Coencapsulation of magnetic nanoparticles and doxorubicin into biodegradable microcarriers for deep tissue targeting by vascular MRI navigation

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Supplementary information

Supplementary methods

FeCo nanoparticle synthesis, annealing and encapsulation preparation

$Co_2(CO)_8$ (1.00 g) (Fisher Scientific, Ottawa, ON, Canada) and $FeCO_5$ (1.3 mL) (Sigma Aldrich, Oakville, ON, Canada) in dichlorobenzene (DCB) (12 mL) (Fisher Scientific) were transferred under inert atmosphere in a solution of DCB (48 mL), tri-n-octylphosphine oxide (TOPO) (0.4 g) (Sigma aldrich) and oleic acid (OA) (1.8 mL) (Sigma Aldrich) under reflux and steering at 200ºC. The temperature was increased until 285ºC for 35 min. The solution was then cooled down to room temperature. The colloidal solution was passed through a 0.2-µm filter (Sigma Aldrich). DCB was evaporated under vacuum rotation and FeCo nanoparticles were suspended in hexane (8 mL) (ACP, Montréal, QC, Canada). Nanoparticles were washed three times by centrifugation (45,000 x *g*, 45 min). The centrifuged particles were resuspended first in hexane (8 mL) and then diluted in ethanol (20 mL) before each centrifugation step. After the last centrifugation, the nanoparticles were dispersed in hexane (14 mL) and transferred into a crucible. After hexane evaporation, the nanoparticles were annealed under argon atmosphere at 650°C during 35 min as described in reference [1]. Annealed nanoparticles were kept under vacuum prior to their use. To clean the nanoparticles after the annealing, FeCo nanoparticles (190 mg) were suspended in dichloromethane (DCM) (10 mL) (ACP) and homogenised (10,000 rpm, 4 min) (PowerGen 700D, Fisher

ThermoScientific). Nanoparticles were placed in an ultrasonic bath during 5 min and then centrifuged (15,000 x *g*, 10 min). These steps were repeated twice. OA (3 mL) was added to the nanoparticles suspended in DCM (8 mL), and the latter were sonicated (2 min, 60 W with 2 s of pulse ON and 1 s of pulse OFF) (Sonic Dismembrator 550, Fisher ThermoScientific). The sonication step was repeated 12 times to suspend nanoparticles. Then nanoparticles were cleaned by 3 centrifugation steps (15,000 x *g*, 15 min) in ethanol (20 mL). Nanoparticles were finally resuspended in DCM (1.8 mL), sealed under an argon atmosphere and stored at 4ºC until use.

In vitro DOX release

In vitro release kinetics were performed under sink conditions. TMMC (10.5 ± 0.2 mg) with phosphate buffered saline (PBS) (5 mL) were placed under rotation at 37ºC to maintain microparticles in suspension. At each time point, TMMC were centrifuged (2000 x *g*, 5 min). The supernatant (600 µL) was taken and replaced by fresh PBS at 37ºC [2]. The DOX concentration in the supernatant was measured by spectrophotometry at 481 nm (Safire plate reader, Tecan, Durham, NC). At the end of the experiment, after centrifugation (2000 x *g*, 5 min), the supernatant was discarded and TMMC were freeze-dried (Freeze Dry System, MI, USA). Dimethyl sulfoxide (DMSO) (1.5 mL) was added on the TMMC to determine the remaining encapsulated DOX content according to the method described in the article.

Theoretical steering method

During the steering (Fig. S2A), TMMC acquired two velocities: V_{mag} corresponded to the magnetophoretic velocity created by the MRI steering, and V_{blood} corresponded to the velocity of TMMC dragged by the blood flow. V_{mag} depends of the concentration of encapsulated magnetic nanoparticles (% *w/w*), the nanoparticle saturation magnetization (Ms) (emu g^{-1}), the

nanoparticle density (kg m^{-3}), TMMC volume (m^3), the applied magnetic gradient (T m^{-1}), the TMMC radius (m) and blood viscosity (0.0035 Pa s) [1]. To be steered to the targeted bifurcation, the TMMC should have crossed half of the hepatic artery radius which represents the longest pathway case for the steering. Accordingly, the time required to cross the artery radius determined from V_{mag} should be lower than the time to reach the bifurcation dragged by the blood flow determined by V_{blood} and the distance between the catheter tip and the bifurcation. The comparison of these two times is used to determine TMMC steering properties such as diameter and Ms.

