_2$-antiplasmin peptide, SPIT holds the potential to be adapted for single-chain monoclonal antibodies raised against activated glycoprotein IIb/IIIa or peptides that bind to existing thrombi, also enabling the assessment of persistent thrombi by $^{19}$F MRI.

The method described here has significant translational potential. Similar to mice, $\alpha_2$AP-targeted PFCs also specifically bind to human thrombi (Figure 6) owing to the species-independent cross-linking of $\alpha_2$-antiplasmin with FXIIIa. Specific targeting, in combination with PEGylation, also ensures that the nanoparticle uptake by monocytes is strongly reduced. Furthermore, perfluorocarbons are biochemically inert as a result of the strong C-F bond, which cannot be cleaved by enzymes, and are therefore nontoxic. Human application of SPIT is feasible with the use of the clinically relevant perfluorooctyl bromide and perfluorodecalin emulsions, which are characterized by short biological half-lives and have been used in human trials. Because of the excellent sensitivity and specificity, we propose that $\alpha_2$AP-PFCs, in conjunction with $^{19}$F MRI, are a suitable future option for the detection of small thrombi even with a clinical MR scanner at 3 T.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

The formation of fibrin-rich deep venous thrombi has a high incidence in the elderly, obese people, and, especially, in patients with tumors, strongly affecting morbidity and mortality. For specific visualization of newly formed thrombi, we used α2-antiplasmin peptide–labeled perfluorocarbon nanoemulsions as the contrast agent, which is cross-linked by factor XIIIa to the developing fibrin network. By simultaneous acquisition of matching anatomic proton (1H) and fluorine (19F) magnetic resonance images, this approach can precisely locate newly formed thrombi as “hot spots.” 19F magnetic resonance imaging is a background-free imaging approach that provides robust signals with an excellent degree of specificity, allowing an imaging scenario in the clinical setting as follows: Without prior knowledge of the thrombus location, a fast, low-resolution, whole-body 19F magnetic resonance imaging scan could be carried out. After identification of 1 or more 19F hot spots, additional high-resolution 1H and 19F scans could be recorded solely at these predefined regions for unambiguous anatomic localization of the developed thrombi. The specific assessment of early thrombus formation versus organized thrombi can improve the selection of patients who benefit from fibrinolytic therapy and help to adjust doses in lysis schemata, reducing serious side effects when chronic thrombi are resistant to therapy. In vivo imaging of factor XIIIa activity via α2-antiplasmin peptide–labeled perfluorocarbon nanoemulsions could further aid in monitoring of imminent thrombus formation after implantation of devices such as pacemakers, valves, or scaffolds and in identifying latent thrombus generation in structures characterized by low blood flow velocities and prone to stasis such as the left atrial appendage.
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SUPPLEMENTAL MATERIAL

EXPANDED METHODS

Perfluorocarbon nanoemulsions

*Formation:* Perfluorocarbon nanoemulsions (PFCs) were prepared as previously reported\(^1,2\). In brief, 2.4% (w/w) phospholipid (Lipoid S75, Lipoid AG, Ludwigshafen, Germany) was dispersed in 10 mM phosphate buffer (isotonized with glycerol), 20% (w/w) perfluoro-15-crown-5 ether (ABCR, Karlsruhe, Germany) was added to the dispersion and a crude emulsion was formed by high shear mixing (Ultra Turrax TP 18/10; IKA-Werke, Staufen, Germany). High pressure homogenization was performed in 10 cycles at 1000 bar (Avestin Emulsiflex C5, AVESTIN Europe, Mannheim, Germany). PFCs were heat-sterilized in glass vials under standard conditions (121 °C, 2 bar, 20 min.). Average particle size was determined by photon correlation spectroscopy to be 149±15 nm. Rhodamine-labelled PFCs were prepared as described previously\(^1,2\). Equal intensities of the fluorescence signal for different rhodamine-labelled emulsions were confirmed by an IVIS spectrometer (Perkin Elmer, Rodgau, Germany, see supplemental Fig. S2A).

*Fluorine content:* Given a spherical shape of these nanoparticles, for 150 nm diameter a volume of ~1.8 zeptoliter (10-21 L) is calculated containing approximately 3250 PFC molecules. Since the perfluoro-15-crown-5 ether has 20 identical \(^{19}\text{F}\) atoms this equals ~65000 \(^{19}\text{F}\) nuclei per nanoparticles. Thus, the concentration of \(^{19}\text{F}\) nuclei within one particle is calculated to be ~60 mol/L.

