Supplementary Materials

Towards nanomaterials for cancer theranostics: A system of DNA-modified magnetic nanoparticles for detection and suppression of RNA marker in cancer cells

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1. Chemicals and Reagents

Custom made DNA were purchased from Integrated DNA technologies (IDT, Coralville, IA USA); see the sequences in Table 1 in the paper and applied concentrations are specified in sections below. Trypsin (E.C. 3.4.21.4), fetal bovine serum (FBS), fetal calf serum (FCS), Trypan Blue (stain dye), (1-ethyl-3[3-(dimethylamino)-propyl] carbodiimide (EDC), *N*-hydroxysuccimide (NHS), Triton-X-100, Tween-20, 2-(*N*-morpholino)ethanesulfonic acid (MES-buffer), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris-buffer), (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES-buffer), silicon tetraethoxide (TEOS), (3-aminopropyl)triethoxysilane (APS), α-bromoisobutyryl bromide (BIB), ethyl α-bromoisobutyrate (EBIB), *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDTA), methane sulfonic acid and other standard organic and inorganic materials and reactants were obtained from Sigma-Aldrich or J.T. Baker and used without further purification. *tert*-Butyl acrylate (TBA) and poly(ethylene glycol) methyl ether acrylate (PEGMA) (average molecular mass 480 g/mol) were purchased from Sigma-Aldrich and purified using a flash-chromatography column containing inhibitor removers (Sigma #311340 and Sigma #311332). All solutions for the experiments were prepared using ultrapure water (18.2 MΩ·cm; Barnstead NANOpure Diamond).

2. Detailed experimental information

Twist mRNA quantification. Twist mRNA downregulation was quantified with qPCR upon treatment with different concentrations of MaBiDz at varying time points. After treatment, total RNA was extracted using RNeasy Mini kit coupled with RNase-free DNase set (Qiagen) and reverse transcribed with Hexanucleotide Mix (Roche). The resulting cDNAs were used for PCR using SYBR-Green Master PCR mix (Applied Biosystem) in triplicates. PCR and data collection were performed on iCycler (BioRad). Twist RT-PCR forward primer is GGAGTCCGCAGTCTTACGAG, and its RT-PCR reverse primer is TCTGGAGGACCTGGTAGAGG (201 bp product size, 55 ^{o}C annealing temperature). All quantitations were normalized to an endogenous control (Ribosomal RNA). The relative quantitation value for each target gene compared to the calibrator for that target is expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to the ribosomal mRNA).

Preparation and culturing of MCF-7 cells. MCF-7 (breast adenocarcinoma) cells were ordered from American Type Culture Collection (ATCC), Manassas, VA, USA. MCF-7 cells were cultured in Eagle's Minimum Essential Medium (EMEM) from ATCC supplemented with 100 U/mL penicillin,

0.05% (w/v), amphotericin B, 0.02% (w/v), gentamicin and 0.1% (w/v) streptomycin, 0.01 mg/mL human recombinant insulin and fetal bovine serum to a final concentration of 10%.

Synthesis of Fe_3O_4 magnetic beads (MaB). Superparamgnetic nanoparticles were synthesized by a co-precipitation methods as described elsewhere.[1] Iron chloride salts, $FeCl_3 \cdot 6H_2O$ (4.43 g) and $FeCl_2 \cdot 4H_2O$ (1.63 g) were dissolved in 190 mL of water with a stoichiometric ratio 2:1 using magnetic stirring at room temperature. Then, 10 mL of 25% (w/w) ammonium hydroxide was added to the solution to yield a black precipitate. The supernatant solution was stirred for additional 10 minutes, then the precipitate was separated with a magnet and rinsed 3 times with water using magnetic separation. The colloidal dispersion of magnetic nanoparticles was stabilized with citrate ions by rapid rinsing of the precipitate with a 2 M nitric acid solution (two consecutive rinsing steps) followed by addition of 5 mL of 0.5 M aqueous solution of trisodium citrate while maintaining pH 2.5 with added sodium hydroxide. After stirring for 1.5 h, the magnetic nanoparticles were magnetically separated, rinsed with water and then diluted to 100 mL (pH 6.0) of the volume of the nanoparticle dispersion. The concentration of the magnetic nanoparticles (15 nm in diameter) in the final stock solution was 2% (w/w).

