Electronic Supplementary Information

Magnetic chromatography improves colloidal and MRI attributes of magnetoliposomes enabling evaluation of the impact of size on bio-distribution in an in vivo model of pancreatic cancer

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Materials and Methods

Materials

Iron (III) acetylacetonate (≥99.9%) ($Fe(acac)_3$), 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (≥98.0%) (DOPG), benzyl alcohol (ACS reagent, ≥99.0%), methanol (ACS reagent, ≥99.8%) (MeOH) and acetone (CHROMASOLV® ≥99.8%) were purchased from Sigma-Aldrich. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG-2000 amine) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids Inc. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate salt (DiD-DS), chloroform (Reagent grade) ($CHCl_3$) and ammonia (35%) were purchased from ThermoFisher. All chemicals were used without further purification. De-ionised water was obtained from a Millipore Milli Q Gradient system fitted with a 0.22 µm Millipak express 20 filter and had a resistivity of < 18.2 MΩ.cm.

Preparation of Primary Magnetic Nanoparticles (MNPs)

Primary MNPs, 8.6 or 12.4 nm, were prepared with slight modifications to the Pinna method [1]. In brief, Fe(acac)3 in benzyl alcohol (20 mL of 141.6 mM) was flushed with N_2 for 20 minutes before refluxing at 205 °C for 7 hours under an N_2 atmosphere. The resultant solution contained 8.6 nm MNPs, as determined by TEM [2], at an γ-Fe_2O_3 concentration of 10 mg/mL (*c.* 90% yield). To prepare 12.4 nm MNPs, 20 mL of 8.6 nm MNPs in benzyl alcohol were washed twice with benzyl alcohol using a standard neodymium (N52-type) magnet and resuspended in Fe(acac)3 in benzyl alcohol (20 mL of 141.6 mM). As with the smaller NPs, the solution was flushed with N_2 for 20 minutes before refluxing at 205 °C for 7 hours under an N_2 atmosphere. The resultant solution contained 12.4 nm MNPs, as determined by TEM [1], at an γ-Fe_2O_3 concentration of 20 mg/mL (*c.* 90% yield).

Preparation of Solid Magnetic Liposomes (SMLs)

SMLs were prepared as we reported previously using a two-step approach [2]. In brief, 5 mL of 8.6 or 12.4 nm MNPs in benzyl alcohol under an N_2 atmosphere and heated to 80 °C with magnetic stirring. 1.5 mL of methanol containing 11% of the surface-equivalent (SE) phospholipid required to complete the inner phospholipid monolayer layer on the MNP surface (see below for amount required for each MNP size) was added to the mixture, followed by 1.5 mL of 35 % NH_4OH. 2 mL of methanol containing the remaining 89% of the phospholipid was then added slowly over 5 minutes. The mixture was kept at 80 °C for an additional 15 minutes before cooling to room temperature and washing the magnetic component on a standard neodymium (N52-type) magnet with 40 mL of a methanol/acetone (50/50) solution four times. The magnetic component was transferred to a clean flask using 10 – 12 mL of deionised H_2O (the volume added can be varied to control the final Fe concentration of the SML suspensions). This suspension was heated to 65 °C with magnetic stirring under an N_2 atmosphere. After 30 minutes at 65 °C, 1.5 mL of methanol containing 30% of the total phospholipid required to complete the outer phospholipid layer (see below) was added to the mixture, followed by 1.5 mL of 35 % NH_4OH. 3.5 mL of methanol containing the remaining 70% of the phospholipid was then added slowly over 5 minutes. The mixture was kept at 80 °C for an additional 15 minutes before cooling to room temperature, sonicating for 30 minutes and magnetically decanting over a standard neodymium (N52-type) magnet for 90 minutes to remove larger aggregates.

As we previously reported [2], it was determined that 0.88 monolayer surface-equivalents (SE) were required to prepare stable suspensions of SMLs, where 1 SE is equivalent to a full coating of phospholipid on the surface of the MNPs used in a preparation, assuming that the MNPs are spherical and completely monodisperse, and that DOPG binds to the MNPs with a headgroup area of 7.1×10^{-15}

cm^2 [3]. The calculation used to determine the phospholipid needed to form an 0.88 SE monolayer on 5 mL of 8.6 nm MNPs (10 mg/mL γ-Fe_2O_3) is described in Table S1.