Sterol-based post-insertion technique (SPIT)

*Generation of the cholesterol-PEG\(_{2000}\)-maleimide anchor:* An equimolar mixture of maleimide-PEG\(_{2000}\)-NH\(_2\) (Jenkem Technology, Plano, TX), cholesteryl chloroformate (Sigma Aldrich, Seelze, Germany) and the activator triethylamine (Carl Roth, Karlsruhe, Germany) in
water-free methylene chloride was prepared. The mixture was stirred for 24 h under exclusion of light in a nitrogen atmosphere. The resulting cholesterol-PEG\textsubscript{2000}-maleimide was purified by chromatography using a sephadex LH20 column and validated by $^1$H NMR spectroscopy. Aliquots were stored at -80 °C under argon.

**Coupling of $\alpha$\textsubscript{2}-antiplasmin peptide to the cholesterol-PEG anchor:** To generate PFCs for site specific targeting of thrombi by $^{19}$F MRI, we used a 14 amino acid peptide derived from $\alpha$2-antiplasmin ($\alpha$\textsuperscript{2}AP) which is known to be cross-linked to fibrin at the glutamine Q3 by factor XIII$\alpha$\textsuperscript{3-5}. As control, Q3 was converted to alanine (Q3A) leading to a low affinity substrate for FXIII$\alpha$\textsuperscript{5}. Both peptides were further functionalized with a cysteine residue at amino acid position 13 (supplemental Fig. S1) for coupling to the cholesterol-PEG anchor. For immunofluorescence studies, carboxyfluorescein (CF) was linked via an additional lysine at the c-terminal tryptophan (W14).

All peptides (Genaxxon, Ulm, Germany) were dissolved in sterile phosphate buffer (10 mM phosphate, isotonized with glycerol, pH 7.4) to 5 mg/ml. The peptides were added to the cholesterol-PEG\textsubscript{2000}-maleimide (Chol-PEG\textsubscript{2000}-Mal) anchor and the mixture was shaken at 17 °C for 20 h at 700 rpm. The cholesterol-anchor was used in 10-fold excess compared to the ligand, thus allowing a quantitative coupling of peptides. During the incubation period the thiol-group of the cysteine residue and the maleimide group form a stable thioether bond\textsuperscript{6}. Free maleimide groups were subsequently deactivated by addition of mercaptoethanolamine. By this way, a mixture of cholesterol-PEG\textsubscript{2000}-peptide and deactivated cholesterol-PEG\textsubscript{2000} (approximate ratio of 1:10) was obtained and used in the following insertion step.

**Post-insertion:** Preformed PFCs were incubated with the obtained mixture of cholesterol-PEG\textsubscript{2000} (chol-PEG) and cholesterol-PEG\textsubscript{2000}-$\alpha$\textsuperscript{2}AP or cholesterol-PEG\textsubscript{2000}-$\alpha$\textsuperscript{2}Q3A on a rotary shaker at 17 °C for one hour. As illustrated in Fig. 1A, this leads to the spontaneous insertion of the cholesterol moiety into the phospholipid layer of the PFC\textsuperscript{7} (molar ratio phospholipid to
cholesterol derivative of 20:1). In addition, PEGylated but non-targeted PFCs were formed by incubation with non-modified cholesterol-PEG\textsubscript{2000} only.

**Characterization of PFCs**

The resulting emulsions were characterized by photon correlation spectroscopy (PCS) on a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) to determine the hydrodynamic diameter, the polydispersity index (PDI) and the $\zeta$-potential. In comparison to non-modified PFCs, we observed a slight increase in size (diameter: non-targeted PFCs = 149±15 nm; $\alpha$\textsuperscript{2AP}-PFCs = 165±13 nm), a similar size distribution (polydispersity index: non-targeted PFCs = 0.14±0.01; $\alpha$\textsuperscript{2AP}-PFCs = 0.16±0.05), but a less negative $\zeta$-potential (non-targeted PFCs = -37.2±4 mV; $\alpha$\textsuperscript{2AP}-PFCs = -11.7±7 mV) for the targeted or PEGylated PFCs. This indicates the successful incorporation of chol-PEG-$\alpha$\textsuperscript{2AP} into the PFC nanoparticles. $^{19}$F MRI measurements confirmed that all nanoemulsions exhibited the same fluorine content (supplemental Fig. S2B).