Synthesis of TMMC with reduced steering properties (Ø = 30 ± 9 µm, Ms = 49 ± 6 emu g^{-1})

These microparticles were synthesized with the same material proportions described in the article. The smaller diameter and nanoparticle loading were obtained by decreasing the penetration depth of the homogenizer probe in the mixture of FeCo-DOX-PLGA-DCM during the emulsion.

***In vitro* steering**

The MRI steering setup was composed of a 1.5-T Siemens Sonata clinical MRI scanner (Siemens, Erlangen, Germany), home-made steering gradient coils, one syringe pump (New Era Pump System Inc, Wantagh, NY), a Plexiglas rabbit hepatic artery phantom with a rectangular cross-section (width = 2 mm and depth = 1.57 mm), and an MRI compatible camera (MRC systems GmbH Heidelberg, Germany) directly above the bifurcation of the phantom [1]. The rabbit common hepatic artery is composed of the caudate bifurcation which leads the blood to the caudate lobes, and the right/left bifurcation which leads the blood to the right lobe and to the left lobes (left median lobe and left lateral lobe) [3]. Accordingly, the

phantom was composed of the caudate bifurcation placed at 13 mm from the TMMC entrance and the right/left bifurcation placed at 20 mm from the caudate bifurcation (Fig. S3). An aqueous solution of 40% (*w/w*) glycerol was used to mimic the blood viscosity [4]. The phantom was placed between two steering coils. This setup was placed in the middle of the MRI tunnel. The flow, obtained with a syringe pump, was set at 7.5 or 12 cm s^{-1}. These values were measured by Doppler ultrasound (US) in the rabbit hepatic artery. To mimic the decrease of the flow velocity during the embolization [5], the velocity was reduced from 12 to 7.5 cm s^{-1} after the injection of half of the glycerol solution (60 mL) used for a steering test. Steering gradients were +/- 400 or +/- 200 mT m^{-1} to target each channel outlet (right or left). Collected TMMC were centrifuged (2000 x *g*, 5 min). The supernatant was discarded and replaced by DMSO (1 mL) to release the encapsulated DOX as previously described.

***In vivo* protocol: complementary information**

Rabbits were anesthetized with isoflurane (2-3%) (Abbott, Saint-Laurent, QC, Canada) delivered with an MRI compatible anesthesia machine (Dispomed, Joliette, QC, Canada). During the experiment, the rabbit body temperature was maintained with a circulating water blanket (Cincinnati Sub-Zero, Cincinnati, OH). The pulse rate and the arterial oxygen saturation were monitored with an MRI compatible pulse oximeter (STARR Life Sciences Corp., Oakmont, PA). An arteriotomy was conducted on the right and left femoral artery and a 4-Fr micropuncture introducer (Cook Medical Inc, Bloomington, IN) was placed on the right side to introduce the microcatheter whereas the balloon catheter was directly placed over a 0.018 wire in the left femoral artery. In the blank animal group, we have observed spasm on the hepatic artery after performing external Doppler US, probably related to the compression of the artery on the catheter induced by the US probe. Hence, we decided to not measure blood flow velocity by US prior to TMMC injection. To minimize the magnetic aggregation

of TMMC during their injection, a tubing (1.27-mm internal diameter) (Cole-Parmer, Montréal, QC, Canada) was connected to the catheter and the injection was done at 1.5 m from MRI scanner. In the steering group, one rabbit received half of the TMMC dose (20 mg in 18 mL of saline solution) to confirm that the steering efficiency was not affected by the injected dose. Blood samples (1 mL) were taken at 15 min before and at 5, 10, 20, 40, 60 and 120 min after the TMMC injection. During the necropsy, the main hepatic artery and the gastroduodenal artery were ligatured to prevent the TMMC reflux. For the determination of the steering efficiency, the TMMC dose found in the left medial lobe and left lateral lobe were added and referred as left lobes because the steering platform used could only target one bifurcation (right/left).

TMMC distribution by histological analysis

Whole slide scanning was performed on stained sections using an automated digital slide scanner (Nanozoomer, Hamamatsu Photonics, Hamamatsu, Japan). In each slide, the TMMC surface was measured in each blood vessel with the NDP.view software (Hamamatsu Photonics). Then, the sum of the TMMC surfaces in one lobe was divided by the weight of the liver sample cut for the slide preparation. The TMMC distribution in each lobe was determined by the sum of the TMMC surfaces in the lobe divided by the total sum of the TMMC surfaces in the right and left lobes.