Table S1. Calculation of the number of moles of phospholipid required to form an SML with 0.88 SE of phospholipid on 5 mL of 8.6 nm MNPs (10 mg/mL γ-Fe_2O_3)

Parameter	Formula	Value
MNP SA (cm^2) – SA_{MNP}	$4\pi.R_{MNP}^2$	2.32×10^{-12}
MNP Volume (cm^3) – V_{MNP}	$4/3(\pi.R_{MNP}^3)$	3.33×10^{-19}
Mass of Fe_2O_3 (g) – $Fe_3O_4(g)$	$Fe_2O_3(moles) \times Mr(Fe_2O_3)$	5.46×10^{-2}
Mass of 1 MNP (g) – $MNP(g)$	$\rho(Fe_3O_4) \times V_{MNP}$	1.71×10^{-18}
Number of MNP Produced - N	$Fe_3O_4(g) / MNP(g)$	2.68×10^{16}
Footprint of PG (cm^2) - SA_{PG}	-	7.10×10^{-15}
PG/NP	SA_{MNP} / SA_{PG}	2.83×10^{2}
Moles of PG for 0.88 SE on 5 mL of MNPs	$0.88*(N*PG/NP)/(6.02 \times 10^{23})$	1.12×10^{-5}

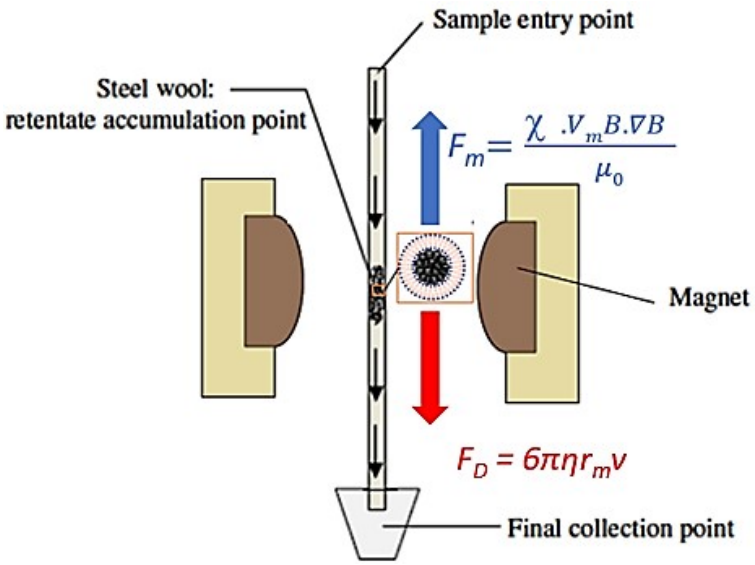
In this work, SMLs were prepared using either 8.6 or 12.4 nm primary MNPs clustered within a DOPG inner phospholipid layer, and either a DOPC or 85/15 DOPC/DSPE-PEG_{2000}) outer phospholipid layer. For SMLs prepared using 5 mL of 8.6 nm MNPs, 11.2 and 56.2 µmoles of phospholipid were required to form the inner and outer phospholipid layers, respectively. For SMLs prepared using 5 mL of 12.4 nm MNPs, 27.9 and 140 µmoles of phospholipid were required to form the inner and outer phospholipid layers, respectively. Our nomenclature for these particles is DOPG // DOPC and DOPG // DOPC/DSPE-PEG.

SML suspensions prepared with 12.4 nm MNPs, a DOPG inner phospholipid layer and an outer phospholipid layer composed of 85/15 DOPC/DSPE-PEG_{2000}, (DOPG // DOPC/DSPE-PEG) were used for both physical characterisation and *in vivo* studies, and have been termed SML-PEG throughout the manuscript. To facilitate *in vivo* monitoring of the biodistribution of SML-PEG, 0.01 mol% DiD-DS was included in the lipid mixture forming the outer phospholipid monolayer. Density gradient centrifugation (DGC) was employed to remove unloaded liposomes from SML-PEG suspensions prior to *in vivo* experiments. Discontinuous density gradients consisting of 0.5 mL of 5% dextran over a cushion of 0.5 mL of 10% dextran were prepared in a centrifuge tube, and 0.5 mL of an SML-PEG suspension was overlaid on top. Samples were centrifuged for 90 minute periods at 6000 g until SML-PEG pelleted. The pelleted SML-PEG was resuspended in varying volumes of H_2O to provide suspensions of desired Fe concentration. We have previously shown that DGC does not alter the d_{hyd} or PDI of SML-PEG suspensions, and that no free liposome reformation occurs up to 8 weeks when stored at 4 °C [2].

