Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Fluorochrome-Functionalized Magnetic Nanoparticles for High-Sensitivity Monitoring of the Polymerase Chain Reaction by Magnetic Resonance**

*David Alcantara, Yanyan Guo, Hushan Yuan, Craig J. Goergen, Howard H. Chen, Hoonsung Cho, David E. Sosnovik, and Lee Josephson**

anie_201201661_sm_miscellaneous_information.pdf

Materials and Methods

Feraheme (FH) was from AMAG Pharmaceuticals and is trademarked by them. Other chemicals were from Sigma-Aldrich (St Louis, MO). PD-10 or NAP-5 columns were from GE Healthcare, Piscataway, NJ. The size and surface charge of FH-TO were obtained on a Nano-ZS Zetasizer (Malvern, Medford, MA), with size expressed as the Z-average. T2 relaxation times were determined on a 20 MHz MiniSpec at 40 °C (Brucker Systems, Billerica, MA). Absorbance was obtained with an Evolution 300 spectrophotometer (Thermo Scientific, Madison, WI), while fluorescence was measured with the GloMax®-Multi Detection System (Promega, Madison, WI). To obtain size and T2 as a function of PCR cycle number (CN), a BioRad Mycycler (Bio-Rad laboratories) was used. Fluorescence as a function of CN with Sybr Green or FH-TO employed an ABI Prism 7000 (Applied Biosystems, Carlsbad CA). Lambda DNA (λ DNA) was from New England Biolabs. Oligonucleotides (10, 18 bp) were from Integrated DNA Technologies.

FH-TO synthesis. FH (30 mg Fe/mL) was transferred to 0.1 M MES, pH 6 by gel filtration using a Sephadex G-25 PD-10 column. To 1 mL of diluted FH (6 mg Fe, 107 µmoles Fe) was added 25 mg of N-(3-dimethyaminopropyl)-N'-ethylcarbodiimide (CDI, MW= 191.7, 130 µmoles) and 5 mg hydroxybenzotriazole (HOBT, MW = 153.1, 32.6 µmoles), and the mixture was incubated for 20 minutes at room temperature. To this was added 40 µL of 1M ethylene diamine (40 µmoles EDA) in 0.1 M MES and the mixture incubated at 50 °C for 90 minutes. The mixture was concentrated by lyophilization and purified on a PD-10 column equilibrated with MES buffer. Some 50 µl of amino-FH (250 ug Fe) was added 3.12 µl (62.4 µg, 79.6 nmoles) of the NHS ester of TO in DMSO (20 mg/ml in DMSO) and allowed to react for 3 h at 25 °C. Purification was on Sephadex G-25 column using water as eluent. Unreacted TO adheres to the column,

eliminating possible contamination of FH-TO with low molecular fluorochromes. The TO/NP ratio was determined spectrophotometrically, using the absorbance at 300 nm and FH standards for iron concentrations, and the absorbance at 501 nm and an extinction coefficient of 63,000 (M*cm)-1 for TO-PRO 1 concentration [1]. There are 5874 iron atoms per crystal (AMAG Pharmaceuticals, FH package insert).

DNA/TO-PRO 1 binding model: A model for TO-PRO 1 bound to DNA was generated based on the experimentally determined NMR structures [2]. Briefly, the MOE 2007.09 docking suite (Chemical Computing Group, Montreal) was used to build 10 base pairs of ideal B-form DNA and an energy minimized structure of TO-PRO 1 (MMF94X force field to a constant of 0.05 kcal/mol). An initial TO-PRO 1/DNA model was generated by superimposing the coordinates for B-form DNA and TO-PRO 1 onto the respective groups in 108D.pdb. Finally, the local interactions between TO-PRO 1 and the model DNA were optimized and minimized using the MOE LigX function.

Reaction of FH-TO and DNA: Phage lambda DNA (48,502 base pairs (bp), MW= 31,500 kDa) was from NEBiolabs (N3013S)). Oligonucleotides of 10 and 18 bp's, (6054.6 and 10997.3 daltons, respectively) were from IDT. 10mM Tris-HCl, 2.5mM MgCl2, 0.5mM CaCl2, pH7.6 (200 µL) was added to a 96 well microtiter plate, then FH-TO (2.5 µL, 0.42 mg Fe/mL) and then 100 µL of DNA in the buffer above (orbital shaker, 2 h, RT). T2 by relaxometry and fluorescence were determined on undiluted samples. For size determinations, duplicate wells were pooled and 300 µL water was added. Kd's, the apparent dissociation constants for changes in T2 or fluorescence produced by increasing DNA concentrations (Figure 2a, 2b), were determined by fitting data to a single binding site, saturation

equation with Prism 4 software (GraphPad Software, San Diego, CA). The size distributions of FH-TO/DNA microaggregates were analyzed using Origin 8.0 Software, (Origin Laboratories).