A modified Stöber method[2] was used to coat the synthesized Fe_3O_4 magnetic nanoparticles with a silica shell. The nanoparticle stock solution (2 mL) was diluted with a mixture of 160 mL of ethanol and 40 mL of water. Then, ammonium hydroxide (25% w/w, 5 mL) was added to the nanoparticle dispersion. After 10 minutes of ultrasonic bath treatment, 1 mL of TEOS was added dropwise to the solution. The synthesis was carried out at 0° C under sonication for 3 h. The reaction was stopped by the addition of several droplets of 10% HCl resulting in precipitation of the silica-coated nanoparticles. The precipitate was collected with a magnet, rinsed 3 times with water using centrifugation and re-suspended in a 50 mL centrifuge tube using ultra-sonication. The resulting product represented a stable dispersion of the core-shell nanoparticles (2 mg/mL). The powder was easily redispersable in water and formed a stable colloidal dispersion with a nanoparticle size of 45 nm and and zeta potential $\xi = -30$ mV (pH 7.4).

PAA-b-PEGMA block copolymer grafting from nanoparticles surface. Grafting of PAA-*b*-PEGMA block copolymer from the surface of the nanoparticles was conducted using activator generated by electron transfer (AGET)–atom transfer radical polymerization (ATRP). The polymerization was conducted in two steps. First, poly-*tert*-butyl acrylate (PTBA) was grafted by polymerization of TBA. The polymerization was followed by grafting of PEGMA blocks. Finally, the post-polymerization treatment was applied to hydrolyze the PTBA blocks and convert them to polyacrylic acid (PAA) blocks. The process was performed according to the steps specified below.

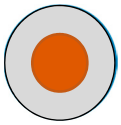
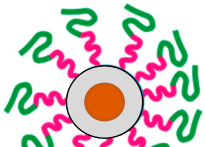
Immobilization of initiator. Silica-coated magnetic nanoparticles were transferred to ethanol medium: the stock nanoparticle solution was mixed with ethanol and the particles were extracted using a magnetic separation. This was repeated several times to decrease concentration of water in the ethanol medium. Finally, the nanoparticles were added to 2% (w/w) (3-aminopropyl)triethoxysilane (APS) solution in ethanol and stirred for 2 h. After the APS immobilization, the particles were rinsed 3 times with ethanol and incubated for 1 h in 100 mL of dry dichloromethane with added 2 mL trimethylamine and 1 mL α-bromoisobutyryl (BIB) bromide. The initiator-functionalized particles were rinsed 3 times with chloroform and ethanol.

Grafting of the copolymer. A TBA monomer solution was purified using a flash-chromatography column containing the inhibitor removers. Then, 320 μL of 0.1 M $CuBr_2$, 320 μL of 0.5 M PMDTA and 10 μL of 0.68 M EBIB ethanol solutions were added to a 30% (w/w) monomer solution in ethanol and loaded with the initiator-functionalized magnetic nanoparticles. EBIB was added to the solution for the synthesis of the polymer in the solution for molecular mass analysis. The reaction mixture was deoxygenated by nitrogen purging for 20 min and then heated to 70° C in an oil bath. Then, 500 μL of 1 M ascorbic acid solution was added to the solution and the reactor was sealed. The polymerization reaction was terminated in 15-30 min by opening vial to air. The non-grafted polymer from the solution was separated from the nanoparticles by centrifugation, re-precipitated 3 times with 30% (v/v) aqueous ethanol and analyzed with gel permeation chromatography (GPC). Grafting

of the second PEGMA block was carried out by a similar procedure: a 10% (w/w) PEGMA solution in ethanol was polymerized for 1 h at room temperature. PTBA-*b*-PEGMA was converted to PAA-*b*-PEGMA by adding methane sulfonic acid. After hydrolysis, the particles were rinsed 3 times with chloroform, ethanol, and water and dried at 50° C in an oven.