Size Selection of SMLs using Magnetic Chromatography (MC)

MC was employed to size select SML suspensions using a WP80 variable field magnet (Bruker) and a variable flow peristaltic pump to transport aqueous SML suspensions though 1/8 inch tubing packed with *c.* 50 mg of steel wool (see **Scheme S1**). The magnetic wool was maintained in the centre of the magnetic field using a polystyrene holder, which ensured that the position was reproducible. For each experiment, 3 mL of the SML suspension was injected onto the WP80 system at room temperature using a flow rate of 5 $mL.hr^{-1}$ and a magnetic field strength of 1.5 T and the eluent was collected. The flow rate was progressively increased from 5 to 200 $mL.hr^{-1}$, while the field strength was progressively decreased from 1.5 to 0.0 T (see **Table 1**). 1.5 mL aliquots of H_2O were subsequently injected at each condition and fractions of the eluent were collected. This approach provides a series of suspensions

of varying cluster size (based upon dynamic light scattering (DLS) analysis) which were retained for further analysis.



Scheme S1. Schematic representation of the equipment employed for magnetic chromatography (MC), with the opposing flow and magnetic forces acting on the SMLs indicated.

Characterisation Techniques

DLS measurements were performed on a NanoZS (Malvern Instruments, Malvern UK) at a temperature of 25 °C. A 3 mW He–Ne laser operating at a wavelength of 633 nm was used as the light source and back scattered light was detected at an angle of 173° to the incident beam. The z-average, or mean hydrodynamic diameter (d_{hyd}) and polydispersity index (PDI) values were calculated using cumulants analysis [4]. It should be noted that it was not possible to record d_{hyd} for dye loaded SMLs and liposomes due to significant absorption of the incident light. For dye loaded SMLs, DLS measurements were carried out on SMLs which were prepared in parallel and did not contain dye molecules.

Total iron content of SML suspensions was determined by atomic absorption spectroscopy for SML suspensions. Measurements were undertaken on a Varian SpectrAA 55B atomic absorption spectrometer fitted with a single slit burner. An Fe-cathode lamp with a wavelength of 248.3 nm was used as the light source and a high temperature air/acetylene flame was used. Acid digestion was employed to prepare MNP samples for analysis. Typically, an aliquot of sample (20 – 100 µL, depending on estimated concentration) was placed in a volumetric flask, to which 1.5 mL of 12 M concentrated HCl was added. The samples were left to stand for 2 hours before quantitatively making up to mark with 1 M HNO_3.

Nuclear magnetic resonance dispersion and T_2 measurements

The ^1H relaxation enhancements due to suspended particles were quantified from the spin-lattice and spin-spin relaxivities, r_1 and r_2:

$$r_{1/2} = \frac{R_{1/2\,(meas)} - R_{1/2\,(solvent)}}{[Fe]}$$ Equation (1)

where $r_{1/2}$ has units $s^{-1}.mM^{-1}$), and $R_{1/2(meas)}$ and $R_{1/2(solvent)}$ are the measured relaxation rates of the suspensions and of the particle-free solvent, respectively. The estimated error for the determination of Fe content by AAS of the dissolved NP suspensions is *c.* 2 % [5]. This uncertainty determines the error in the reported r_1 values, as the error in R_1 measurement is significantly lower (<1 %) [6].

The ^1H Larmor frequency (ν_L) dependence of the spin-lattice relaxivity (r_1) for the aqueous nanoparticle suspensions was recorded over the frequency range 0.01–20 MHz using a Stelar Spinmaster Fast Field Cycling NMR Relaxometer (Stelar SRL, Mede, Italy). The system operated at a measurement frequency of 9.25 MHz for ^1H, with a 90° pulse of 7 µs. r_1 measurements were performed as a function of external field, B_0, with standard pulse sequences incorporating B_0 field excursions [7]. The temperature was set to 25.0 ± 0.1 °C and allowed to equilibrate thermally for 10 minutes prior to measurement. For $10 < \nu_L < 25$ MHz a non-polarised experiment (NP/S) was employed, while in the lower frequency range $0.01 < \nu_L < 10$ MHz a pre-polarised experiment (PP/S) was employed [8]. r_2 measurements were carried out on a Maran Ultra 23.4 MHz NMR Analyser (Oxford Instruments, United Kingdom) at 25.0 ± 0.1 °C, using a Carr-Purcell-Meiboom-Gill sequence (CPMG/S).