**Animal experiments**

Animal experiments were approved by the „Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen” and were performed in accordance with the national guidelines on animal care. Male mice (C57BL/6; 25-30 g body weight; 8-10 weeks of age) used in this study were bred at the central animal facility of the Heinrich Heine University (Düsseldorf, Germany) and fed with a standard chow diet and received tap water *ad libidum*.

**Induction of venous thrombi and pulmonary thromboembolism**

During surgery, mice were kept under anaesthesia with 1.5% isoflurane. Buprenorphin was injected for analgesia. A median laparotomy was performed, and the inferior *vena cava* was exposed at the anatomical level of both kidneys. Subsequently, a filter paper (1×4 mm\textsuperscript{2})
soaked with 10% FeCl₃ was placed on the top of the vessel and incubated for 8 min. To assure the location of the FeCl₃-soaked filter paper on top of the vessel surface, two stretches of parafilm were placed on both sides of the vessel. After removal of the filter paper the vessel was washed with 0.9% NaCl to remove residual FeCl₃. PFCs (3 mmol/kg body weight) were injected into the tail vein approximately 5 min prior to thrombus induction or 5, 15, 30, 60 or 90 min post thrombus induction. Subsequently, MRI scans were performed at 2, 8 or 24 h after surgery. To induce pulmonary thromboembolism a mixture of human thrombin (Sigma-Aldrich, Seelze, Germany, 10U/25 g body weight) and α₂AP-PFCs (or unmodified PFCs or Q3A-PFCs as control) was injected which resulted in an 80% survival rate. ¹H/¹⁹F MRI measurements were performed 24 h later.

**MRI studies**

Experiments were performed at a vertical 9.4 T Bruker AVANCE III Wide Bore NMR spectrometer (Bruker, Rheinstetten, Germany) operating at frequencies of 400.21 MHz for ¹H and 376.54 MHz for ¹⁹F measurements using microimaging units as described previously.¹²⁻¹¹ Mice were anaesthetized with 1.5% isoflurane and were kept at 37 °C during the measurements. Data were acquired using a 25-mm birdcage resonator tuneable to ¹H and ¹⁹F. After acquisition of the morphological ¹H images, the resonator was tuned to ¹⁹F and anatomically matching ¹⁹F images were recorded. An overview over all imaging parameters used for ¹H/¹⁹F MRI is given in supplemental table 1.

For *in vivo* imaging of thrombi, ¹H MR reference images from the abdomen were acquired using a rapid acquisition and relaxation enhancement sequence (RARE; field of view (FOV) = 2.56×2.56 cm², matrix 256×256, 0.1×0.1 mm² in plane resolution, 1 mm slice thickness; TR = 3000 ms; RARE factor = 16, 6 averages, scan time = 4 min). ¹H MR angiography for visualization of stenosis and restricted blood flow was carried out by a ¹H FLASH-2D flow compensated sequence (fast low angle shot = FLASH; FOV = 2.56×2.56 cm², matrix
256×256, 0.1×0.1 mm² in plane resolution, 0.4 mm slice thickness; TR = 10 ms; 6 averages; scan time = 4 min). For imaging of the thorax a retrospective-gated gradient echo sequence (Intragate®, Bruker. Rheinstetten, Germany) was used (2.56×2.56 cm², 256×256 matrix, 0.1×0.1 mm² in plane resolution, 1 mm slice thickness, TR = 5.8 ms, TE 1.3 ms, scan time 2 min). Corresponding ¹⁹F images were recorded from the same FOV with a ¹⁹F RARE sequence (matrix 64×64, 0.4×0.4 mm² in plane resolution, 1 mm slice thickness; TR = 4000 ms; RARE factor = 32; 256 averages; scan time = 34 min).

For analysis of PFC washout kinetics, blood flow was visualized by a ¹H FLASH-2D flow compensated sequence (fast low angle shot = FLASH; FOV = 2.56×2.56 cm², matrix 256×256, 0.1×0.1 mm² in plane resolution, 2 mm slice thickness; TR = 10 ms; 2 averages; scan time = 1 min). Thereafter, circulating PFCs in the blood (i.e. vena cava inferior) were determined by a ¹⁹F FLASH sequence (FOV = 2.56×2.56 cm, matrix 32×32, 0.8×0.8 mm² in plane resolution, 2 mm slice thickness; TR = 50 ms; 375 averages; scan time = 10 min). To compare the half-life of the individual PFCs in the blood, the signal-to-noise ratio of the ¹⁹F signal was determined for each time point, which was used to calculate the area under the curve (AUC) over the entire observation period (24 h).