Plasmatic DOX concentration analysis

Fresh blood sample in EDTA coated tube (1.6 mg EDTA per mL of blood) was centrifuged (1550 x *g*, 3 min). Plasma was collected and stored at -20ºC. DOX was extracted from the plasma according to the following procedure: plasma (50 µL) was added to the internal standard solution (100 µL) composed of daunorubicin in methanol (10 ng mL^{-1}). The solution

was centrifuged (15,680 x *g*, 10 min) and was kept at 4ºC prior to its analysis. It was injected (17 µL) in a liquid chromatography-mass spectrometry system (LC-MS/MS). The system was composed of a high-performance liquid chromatography (HPLC) (Prominence, Shimadzu Prominence system, Columbia, MD), an MS equipped with an electrospray source in a positive mode of ionization (API 4000, AB Sciex Instruments, Concord, ON, Canada), a guard column (Zorbax XDB-8, 12.5 x 2.1 mm, Agilent Technologies Canada Inc, Mississauga, ON, Canada), and an analytical column (Zorbax SB-C18, 3.5 µM, 2.1 x 50 mm, Agilent Technologies Canada Inc). An acetonitrile (ACN) (mobile phase B) gradient (7-50%) in water containing 0.05% (*v*/*v*) formic acid (mobiles phase A) were used as mobile phases at a flow rate of 0.4 mL min^{-1}. An equilibration time of 4 min was set between each injection. DOX and daunorubicin were determined by multiple reaction monitoring (MRM) scan mode (in a positive mode of ionization). MRM transitions were 544 → 397 (DOX) and 528 → 321 (daunorubicin). For the calibration standard curve, DOX concentrations in plasma spanned between 100 to 10,000 pg mL^{-1}. The method was validated with quality control standards and system suitability prior and after the sample injection. MRM chromatograms were integrated using Analyst software v.1.4.2. (AB Sciex Instruments).

DOX quantification in the liver

Liver samples dedicated to DOX analysis were weighed (4.9 ± 0.6 g per lobe) and homogenized (2-3 min) (PT 10/35, Brinkmann, Mississauga, ON, Canada) with Milli-Q-water (2 mL per g of liver). Formic acid (100 µL per g of liver), daunorubicin as internal standard (18 µL per g of liver), and ACN (5.6 mL per g of liver) were added and homogenized (2-3 min). 66%-methanol-water solution (*v*/*v*) (1 mL per g of liver) was added and the mixture was vortexed (30 s). The homogenized liver solutions were centrifuged (3500 x *g*, 10 min). A sample of the supernatant (1000 µL) was evaporated (24 h) to remove the organic solvent.

Then, formic acid (88%) (6 µL) and methanol (164 µL) were added to each sample. The samples were centrifuged (15,680 x *g*, 5 min) and stored at 4 ºC. The sample (15 µL) was then analysed with the same system described for the plasmatic DOX concentration analysis. The standard curve was obtained by added free-DOX to blank liver (non-treated with TMMC) and submitted to the same protocol as previously described. The DOX concentration was calculated per g of liver and the standard curve concentration range was between 1 to 270 ng of DOX per g of liver. The extraction and analysis method were validated by measuring blank liver containing a known amount of TMMC. For the statistical analysis, in the control group, one lobe was lost during the analysis process (n = 2 for the control group); thus no statistical analysis was possible.