TEM: For TEM of FH-TO, five microliters of 300 ug Fe/mL in aqueous dextran T-10 (5 mg/mL) was placed on a TEM grid (Copper-Carbon coated grids, CF300-Cu50, Electron Microscopy Sciences, Hatfield-PA) and dried in a hood for 3 hours. TEM was at 80 kV (Zeiss, Libra120 Energy Filtered Transmission Electron Microscope). For TEM of microaggregates, FH-TO (1.6 μL, 300 ug Fe/mL) was reacted with λ DNA (50 μL, 0.25 ug/mL, 1h, RT). One μL of a dextran solution (2.5mg/ml) was added to 9 μL microaggregates, loaded on TEM grids, and imaged as above.

Model PCR reaction: A 100 base pair synthetic dsDNA with a sequence of 5- CGC-GGA-TCC-GTA-AAA-CGA-CGG-CCA-GTG-CCG-ATT-ACA-TAC-AGA-TTA-CAG-ATT-ACA-GAT-TAC-AGA-TTA-CAC-TAG-CTA-GCG-AAA-CAG-CTA-TGA-CCA-TGA-TTA-C-3 (IDT, Coralville, IA, USA) was used as a template with a forward primer (5'-CGC-GGA-TCC-GTA-AAA-CGA-CGG-CCA-GTG-CC-3', Tm=69.2'C, MW=8897.8 daltons) and reverse primer (5'-GTA-ATC-ATG-GTC-ATA-GCT-GTT-TCG-CT-3', Tm=57.2'C, MW=7967.2 daltons). All were from IDT, primers were designed with PrimerQuest® online tool (IDT). The PCR reaction employed 5 μL of 10x Thermopol Reaction Buffer (NEBiolabs, #B9004S), 1 μL of each for forward and reverse primers (1 μg/μl), 1 μl VentR polymerase, (2000units/mL NEBiolabs, #M0254L), 1μL of template with concentrations ranging from 10^{-3} to 10^{-9} picomoles and 1 μL dNTP nucleotide mix (Thermo-Fischer, BP2565-1), Carlsbad, CA). Some 36.2 μl of RNase free water was then added followed by 3.8 μL of FH-TO (0.1 mg Fe/mL). For T2 by relaxometry measurements were made in a 50 μL capillary tube.

MRI of Model PCR Reaction: Measurements were made using a 21 cm horizontal-bore 9.4T scanner (Biospec Bruker, Billerica, MA, USA). PCR tubes were immersed in water with 5 mM Gd-DTPA (Bayer Schering Pharma, Berlin, Germany). A multi-echo spin echo sequence was used to create a T2 maps of the tubes. Parameters included: Field of view (FOV) = 8 x 3 cm; slice thickness = 1 mm, matrix size (320 x 100); TR = 2500 ms; TE 4.3-52 ms (12 equally spaced echoes at 4.3 ms intervals ranging from 4.3 ms to 52 ms); T2 maps were generated using a 12-point mono-exponential fitting algorithm in a freeware DICOM viewer (OsiriX, University of Geneva).

MR of PCR generated DNA with an apoptosis gene array: RT² Profiler PCR 96 well arrays (SAB Biosciences, Frederick, MD) for human apoptosis gene expression were employed according to the manufacturer's instructions. Jurkat T cells (Clone E6-1, ATCC# TIB-152) were exposed camptothecin to induce apoptosis (7uM camptothecin, 6h)). Cells were pelleted by centrifugation, lysed, and RNA was isolated using the Qiagen RNeasy Plus Mini Kit (Qiagen, Hillden, Germany). The cDNA was subsequently generated from the purified total RNA using the RT² First Strand Kit (SABiosciences). The synthesized cDNA was then aliquoted and stored at -20 °C for PCR array applications. Real-time qPCR was carried out by combining the cDNA with the RT2 SYBR Green Master Mix (SABiosciences) in the ABI 7000 system (Applied Biosystems, Foster City, CA) for fluorescence-based detection of amplicons (1-40 cycles, see Figure S2). To measure relaxation rates and obtain T2 or MR images of the array, the PCR reaction was stopped at the indicated cycle number, water added (75µL), and 50 µL placed in two separate PCR plates to which either FH or FH-TO (250 ng Fe/tube) was added. After an additional PCR cycle, T2's were measured (0.47 T relaxometry, Figure 4b) with ΔT2 taken as the