The full protocol for in vivo MRI studies including both ¹H and ¹⁹F imaging took around 20-30 minutes (detection of ¹⁹F signal in flowing blood) or 50-60 minutes (venous thrombi and pulmonary emboli) and was well tolerated by all mice which recovered from anaesthesia within five minutes.

For high-resolution images, thrombi were excised, PFA fixed, embedded in 1% agarose and analysed by MRI. 3D ¹H/¹⁹F RARE sequences with an FOV of 1×1×1 cm³ and a matrix of 128×128×128 (¹H = 0.04×0.04 mm² in plane resolution, 80 averages, TR = 2500 ms, 35 h scan time; ¹⁹F = 0.04×0.04 mm² in plane resolution, 170 averages, 67 h scan-time) were used to obtain isotropic data sets. Excised and PFA-fixed lungs were measured by 3D ¹H/¹⁹F
RARE sequences with an FOV of 2.2×2.2×0.8 cm³ and a matrix of 256×256×92 (¹H = 0.09×0.09 mm² in plane resolution, 32 averages, TR = 2500 ms, 19 h scan time; ¹⁹F = 0.09×0.09 mm² in plane resolution, 128 averages, 65 h scan-time). 3D reconstruction was performed with Amira (FEI Visualization Sciences Group, Burlington, MA) by segmentation of the lung tissue and the pulmonary branches which were combined with a maximum intensity projection of the fluorine signal (supplemental Movie 1).

An overview of the different imaging parameters for individual ¹H/¹⁹F scans is given in supplemental Table 1.

**In vitro thrombus generation and PFC uptake**

Human blood was obtained by venous puncture and collected on ice. 100 µl of blood were transferred to a round-bottom 96-well plate and incubated at 37 °C for 15 min. Next, 25 µl of PFCs (non-targeted PFCs or α2AP-PFCs) were added to each well and the plate was further incubated for 90 min at 37 °C under constant motion. The blood clots were extensively washed with cold PBS and subjected to ¹H/¹⁹F MRI.

For PFC uptake studies, blood was obtained as above and subjected to erythrocyte lysis. Cells were resuspended to a density of 10⁶ cells/ml in DMEM (Sigma Aldrich, Seelze, Germany) containing 10% FCS (Biochrom GmbH, Berlin, Germany) pre-warmed to 37 °C. 5 µl of rhodamine-labelled non-targeted PFCs or PEG-PFCs were added to 500 µl cell suspension and incubated for 5, 30, 60 and 120 min. 5 ml ice-cold PBS was added to stop the reaction, cells were subsequently fixed with 0.5% PFA and resuspended in MACS buffer. Cells were analysed with a LSR-Fortessa flow cytometer (BD Biosciences, Heidelberg, Germany) and data were processed with FCS Express 4 (Denovo Software, Glendale, CA). Monocytes were identified by SSC/FSC profile and CD11b expression. Additionally, THP-1 cells (a human monocytic cell line) was also incubated with rhodamine-containing non-targeted PFCs or PEG-PFCs for 120 min and analysed by flow cytometry.
Cytotoxicity assay
Murine splenocytes (approx. $10^6$ cells/200 µl) from C56BL/6 mice were incubated with PFCs, PEG-PFCs, α2AP-PFCs or Q3A-PFCs (1 µl PFC per 100 µl cell suspension) in DMEM (10% FCS, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml sodium pyruvate) for 5 h at 37 °C. Subsequently, cells were washed and stained with CD45 mAb (see below) and DAPI to calculate the amount of viable cells. The same protocol was used to determine the cytotoxic effect of PFCs on THP-1 cells, except for the incubation period, which was 24 h. Flow cytometry confirmed the non-cytotoxicity of SPIT modified PFCs (supplemental Fig. S2C).

