Non-invasive imaging of deep venous thrombi by 19F MRI using targeted perfluorcarbon nanoemulsions

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Purpose: Detection of deep venous thrombi which are not accessible by ultrasound is still a serious challenge. Therefore a non-invasive technique for unequivocal identification of those thrombi for diagnosis and subsequent monitoring of antithrombotic therapy would be highly desirable. Up to now ^{19}F MRI has been widely used for cell tracking purposes either after *ex-vivo* or *in-vivo* loading of target cell populations. However, intravenous injection of perfluorocarbon nanoemulsions (PFC-NE) results in rapid clearance of circulating PFC-NE particles, since they are avidly taken up by blood monocytes and organs of the reticuloendothelial system. Therefore, a minimization of this phagocytic uptake is a prerequisite for an efficient targeting of PFC-NE to other cells or structures. Moreover, generation of targeted PFC-NE is hampered by the high-pressue homogenization process (about 1000 bar) required for preparation of PFC-NE which may easily destroy thermodynamically labile ligands. Thus, in the present study, we aimed at evaluating a novel post-insertion technique for generation of targeted PFC-NE, which makes use of a cholesterol-polyethylenglycol$_{2000}$-maleimide anchor (Chol-PEG$_{2000}$). Here, the targeting ligand can be coupled to the maleimide group under very mild conditions while the cholesterol-anchor smoothly inserts into the lipid layer of preformed PFC-NE and the PEG moiety concomitantly impairs the PFC uptake by monocytes (Fig. 1a).

Methods: For targeting of freshly induced thrombi, we used the 14 amino-acid α2-antiplasmin ($\alpha 2^{AP}$) peptide which is known to be cross-linked to fibrin *via* the glutamine (Q3) by active factor XIII (Miserus *et al., JACC Cardiovasc. Imaging* ***2****, 8* (2009)). As control Q3 was mutated to alanine (Q3A). Peptides were conjugated to the Chol-PEG$_{2000}$ anchor *via* an additional cysteine residue in the peptide to obtain Chol-PEG-$\alpha 2^{AP}$ or the Chol-PEG-$\alpha 2^{APQ3A}$ (control) conjugate. Both constructs were inserted into preformed PFC-NE (containing 20% perfluoro-15-crown-5 ether) by incubation at 37 °C for 1 h and were subsequently characterized by photon correlation spectroscopy (PCS). Thrombi were induced *in-vivo* using a filter paper soaked with 10% $FeCl_3$ which was applied to the external side of the *vena cava inferior* for 8 min and resulted in a non-occlusive thrombus. Since factor XIII is active during the early phase of the thrombus formations, we injected $\alpha 2^{AP}$-targeted-NE (or NE with $\alpha 2^{APQ3A}$ as control) 5 min prior to $FeCl_3$ application. Combined ^{1}H/^{19}F MRI was performed 2 h, 8 h or 24 h after PFC injection at a vertical 9.4 T Bruker Avance^{III} Wide Bore NMR spectrometer using a microimaging unit (Micro 2.5) with an actively shielded 40-mm gradient set (1 T/m maximum gradient strength and 110 µs rise time at 100 % gradient switching. Mice were placed in a 25-mm ^{1}H/^{19}F birdcage resonator and analyzed using standard ^{1}H/^{19}F multi-slice RARE sequences (^{19}F RARE: 2.56×2.56 cm^2 FoV, 64×64 matrix, 1 mm slice thickness, TR 4000 ms, 256 averages, 34 min acquisition time). Subsequently, excised thrombi (fixed and embedded in agarose) were examined *ex-vivo* by high resolution ^{1}H/^{19}F MRI (^{19}F 3D RARE: 1×1×1 mm^3 FoV, 128×128 matrix, 67 h scan time). For further *ex-vivo* analysis, FITC-labelled α2-NE were applied, thrombi were excised and processed for histology or flow cytometry.