difference between FH and FH-TO. Plates were imaged on a commercial 3T clinical MRI scanner (TRIO, Siemens, Erlangen Germany, Figure 4c). A multi-echo spin echo readout of the arrays was performed with the following parameters: Field of view (FOV) = 169 x 120 cm; slice thickness = 2 mm, matrix size (192 x 136); TR = 3000 ms; TE 13.8-165.6 ms (12 equally spaced echoes at 13.8 ms intervals ranging from 13.8 ms to 165.6 ms). T2 maps of the array were created as described above.

Supplementary Data and Figures

Figure S1 shows the MRI phantom consisting of PCR reaction tubes (50 microliters per tube) in Gd-DTPA solution use to make the MRI image shown in Figure 3a.

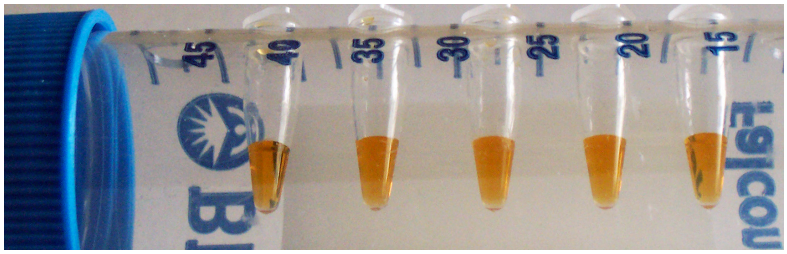

Figure S1: Phantom used for Figure 3a.

Table S1 provides the data points used to generate four parameter logit fits shown in Figure 3. Values in red were taken as 100% and green as 0%.

Table S1: Raw data for Figures 3a, 3b and 3c Data were fit the logit equation, Figure 3c. Minima (green) and maxima (red) are indicated.					
Probe:	**FH-TO (3b,3c)**	**FH-TO (3a,3c)**	**FH-TO (3c)**	**Sybr Green (3c)**	**FH-TO (3b)**
Detection Method:	**T2@0.47T, msec**	**T2 @9.4T, msec**	**Fluores., A.U.**	**Fluores. A.U.**	**Size, nm**
Cycle 1	63.8	75.1	0.000	0.001	402
2			0.016	0.001	
3			0.013	0.002	
4			0.006	0.002	
5	80.4	94.4	0.010	0.002	437
6			0.012	0.001	
7			0.004	0.001	
8			0.015	0.000	
9			0.017	0.002	
10	105		0.014	0.002	550
11			0.000	0.005	
12			0.000	0.002	
13			0.007	0.002	
14			0.040	0.000	
15	133	121.4	0.010	0.000	707
16			0.000	0.000	
17			0.000	0.004	
18			0.000	0.006	
19			0.000	0.004	
20	162	143.0	0.000	0.003	1500
21			0.006	0.009	
22			0.013	0.021	
23			0.014	0.040	
24			0.008	0.068	
25	190		0.025	0.112	1867
26			0.046	0.178	
27			0.067	0.266	
28			0.089	0.385	
29			0.140	0.533	
30	214.5	174.5	0.185	0.698	2600
31			0.213	0.866	
32			0.228	1.025	
33			0.259	1.178	
34			0.283	1.324	
35	212		0.295	1.471	
36			0.282	1.608	
37			0.282	1.728	
38			0.305	1.838	
39			0.325	1.958	
40	195		0.325	2.001	

Figure S2 shows the fluorescence response for all genes in the 96 wells and over the entire cycle range using RT-PCR arrays for the expression of apoptosis related genes. Cycling was stopped at CN=18 or CN =32 to obtain images in Figure 4c.