Characterization of the nanoparticles. The nanoparticle size analysis was carried out using a combination of dynamic light scattering (Malvern Zetasizer Nano) and AFM (Icon, Bruker) methods. The summary of the nanoparticle dimensions and molecular characteristics of the grafted brush are presented in the preliminary short communication (supporting information).[3] In addition, the most important features of the functionalized magnetic nanoparticles are summarized in Table SM1. The experimental characterization of the functionalized magnetic nanoparticles is additionally illustrated with Figures SM1-SM3.

Table S1. Structure and features of the polymer brush-decorated magnetic nanoparticles.

Schematics	 **Iron oxide core**	 **Iron oxide core coated with a silica shell**	 **Core-shell particle with grafted PAA-*b*-PEGMA** [a]
Diameter (DLS) at pH 7.4, ± 5 nm	16	50	115
ζ-potential, ± 10 mv	-10	-40	-5
Brush thickness swollen/dry, ± 5 nm	-	-	32.5/9.3
Number average molecular mass Mn of the block-copolymer, g/mol, ± 5%	-	-	17000
Grafting density, ±0.06 nm^{-2}	-	-	0.36

[a] The features of the magnetic nanoparticles have not been measurably changed after additional modification step with DNA. This was attributed to the small fraction of the DNA bound to the polymer-brush layer.

Preparation of MaBiDZ. To conjugate NH_2-modified DZa and Hook oligos (see all DNA abbreviated names and sequences in Table 1 in the paper) to the polymer-functionalized Fe_3O_4 magnetic nanoparticles, EDC/NHS carbodiimide coupling was employed. Carboxyl groups on the MaB polymeric brush surface were activated using 20 mM EDC and 50 mM NHS for 25 minutes in a mixture containing 0.05% Tween-20 and pH 4.5 MES-buffer, 50 mM, on a slow tilt shaker. Unreacted EDC, NHS and their reaction low-molecular products were removed through centrifugation for 10 min at 14,000 r.p.m. and the pellet was re-suspended in pH 5.5 HEPES-buffer, 50 mM, containing 50 mM $MgCl_2$. DZa and Hook, both modified respectively with amino groups at the 5'-ends were then separately incubated with the magnetic beads containing activated carboxylic groups for 1.5 h. Unbound DNA was removed through centrifugation for 10 min at 14,000 r.p.m. and MaB were re-suspended in pH 7.4 HEPES-buffer, 50 mM, containing 50 mM $MgCl_2$. Specific analyte strand (Twist), DZb and the prepared DZa-bound MaB conjugates were pre-incubated in a thermostated water bath at 30° C for 20 min. F-sub was incubated with the prepared Hook-MaB conjugates for 1 h. The F-sub-Hook-MaB conjugates were then centrifuged at 14,000 r.p.m. for 10 min, the supernatant containing unbound F-sub was discarded, and the pellet was re-suspended in pH 7.4 HEPES-buffer, 50 mM.

Attachment of BiDZ was confirmed using Diamond Nucleic Acid dye (Promega). A calibration line was developed by measuring fluorescence of known concentrations of DZa bound to the dye. Fluorescence of the dye bound to a known quantity of MaB was then measured in order to find the average fluorescence per MaB. Lastly, the calibration plot was used to relate the fluorescence per MaB to DZa strands per MaB. The measurements of fluorescence per MaB were repeated three times and a value of 120 DZa strands ± 11/MaB was obtained.

Magnetic field applied. For magnet-controls, we placed NdFeB, grade 52, magnets measuring 5/16" diameter × 1/8" thickness (K & J Magnetics, Inc.) under each cell dish providing the magnetic field intensity ca. 0.3 T.

Table S2. Diffusion (V_d) and sedimentation (V_s) velocities of the Fe_3O_4 nanoparticles used in this study.