Supplementary figures

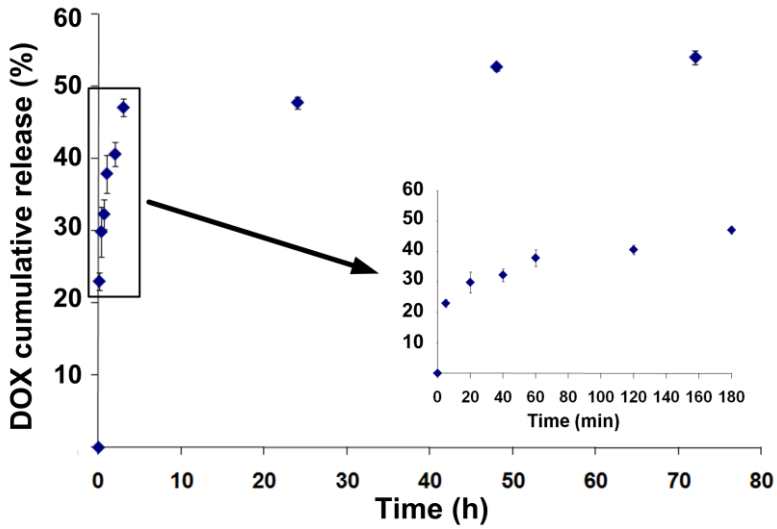

Fig. S1: *In vitro* DOX release from TMMC in PBS buffer at 37ºC under sink conditions. At the end of the assay, TMMC contained 54 ± 1% of the initial encapsulated DOX amount. Mean ± SD (n=3).

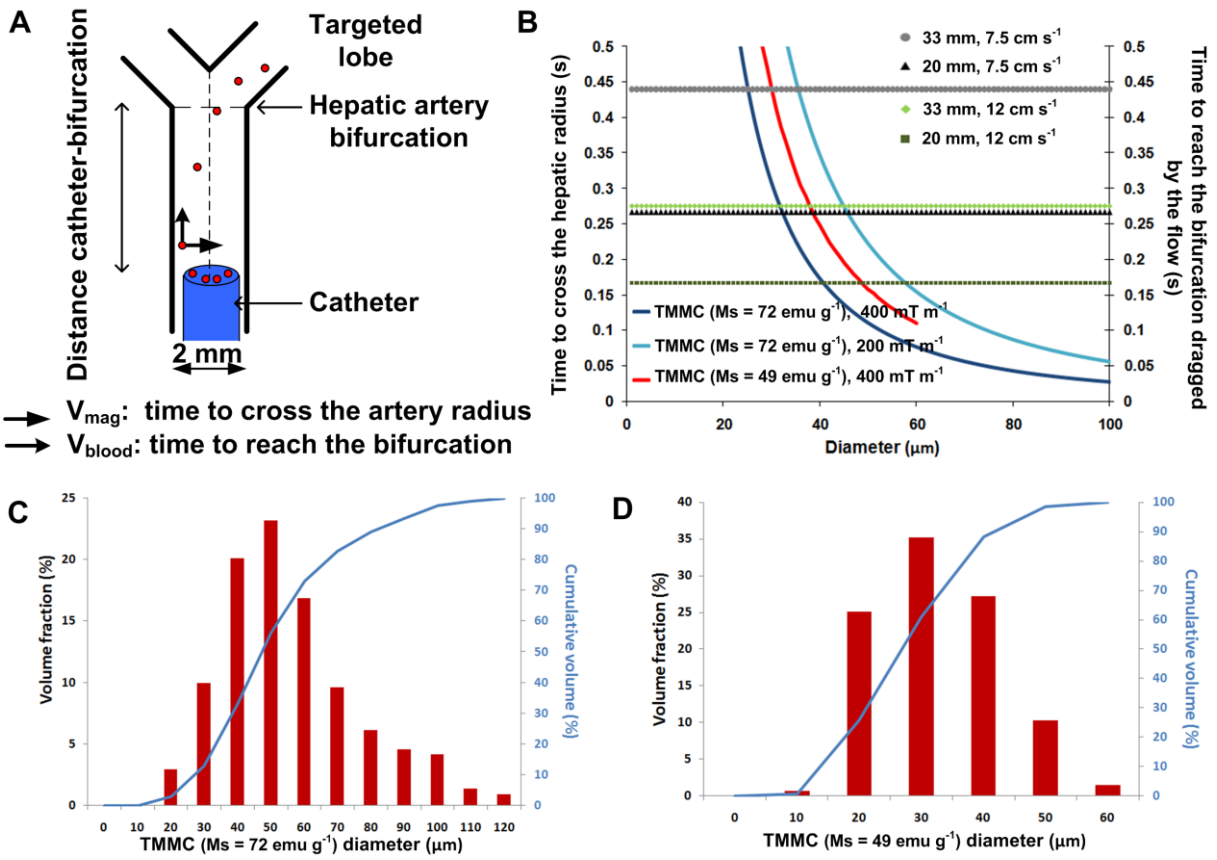

Fig. S2: Theoretical steering of the TMMC as a function of diameter, magnetic gradient applied, flow velocity and distance between the catheter and the bifurcation. Image A displays a schematic view of the TMMC steering in the rabbit hepatic artery. B) Comparison of the time required to cross the hepatic artery radius and the time to reach the bifurcation dragged by the flow according to the distance between the catheter and the bifurcation, the magnetic gradient applied, TMMC characteristics (diameter and Ms) and the flow velocity (12 or 7.5 cm s^{-1}). C) and D) images display the size distribution and the cumulative distribution of TMMC with Ms = 72 emu g^{-1} and with Ms = 49 emu g^{-1}, respectively.