Flow cytometry
Mice were killed by cervical dislocation thrombi were excised and digested in streptokinase/plasmin (150 U/ml and 2 U/ml) for 30 min at 37 °C under constant shaking. To generate a single cell suspension, thrombi were passed through a 70 µm cell strainer (BD Biosciences, Heidelberg, Germany), washed with FACS buffer (PBS, 5% BSA, 0.5 mM EDTA and 0.1% NaN₃) and stained for 30 min with fluorochrome-coupled antibodies: CD45 (PE; 3F11.1), CD11b (APC; M1/70), CD3 (APC; 145-2c11) (all Miltenyi Biotec, Bergisch Gladbach, Germany), Ly6G (FITC; 1A8; BD-Pharmingen, Heidelberg, Germany), B220 (APC.Cy7; RA3 6B2) and CD41 (PE-Cy.7; MWReg30) (both: eBiosciences, San Diego, CA).

Histology and fluorescence microscopy
Excised thrombi and lungs were fixed in 4% PFA or embedded in Tissue-Tek (Weckert Labortechnik, Kitzingen, Germany) and frozen at -20 °C. Sections of 8 µm or 14 µm were cut and processed for immunohistochemical staining as described previously*. In brief, thrombus sections were fixed in Zamboni's fixation for 10 min and then incubated with
hematoxylin and eosin or picrosirius red (Sigma Aldrich, Seelze, Germany) according to the manufacturer’s instructions. For fluorescence microscopy, samples were mounted in ProLong® Gold (Life Technologies GmbH, Darmstadt, Germany). Slides were analyzed with an Olympus BX61 fluorescence microscope and processed by “cellSens Dimension” digital imaging software (Olympus, Hamburg, Germany) or a Zeiss LSM710 confocal laserscanning microscope and analyzed with ZEN 2012 light (both Zeiss, Jena, Germany).

Statistics

Statistical tests were performed with „R“ (http://www.r-project.org/index.html). All data were evaluated for normal distribution using the Shapiro-Wilk test and values are given as mean ± standard deviations (SD). Statistical difference was assessed by the Welch’s test (for unequal variances) and a level of p<0.05 was considered statistically significant.

REFERENCES


**SUPPLEMENTAL TABLE 1**

Summary of acquisition parameters and n-numbers for individual $^1$H/$^{19}$F MR experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nucleus</th>
<th>FOV (mm$^2$)</th>
<th>Matrix</th>
<th>Slice Thickness (mm)</th>
<th>Averages</th>
<th>In plane Resolution (mm$^2$)</th>
<th>Acquisition time</th>
<th>n-numbers</th>
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<td>$^1$H</td>
<td>25.6x25.6</td>
<td>256x256</td>
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<td>6</td>
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<td></td>
<td>$^{19}$F</td>
<td>25.6x25.6</td>
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<td>256</td>
<td>0.4x0.4</td>
<td>34 min</td>
<td>8/6/24*</td>
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<td>25.6x25.6</td>
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<td>6</td>
<td>0.1x0.1</td>
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<td>0.04x0.04</td>
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<td></td>
<td>$^{19}$F</td>
<td>10x10</td>
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<td>0.08x0.08</td>
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<td><em>Ex vivo</em> human thrombus generation</td>
<td>$^1$H</td>
<td>20x20</td>
<td>128x128</td>
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* n=8 for specific α2AP-PFC, n=6 for control Q3A-PFC and n=4 per time point for temporal analysis of α2AP-PFC accumulation within the thrombus when injected -5, 5, 15, 30, 60, and 90 min injection after induction.

** Retrospective gating for 16 frames per heart cycle.
FIGURE AND MOVIE LEGENDS

Figure S1:
Schematic structure of the cholesterol-PEG$_{2000}$-maleimide and the $\alpha_2$-antiplasmin peptide [peptide structure adapted from Miserus et al. (2009)]. In control experiments, glutamine (Q3) was changed to alanine (Q3A) to impair factor XIIIa-mediated cross-linking with fibrin. Cholesterol-PEG$_{2000}$-maleimide was linked to the peptides via an additional cysteine by formation of a stable thioether bond (arrow).

Figure S2:
A) Comparison of the fluorescence signal of rhodamine-labelled neat PFCs (Neat) and PEG-PFCs (PEG). Data are mean values ± SD of four experiments. B) $^{19}$F signal (SNR) of neat PFCs (Neat), PEG-PFCs (PEG), $\alpha_2$AP-PFCs (a2) or Q3A-PFCs (Q3A) showing that the $^{19}$F signal is similar for all nanoemulsions. Data are mean values ± SD of four individual measurements. C) Viability of murine splenocytes and THP-1 cells after incubation (5 h for splenocytes; 24 h for THP-1 cells) with neat PFCs, PEG-PFCs, $\alpha_2$AP-PFCs or Q3A-PFCs. The amount of DAPI-negative cells (= viable cells) determined by flow cytometry is displayed. Data are mean values ± SD of three experiments.