$V_d = 2D/x$ (m/s)	$V_s\ 2g(\rho_{MNP,a} - \rho_m)d_h^2/9\eta$ (m/s)	V_s/V_d
5.08511E-10	6.91378E-09	13.6

Table S3. Diffusion (D) and sedimentation (S) coefficients of the gold nanoparticles used in this study.

Diffusion coefficient (from Stokes-Einstein equation) $D = k_BT/6\pi\eta r$ (m^2/s)	Sedimentation coefficient Mason Weaver equation $S=m/f\ x\ [1 - \rho_m/\rho_{mnp,a}]$ (s)
4.32235E-12	6.77548E-10

Table S4. Parameters used to calculate D and S for the Fe_3O_4 nanoparticles used in this study.

Apparent density $\rho_{MNP,a} = 1x(1-V_f) + (\rho_{MNP}\ x\ V_f)$	v_f	f = drag coefficient k_BT/D (g/sec)
178574.8	0.364431	9.52E-07

Table S5. RLU counts per cell in the presence and absence of a magnetic field at t= 1hr. 2hr, 3hr.

1 hour		2 hours		3 hours	
Magnet	**No Magnet**	**Magnet**	No Magnet	Magnet	No Magnet
18.08	20.877	66.713	13.687	67.713	26.368
21.457	23.299	59.035	17.487	84.653	54.805
30.917	25.596	86.091	11.696	87.253	44.445
27.595	22.891	71.017	14.315	59.958	54.198
13.722	21.859	50.892	7.833	90.475	77.581
29.723	21.062	62.599	11.263	89.063	51.797
33.46	26.602	68.805	8.419	66.168	63.36
21.306	26.634	54.212	12.154	77.271	42.405
20.713	33.384	79.887	9.878	50.744	58.436
24.999	20.276	60.001	8.439	89.015	57.058
23.23	23.353	80		58.623	54.141
26.635	24.827	60		75.19	67.057
31.056	23.54	60		66.429	82.565
24.404	23.856	60		94.777	73.347
21.77	22.954	100		89.304	91.708
26.635				66.755	60.603
31.056				62.027	59.625
24.404				66.676	
21.77				81.753	
				82.882	

Table S6. Pivot tables for RLU counts per cell in Table 4.

1 hour			2 hours			3 hours		
Counts	**Magnet**	**No Magnet**	Counts	Magnet	No Magnet	Counts	Magnet	No Magnet
10	0	0	0	0	0	0	0	0
12	0	0	20	0	10	20	0	0
14	0	1	40	0	0	40	0	1
16	0	0	60	6	0	60	3	9
18	0	0	80	7	0	80	8	5
20	0	1	100	2	0	100	9	2
22	4	5						
24	6	1						
26	2	3						
28	2	3						
30	0	1						
32	0	3						
34	1	1						

Table S7. Raw qPCR data collected at 2 hours.

Control	**Concentration**	Ct Twist (A)		Ct Ribosomal (B)	
		Magnet	No magnet	Magnet	No magnet
Twist	0.5 pM	27.3	26.5	12.5	12.2
		27.3	26.5	12.4	12.3
		27.3	26.5	12.4	12.3
	1.00 pM	27.4	26.6	12.4	12.3
		27.4	26.6	12.4	12.2
		27.4	26.6	12.4	12.2
	2.00 pM	27.7	27.1	12.4	12.3
		27.7	27.1	12.4	12.3
		27.7	27.1	12.4	12.3
Untreated		26.2		12	
		26.2		12.1	
		26.2		12	

Table S8. Raw qPCR data collected at 4 hours.

Control	**Concentration**	**Ct Twist (A)**		**Ct Ribosomal (B)**	
		Magnet	No magnet	Magnet	No magnet
Twist	0.5 pM	26.9	26.2	12.2	12.1
		26.9	26.2	12.2	12
		26.9	26.3	12.2	12
	1.00 pM	27	26.3	12.3	12
		27	26.3	12.3	12
		27.1	26.2	12.3	12.1
	2.00 pM	27.1	26.3	12.3	12.1
		27.1	26.3	12.3	12.1
		27.1	26.4	12.3	12.2
Untreated		26.2		12.1	
		26.2		12	
		26.2		12	

Table S9. Raw qPCR data collected at 6 hours.