According to our theoretical steering model based on V_{mag} and V_{blood} (Fig. S2*B*), to steer a TMMC (Ms = 72 emu g^{-1}) with a gradient of 400 mT m^{-1} with a flow of 7.5 cm s^{-1} with a catheter placed at 20 mm from the bifurcation, the TMMC diameter should be at least of 32 µm. When the flow was set up at 12 cm s^{-1}, this value increased to 42 µm. Consequently based on the size distribution, the volume fraction of TMMC suspension which could not be

steered can be determined for different catheter tip positions and different flow velocities. For the two examples previously described, the cumulative volume which could not be steered reached 17 and 40%, respectively. By taking into consideration in our model the *in vitro* steering parameters, a significant difference in steering efficiency (Fig. 3) was achieved when the cumulative volume, which could not be steered, reached at least 20%. Our basic steering model could help to choose the appropriate TMMC formulation depending on the vascular network targeted.

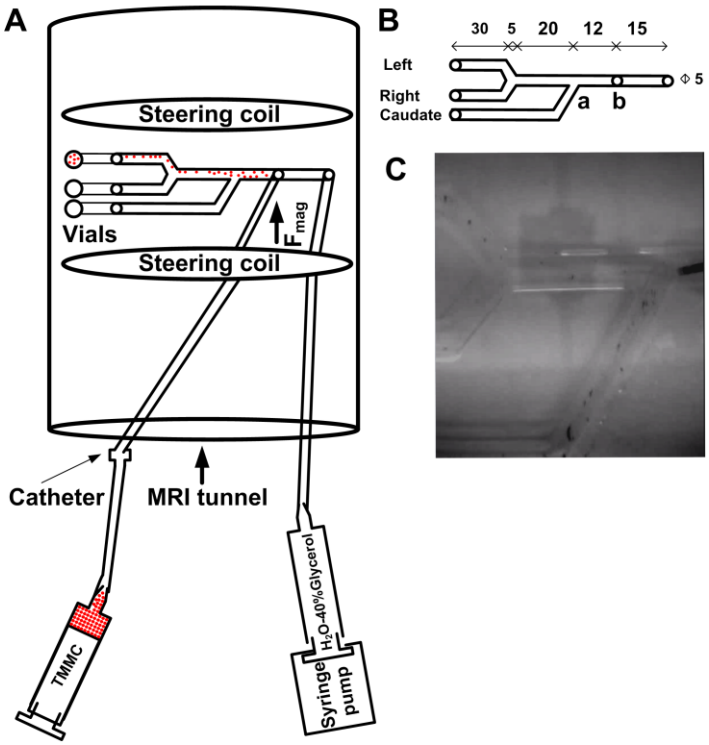

Fig. S3: *In vitro* steering MRI setup. Image A represents a schematic view of the MRI steering setup. Image B is a schematic view of the hepatic artery phantom with its dimensions. On the image, a and b refer to the position of the catheter tip. Image C displays a frame extracted during the recording of a TMMC steering test when the catheter was placed at 20 mm from the bifurcation.