In vivo imaging of SML biodistribution

MIA PaCa-2 pancreatic cancer tumours were obtained from the American Type Culture Collection and used in this work. Small tumour fragments (*c.* 8 mm^3) from donor mice were implanted subcutaneously on the abdominal wall of anesthetized 18 to 20 g CB17 SCID mice. Once tumours reached a volume greater than *c.*200 mm^3, mice were randomized into groups and studies were initiated. All procedures were approved by the Roswell Park Institutional Animal Care and Use Committee.

Mice (n=3/group) were treated with DiD-DS labelled (0.1 mol%) SML-PEG formulation in 10% dextrose at a dose of 0.73 $mg.kg^{-1}$ (mg of Fe per mass of mouse), using tail vein injections at volumes *c.* 0.1 mL. Near-infrared fluorescence imaging was carried out on an IVIS® Spectrum in vivo imaging system (Perkin-Elmer) to determine biodistribution of DiD-DS labelled SML-PEG at varying timepoints. Regions of interest (ROIs) were drawn on the recorded images using Living Image Software (Perkin-Elmer) and radiant efficiencies were calculated at 640/680 nm (ex/em). Statistical analysis was carried out using unpaired two tailed t-tests and differences were considered significant when **** $p < 0.0001$, *** $p < 0.0002$, ** $p < 0.0021$, * $p < 0.0332$.

Results

Table S2. Summary of MC results for a range of SML suspensions prepared with varying primary MNP size and phospholipid bilayer composition. The inner phospholipid layer was DOPG in all cases.

MNP Size (nm)	Outer Monolayer	SML d_{hyd} (nm)	d_{hyd} range of Fractions (nm)*	[Fe] range of Fractions (mM)*
8.6	DOPC	63	60 – 68	0.03 – 2.50
12.4	DOPC	95	94 – 140	0.20 – 8.00
8.6	DOPC/DSPE-PEG-NH_2	90	80 – 120	0.03 – 1.00

**The procedure used in these experiments is as per Table 1 in the main text, with 6 fractions collected under identical flow rate and magnetic field strength*

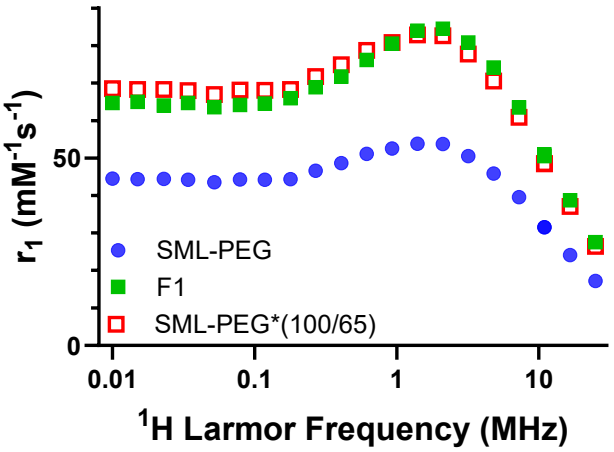


Figure S1. FFC-NMR profiles at 25 °C of (●) an SML-PEG suspension; (■) fraction F1, and (□) SML-PEG scaled by 100/65, which corresponds to F1 accounting for 65% of the Fe content of the SML-PEG suspension.