Control	Concentration	Ct Twist (A)		Ct Ribosomal (B)	
		Magnet	No magnet	Magnet	No magnet
Twist	0.5 pM	29.9	28	12.8	12.8
		30	28	12.8	12.8
		30	28	12.8	12.8
	1.00 pM	30.4	30	12.8	12.8
		30.4	30	12.8	12.8
	2.00 pM	32.3	32	13.2	13
		32.3	31.7	13.6	13.6
		32.3		13.6	13.4
Untreated		26.1		12.2	
		26.1		12.1	
		26.1		12.5	

Table S10. Raw qPCR data collected at 8 hours.

Control	Concentration	Ct Twist (A)		Ct Ribosomal (B)	
		Magnet	No magnet	Magnet	No magnet
Twist	0.5 pM	36.6	33.3337	14.7	16.5
		35	33.3337	16.4	16.2
	1.00 pM	36.1	33.7	14.7	16.5
		35	35.1	16.4	16.2
		37.3	33.9	17.6	16.1
	2.00 pM	37.3	36.1	16.9	15.9
		37.3	36.5	16.8	15.8
		37.4	36.5	17	16
Untreated		26.6		12.2	
		26		12.7	
		26.9		12.5	

Table S11. Sample calculations (2 hours) done to obtain relative percentages of Twist mRNA in treated vs untreated cells.

Control	**Conc (pM)**	**Ct Twist (A)**		**Ct Ribosomal (B)**		Δ Ct (A - B) M	Δ Ct (A - B) NM	RQ: 2^-ΔCt (x 10^{-5})		Average RQ (× 10^{-5})		ΔΔCt expression st dev (Normalized to untreated)		ΔΔCt expression (Normalized to untreated)	
		M	**NM**	**M**	**NM**	M	NM	M	NM	M	NM	M	NM	M	NM
Twist	0.5	26.9	26.2	12.2	12.1	14.7	14.1	3.75	5.69	3.75	5.321	0	0.0677	69.058	97.819
		26.9	26.2	12.2	12	14.7	14.2	3.75	5.3134						
		26.9	26.3	12.2	12	14.7	14.3	3.75	4.9576						
	1.00	27	26.3	12.3	12	14.7	14.3	3.75	4.9	3.673	5.203	0.0267	0.07823	67.516	95.639
		27	26.3	12.3	12	14.7	14.3	3.75	4.9						
		27.1	26.2	12.3	12.1	14.8	14.1	3.5	5.694						
	2.00	27.1	26.3	12.3	12.1	14.8	14.2	3.5	5.313	3.505	5.31	0	0	61.557	93.3033
		27.1	26.3	12.3	12.1	14.8	14.2	3.5	5.31						
		27.1	26.4	12.3	12.2	14.8	14.2	3.5	5.3						
Untreated		26.2		12.1		14.1		5.7		5.440		0.040470199			
		26.2		12		14.2		5.3							
		26.2		12		14.2		5.3							