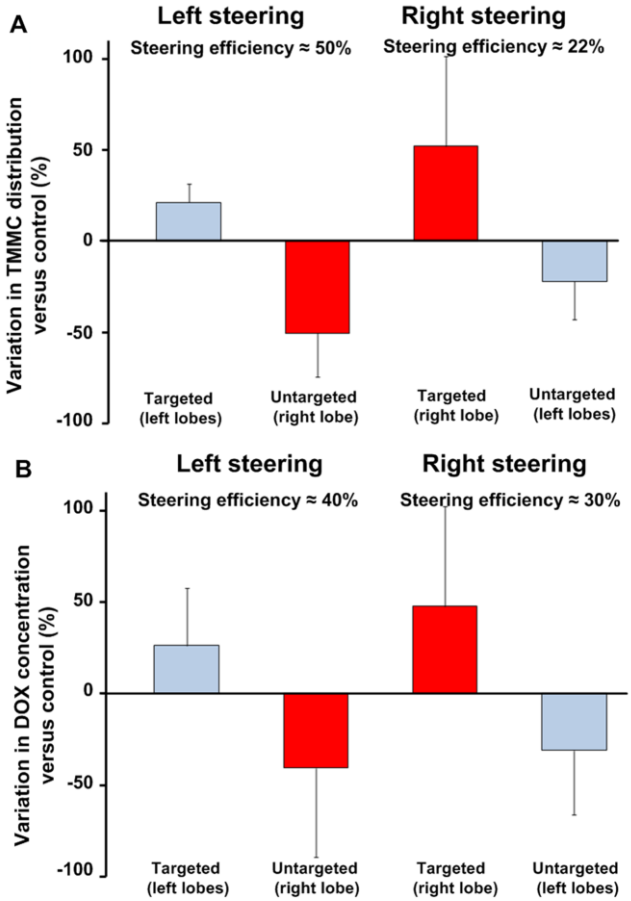

Fig. S4: *In vivo* variation in TMMC distribution determined by histology (A) and in DOX concentration (B) *versus* control. The same TMMC distribution profile obtained with the Co analysis was achieved with these two analyses. Good correlation coefficients were found between the TMMC/DOX distribution obtained with the three methods: r = 0.99 for AAS and histology results, r = 0.99 for AAS and DOX results, and r = 0.98 for DOX and histology results. These data confirm that MRN can significantly control the TMMC distribution in the liver. Mean ± SD (n=4).

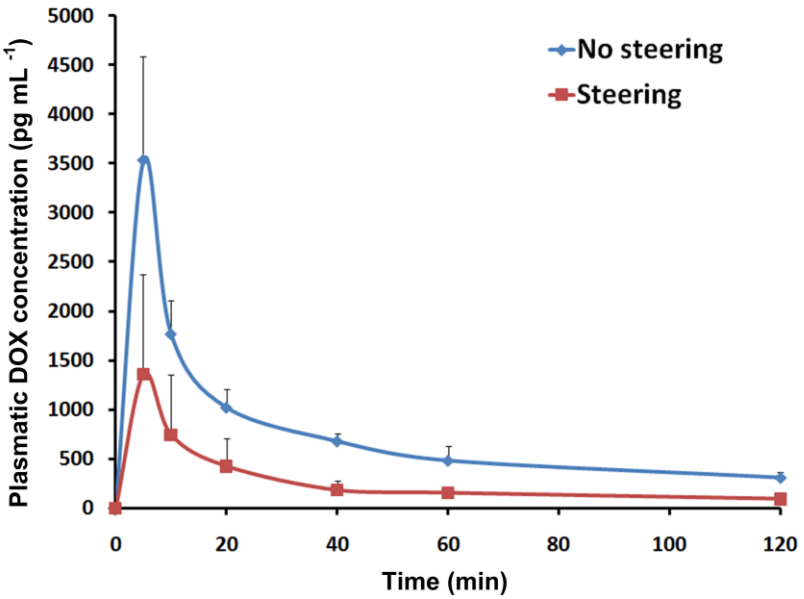

Fig. S5: Plasmatic DOX concentration after TMMC injection. The values were rationalized by the injected dose. The burst effect occurred at 5 min after the injection in both groups. The area under the plasmatic concentration *vs.* time curve (AUC) in the steering group (AUC = 8505 ± 4975) was significantly lower than the one in the control group (AUC = 23485 ± 5171).

Accordingly, the steering could reduce the plasmatic DOX concentration. This result could be attributed to the slight aggregation of the TMMC occurring during the injection due to the MRI scanner magnetic field [1]; the aggregation reduced the contact surface with the blood. It was shown that when DEB diameter increased, the plasmatic DOX concentration was reduced because of the reduction of the contact surface [6]. The steering could have an additional impact on the plasmatic concentration by reducing the contact with the blood; TMMC deposited mainly in one lobe. Additional experiments are required to determine if this effect was due to the steering or to the magnetic TMMC aggregation during the injection. Mean ± SD (n=3).

Supplementary References

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