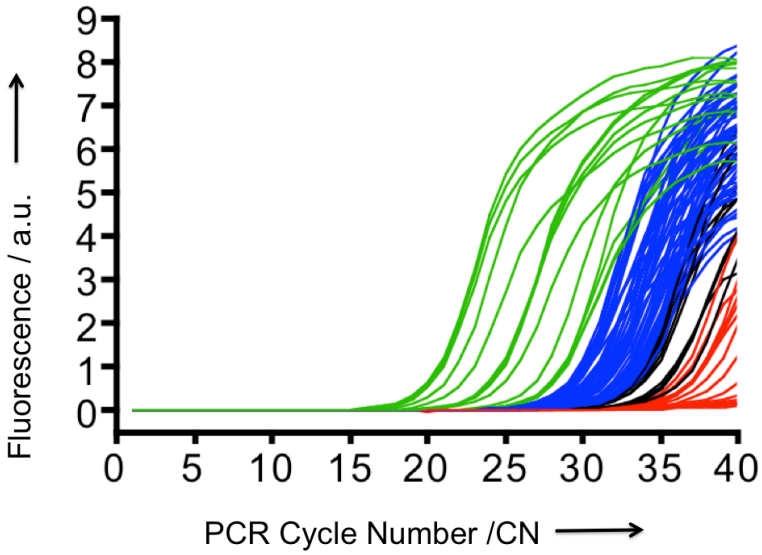

Figure S2: Fluorescence versus CN for the apoptosis gene expression array. Color coding is for high DNA (green Ct's of < 25), medium DNA (blue, 26-29), low DNA (black, 30-32), and very low DNA (red> 32).

Figure S3 provides additional TEM's of FH-TO/lambda DNA aggregates.

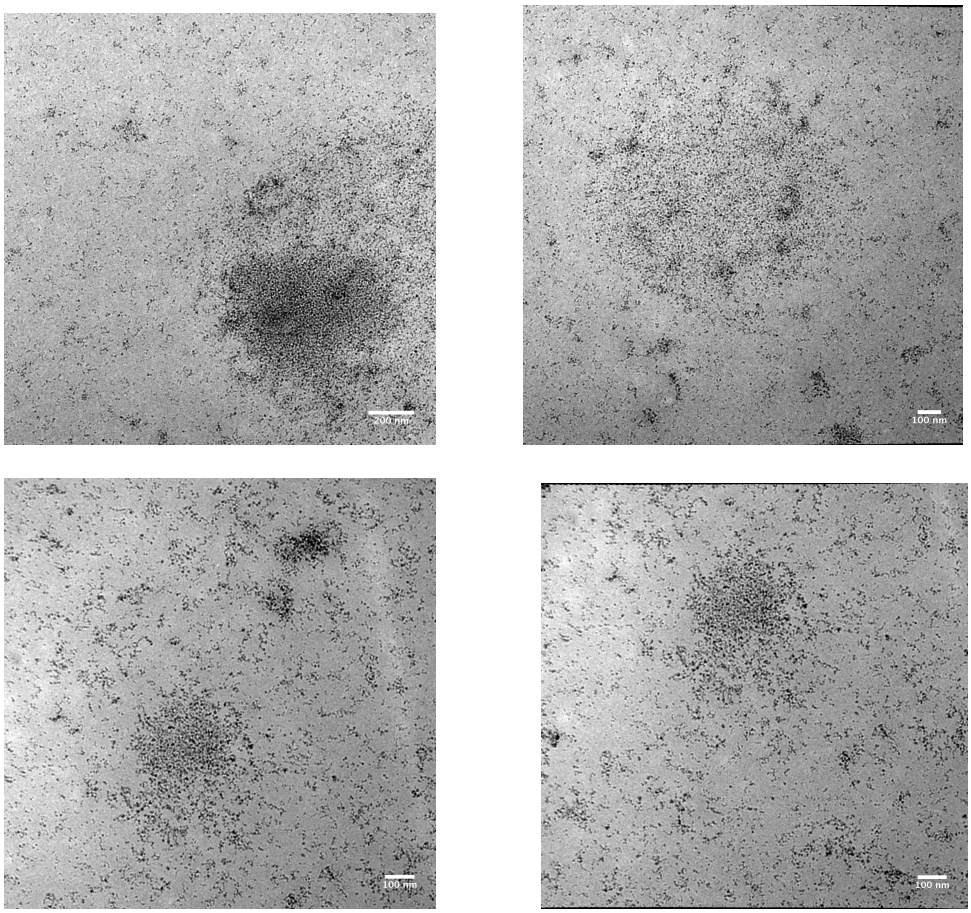

Figure S3: Additional TEM's of microaggregates of FH-TO incubated with λ-DNA.

References

[1] R. Pei, M. N. Stojanovic, *Anal Bioanal Chem* **2008**, *390*, 1093.
[2] a)H. P. Spielmann, D. E. Wemmer, J. P. Jacobsen, *Biochemistry* **1995**, *34*, 8542; b)S. Prodhomme, J. P. Demaret, S. Vinogradov, U. Asseline, L. Morin-Allory, P. Vigny, *J Photochem Photobiol B* **1999**, *53*, 60.