Figure S3:
A) MR angiography of control animals (upper panel) and mice with FeCl$_3$-induced venous thrombi (lower panel). Left: Maximum intensity projection (MIP) visualizing the blood flow in the vena cava (VC) and the aorta abdominalis (AA; right). Middle: Axial slices of angiographic $^1$H FLASH scans at the level of thrombus induction. Right: $^1$H RARE scans demonstrating the absence (upper row) or the presence (lower row) of a venous thrombus. Yellow arrows indicate the location of the thrombus. B) Contrast to noise ratio of $^1$H/$^{19}$F
signals between thrombi and vessel lumen, muscle and connective tissue, respectively, next to
the location of the thrombus. (Data are mean values of n=8; *p<0.05)

**Figure S4:**

(A) *In vivo* $^{19}$F signal after application of $\alpha_2$AP-PFCs at different times after induction of
venous thrombi in mice and (B) *ex vivo* generated human thrombi. Data are mean values ±
SD of four experiments.

**Figure S5:**

Combined $^1$H/$^{19}$F MRI of animals after thrombin + PFC or thrombin + Q3A-PFC injection.
Note the absence of any $^{19}$F signal within the dark lung tissue, even for neat PFCs, which
indicates the absence of inflammation.

**Figure S6:**

Detection threshold for $^{19}$F MRI using $\alpha_2$AP-PFCs at a voxel size of 0.16 mm$^2$. Emulsions
were diluted and measured by $^1$H/$^{19}$F MRI using identical settings as for the *in vivo* MRI. The
arrow indicates the observed SNR within the thrombus in our study. The SNR of ~70
observed *in vivo* corresponds to approximately 150 nmol $^{19}$F atoms/voxel (with a voxel-size
of 0.16 mm$^3$), while the detection threshold was around 10 nmol/voxel. With 150 nmol $^{19}$F
atoms (i.e. $150\times10^{-9}$ mol) in a volume of 0.16 mm$^3$ (i.e. $0.16\times10^{-6}$ liter), a $^{19}$F molarity of
$\sim940\times10^{-3}$ mol/L ($150\times10^{-9}/0.16\times10^{-6}$ mol/liter) or 940 mmol/L can be calculated. The
perfluoro-15-crown-5 ether contains 20 equivalent $^{19}$F atoms and therefore the concentration
of the PFC within the thrombus is in the range of 40-50 mmol/L (940/20) and the detection
threshold under the current setting would be around 0.3 mmol/L PFC (40/15). Thus, the ratio
between accumulated (40 mmol/L) and injected dose (3 mmol/kg BW) is approximately 13.
Supplemental Movie 1:

Animated 3D reconstruction of an *ex vivo* high resolution $^1$H/$^{19}$F MRI data set of the thromboembolic lung displayed in Fig. 5 showing the surface of the pulmonary lobes (blue), the internal structures (pulmonary branches; grey) and the $^{19}$F signal (red). Note that the $^{19}$F signal is located in vicinity to pulmonary branches and that signals can be also identified in the periphery of the lung.
Figure S2

A  Fluorescence

B  \(^{19}\)F signal

C  

Murine splenocytes

THP-1 cells
Figure S3

A

<table>
<thead>
<tr>
<th>Control</th>
<th>MIP</th>
<th>$^1$H FLASH</th>
<th>$^1$H RARE</th>
</tr>
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<td>VC</td>
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<tr>
<td>FeCl$_3$</td>
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<td>AA</td>
<td>VC</td>
</tr>
</tbody>
</table>

B

Comparison of contrast to noise ratio for different tissues:

- **Vessel**: $^1$H MRI > $^{19}$F MRI
- **Muscle**: $^1$H MRI > $^{19}$F MRI
- **Tissue**: $^1$H MRI > $^{19}$F MRI

*p* values indicate statistically significant differences.
Figure S4

*In vivo:*
Murine DVT

*Ex vivo:*
Human thrombi
Figure S5

$\text{PFC}$  $\text{Q3A-PFC}$

$^{1}\text{H}/^{19}\text{F MRI}$
Figure S6