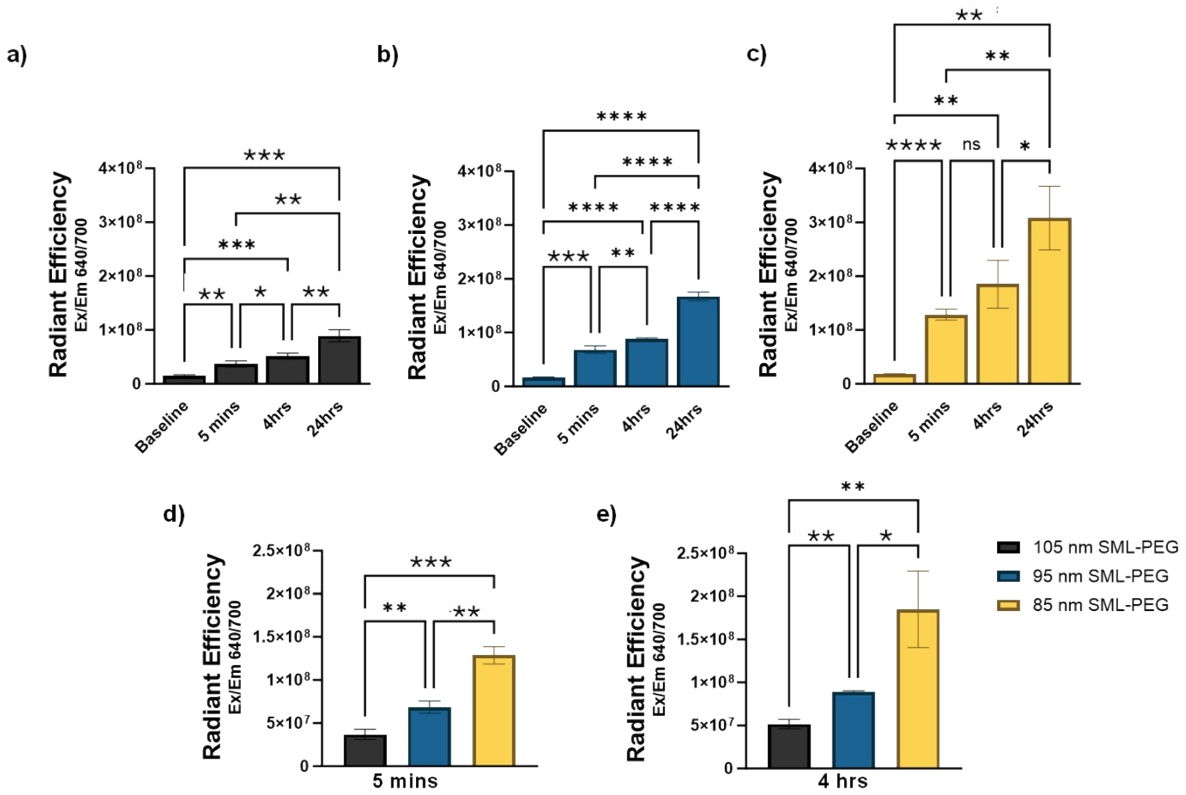


Figure S2. Radiant efficiencies from IVIS imaging of MIA PaCa-2 tumours (n=3 mice/group) post iv injection of SML-PEG of varying size at an Fe dose of 0.73 mg.kg^{-1}. Recorded radiant efficiencies at baseline, 5 minutes, 4 and 24 hours post injection for MC-processed F1 SML-PEG suspensions of **(a)** 105 nm, **(b)** 95 nm and **(c)** 85 nm. Comparison of recorded radiant efficiencies for 105, 95 and 85 nm SML-PEG suspensions at **(d)** 5 minutes and **(e)** 4 hours post injection.