3. Additional Figures and Short Comments.

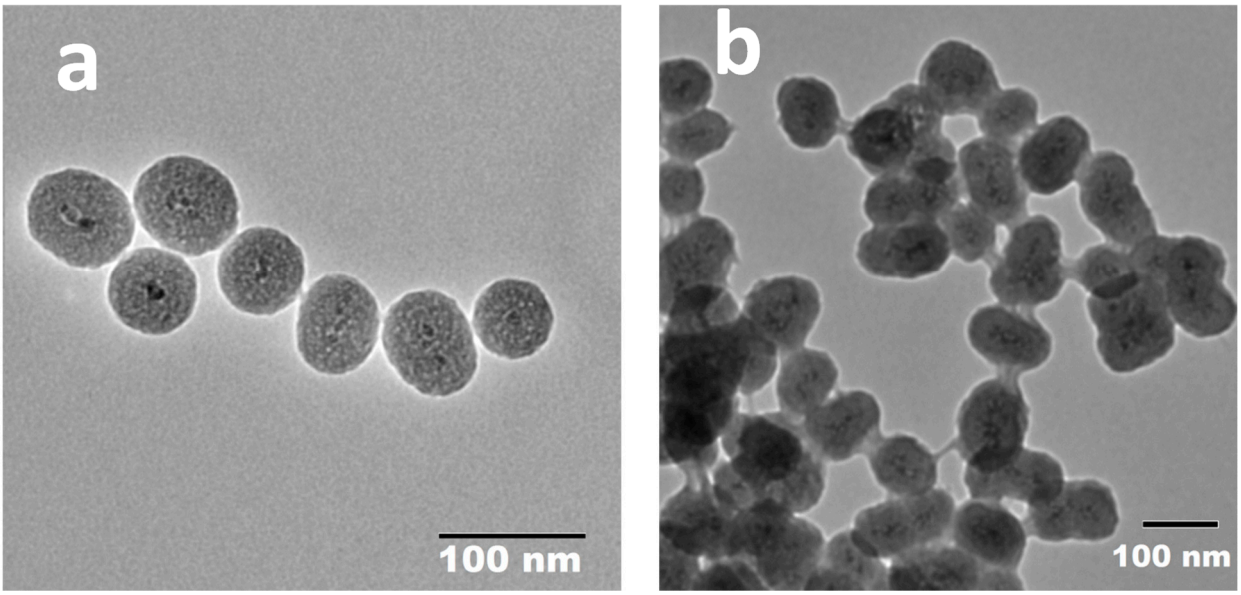


Figure S1. Transmission electron microscopy (TEM) images of magnetic nanoparticles: a) 50 ±5 nm silica-coated magnetic nanoparticles reveal a 15 nm iron oxide magnetic core and a 20 nm thick silica shell; b) magnetic nanoparticles with 12.5±5.0 nm grafted poly(acrylic acid)–poly(ethylene glycol) block-copolymer (PAA-*b*-PEGMA); the latter is observed as polymer structures bridging between two adjustment nanoparticles. Note that the aggregated state of the nanoparticles was obtained during the TEM imaging and it does not mean the nanoparticle aggregation in the solution state. The TEM images in this figure are shown to present size, shape and structure of individual particles. The particles appear as 2D agglomerated structures. TEM images are obtained for the samples prepared by deposition of a droplet of an aqueous particle suspension on the TEM-grid. Evaporation of water results in receding of the contact line when the capillary forces translocate particles that are "shoveled" by the contact line. The beads form 2D-aggolomerates with loosely assembled individual particles. This is very typical for TEM-imaging of nanoparticles. If particles were aggregated in solution, they would appear in TEM images as dense 3D-aggregates.