Results and Discussion: Chol-PEG$_{2000}$-$\alpha 2^{AP}$ nanoemulsions (a2^{AP}-NE) were analyzed by PCS measurements and compared to the pre-formed PFC-NE stock solution. We observed a slight increase in size (diameter: NE = 149±15 nm; a2^{AP}-NE = 165±13 nm), a similar size distribution (polydispersity index: NE = 0.136±0.01; a2^{AP}-NE = 0.164±0.05), and a profoundly reduced ζ-potential (NE = -37.2±4 mV; a2^{AP}-NE = -11.7±7 mV) indicating the successful incorporation of Chol-PEG$_{2000}$-$\alpha 2^{AP}$ into PFC-NE. PEGylation of particles resulted also in significantly altered *in-vivo* distribution when intravenously injected immediately prior to thrombus induction. Unmodified PFC-NE were quickly phagocytosed, which gave rise to a strong ^{19}F signal in the area of the surgery due to infiltration of PFC-loaded immune cells, but no PFC deposition was noticed within the thrombus. However, upon PEGylation almost no ^{19}F signals were detected within the area of interest (Fig. 1b). The absence of untargeted PFC-NE in thrombi was further confirmed by flow cytometry which revealed infiltration of a negligible amount of monocytes and some neutrophils into the plug.

In the next step, we explored the suitability of the PEGylated PFC-NE, which were additionally equipped with the ligand $\alpha 2^{AP}$, for specific thrombus detection. To this end, a2^{AP}-NE (or $\alpha 2^{APQ3A}$-NE) were injected prior to thrombus induction and animals were analyzed by non-invasive ^{1}H/^{19}F MRI after 24 h. In animals which received a2^{AP}-NE strong ^{19}F signals could be detected within the thrombus which were absent when NE with control peptide was injected (Fig. 1c). Importantly, $\alpha 2^{AP}$-NE clearly delineated the thrombus but were not found in the area of the surgery. The location of the ^{19}F signal within the thrombus was further confirmed by analyzing isolated thrombi *ex-vivo* by high resolution ^{1}H/^{19}F MRI as well as by histology. To test whether ^{19}F MRI can also be performed quite shortly after $\alpha 2^{AP}$-NE administration, we analyzed the ^{19}F signal after 2 h, 8 h and 24 h and found that ^{19}F signal reached 80% of the 24 h signal even after 2 h which mirrors the fast completion of thrombus formation in this model.

Conclusion: ^{19}F MRI using stable α2-antiplasmin targeted PFC-NE is a valuable tool for the rapid, highly specific and unequivocal identification of freshly developed deep venous thrombi, which might have a potential clinical application in detection of rethrombosis or lung thromboembolism after surgery. Moreover, we propose that the sterol-based post-insertion technique is a promising platform to equip PFC-NE with different ligands (peptides, antibodies etc.) for *in-vivo* ^{19}F MRI of specific targets.

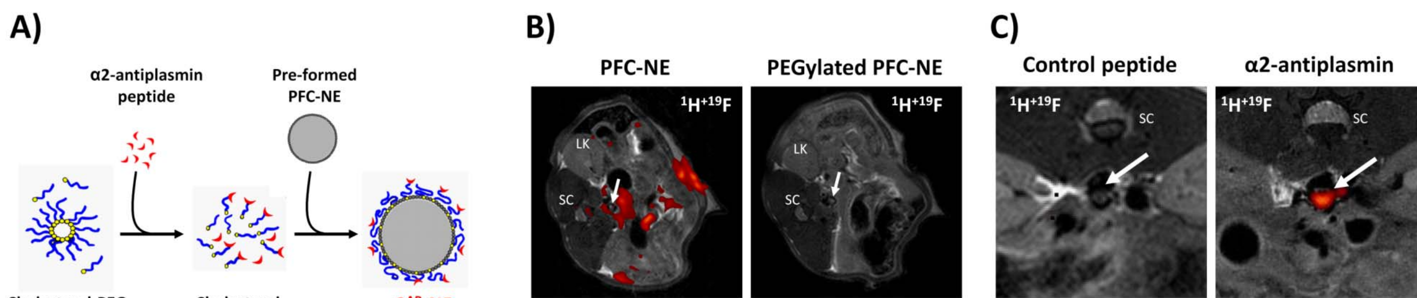

Figure 1: A) Scheme showing the principle of the sterol-based post-insertion technique for generation of α2-antiplasmin targeted PFC-NE (a2^{AP}-NE). **B)** *In vivo* ^{1}H/^{19}F MRI of mice which received intravenously administered untargeted PFC-NE (left) or PEGylated PFC-NE (right). Arrows show the location of the thrombus. **C)** Magnification of *in vivo* ^{1}H/^{19}F MRI scans of mice which received $\alpha 2^{AP}$-NE (right) or NE with a control peptide (left). Arrows indicate the location of the thrombus.