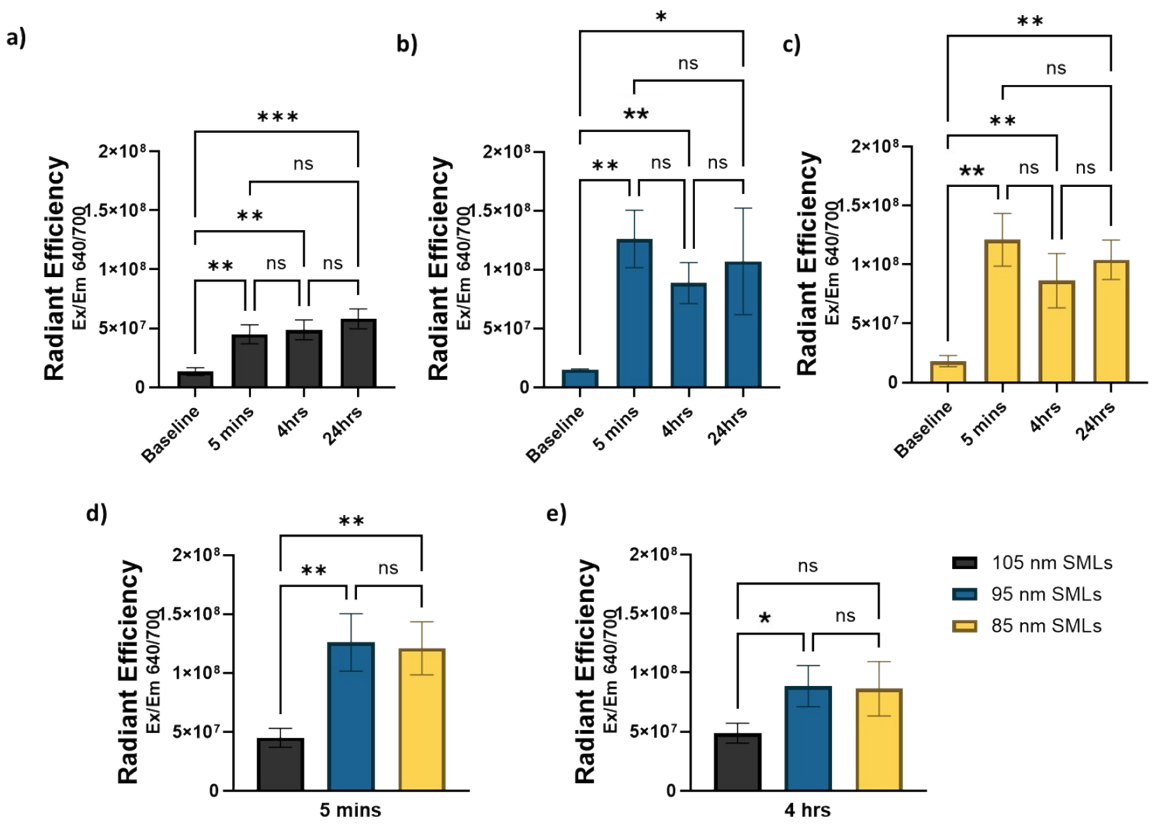


Figure S3. Radiant efficiencies from IVIS imaging of the liver (n=3 mice/group) post iv injection of SML-PEG of varying size at an Fe dose of 0.73 mg.kg^{-1}. Radiant efficiencies at baseline, 5 minutes, 4 and 24 hours post injection of SML-PEG suspensions of **(a)** 105, **(b)** 95 and **(c)** 85 nm. Comparison of recorded radiant efficiencies for 105, 95 and 85 nm SML-PEG suspensions at **(d)** 5 minutes and **(e)** 4 hours post injection.

References

[1] N. Pinna, S. Grancharov, P. Beato, P. Bonville, M. Antonietti, M. Niederberger. Magnetite Nanocrystals: Nonaqueous synthesis, characterization, and solubility. *Chem. Mater.*, 2005, 17, 3044 - 3049.
[2] C. Moloney, T.R. Chaudhuri, J.A. Spernyak, R.M. Straubinger, D.F. Brougham. Long-circulating magnetoliposomes as surrogates for assessing pancreatic tumour permeability and nanoparticle deposition. *Acta Biomaterialia*, 2023, 158, 611–624
[3] A. Liljas, L. Liljas, J. Piskur, G. Lindblom, P. Nissen, M. Kjeldgaard, Textbook of Structural Biology, World Scientific Publishing, USA, 2017.
[4] ISO, ISO 22412:2017 Particle size analysis — Dynamic light scattering (DLS), 2017.
[5] P. Monks, J.K. Wychowaniec, E. McKiernan, S. Clerkin, J. Crean, B.J. Rodriguez, E.G. Reynaud, A. Heise, D.F. Brougham. Spatiotemporally resolved heat dissipation in 3D patterned magnetically responsive hydrogels. *Small*, 2021, 17, 2004452
[6] E. MacMahon, D.F. Brougham. pH dependence of MRI contrast in magnetic nanoparticle suspensions demonstrates inner-sphere relaxivity contributions and reveals the mechanism of dissolution. *Langmuir*, 2023, 39, 2171-2181.
[7] R. Kimmich, E. Anoardo, Field-cycling NMR relaxometry. *Prog. in Nuclear Magn. Reson. Spec.* 2004, 44, 257-320.
[8] C.J. Meledandri, D.F. Brougham, Low field magnetic resonance techniques in the development of nanomaterials for biomedical applications, *Anal Methods*, 2012, 4, 331 - 341.