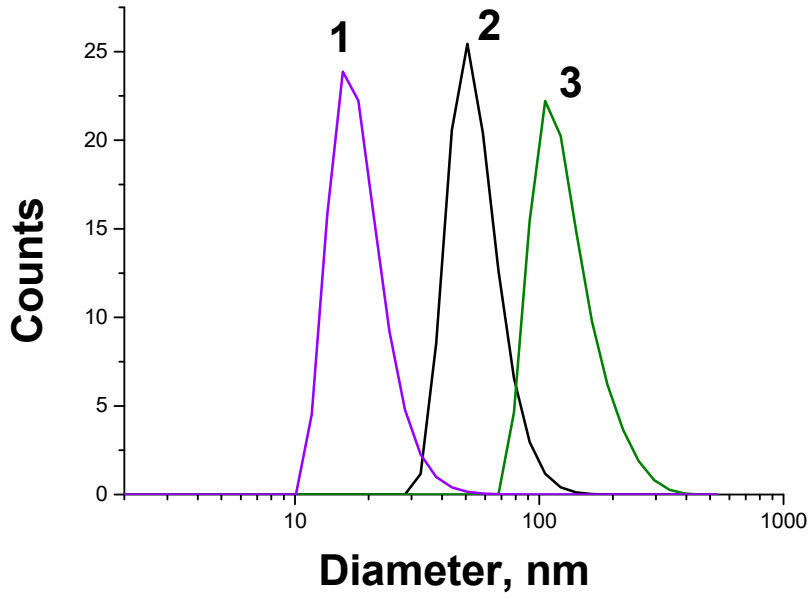


Figure S2. Dynamic light scattering (DLS) analysis of the nanoparticles in aqueous dispersions at pH 7.5: 1) Fe_3O_4 magnetic nanoparticles, 2) silica-coated magnetic nanoparticles, 3) magnetic nanoparticles with the grafted PAA-*b*-PEGMA polymer layer. Distributions by size of the magnetic beads in aqueous environment are shown in this figure when the measurements followed each step of the surface modification. The distribution functions demonstrate that a fraction of particles that exceeds the average diameter by 2-fold is less than 5%. This is a solid evidence that in the absence of the magnetic field the nanoparticle either not aggregated in aqueous solutions or a number of aggregates is negligible.

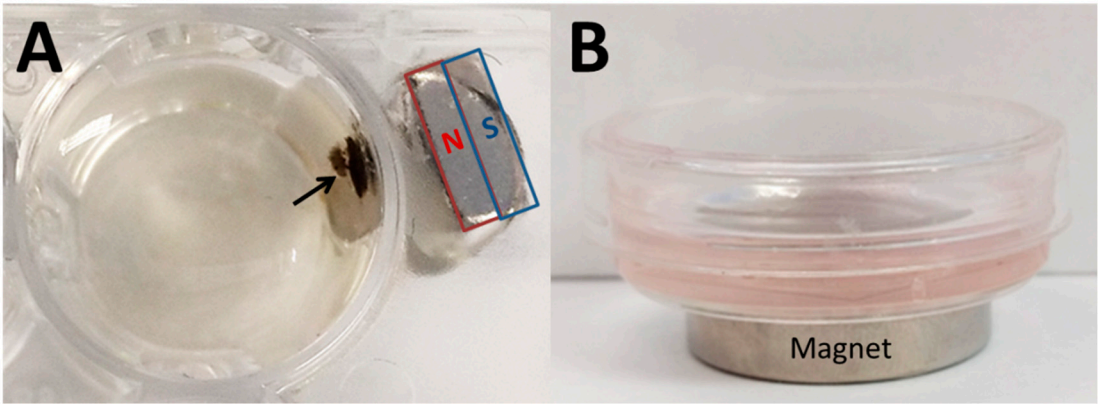


Figure S3. Photos of the experimental setups: (A) *ex vivo* experiments and (B) *in vitro* experiments. Aggregate of MaB is indicated by arrow in (A).

References

1. Bumb, A.; Brechbiel, M.W.; Choyke, P.L.; Fugger, L.; Eggeman, A.; Prabhakaran, D.; Hutchinson, J.; Dobson, P.J. Synthesis and characterization of ultra-small superparamagnetic iron oxide nanoparticles thinly coated with silica. *Nanotechnology* **2008**, *19*, art. No. 335601.
2. Deng, Y.-H.; Wang, C.-C.; Hu, J.-H.; Yang, W.-L.; Fu, S.-K. Investigation of formation of silica-coated magnetite nanoparticles via sol–gel approach. *Colloids Surf. A* **2005**, *262*, 87–93.

3. Bakshi, S.F.; Guz, N.; Zakharchenko, A.; Deng, H.; Tumanov, A.; Woodworth, C.D.; Minko, S.; Kolpashchikov, D.M.; Katz, E. Magnetic field-activated sensing of mRNA in living cells. *J. Am. Chem. Soc.* **2017**, *139*, 12117–12120.