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Nanoreactors Based on DNAzyme-Functionalized Magnetic Nanoparticles Activated by Magnetic Field

Saira F. Bakshi,[†] Nataliia Guz,[†] Andrey Zakharchenko,[§] Han Deng,[‡] Alexei V. Tumanov,[‖] Craig D. Woodworth,[‡] Sergiy Minko,[§]* Dmitry M. Kolpashchikov,[◊⊥]* Evgeny Katz[†]*

[†] Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, NY 13699-5810, USA
[§] Nanostructured Materials Lab, University of Georgia, Athens, GA 30602, USA
[‡] Department of Biology, Clarkson University, Potsdam, NY 13699-5810, USA
[‖] Department of Microbiology, Immunology, and Molecular Genetics, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900
[◊] Chemistry Department, University of Central Florida, 4000 Central Florida Boulevard, Orlando, FL 32816-2366
[⊥]ITMO University, Laboratory of Solution Chemistry of Advanced Materials and Technologies, Lomonosova St. 9, 191002, St. Petersburg, Russian Federation

*** Corresponding Authors:**
Evgeny Katz: ekatz@clarkson.edu,
Dmitry M. Kolpashchikov: dmitry.kolpashchikov@ucf.edu,
Sergiy Minko: sminko@uga.edu

Supporting Information

The major experimental steps and conditions have been detailed in the preliminary short publication.[1] Now they are collected in the Supporting Information below for the readers' convenience.

***Isolation and culturing of ectocervical cell strain HCX from human tissue*.** All cell culture preparations were performed following published procedure.[2] Ectocervical cells were cultured in Keratinocyte Serum Free Medium (KSFM) (Gibco).

***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, 100 µg/mL streptomycin, 0.5 µg/mL amphotericin B, 20 µg/mL gentamicin and 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). Superparamagnetic nanoparticles were synthesized by a co-precipitation methods as described elsewhere.[3] 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 hydrochloric acid. 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[4] 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 re-dispersible in water and formed a stable colloidal dispersion with a nanoparticle size of 45 nm and zeta potential ξ = -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 2 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 dichloromethane 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 60° 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 45-80 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).[1]

Preparation of MaBiDZ. To conjugate NH_2-modified DZa and Hook oligos (see all DNA abbreviated names and sequences in Table 1) 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. MaB conjugates were washed three times after each modification.

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.

DNase Assay. To prepare the particle-modified F-Sub solution, 5 µL of 1 mg/mL F-sub modified MaBs, 2 µL of 2 U/µL RNase-free DNAse I solution (New England Biolabs, Ipswich, MA) and 10 µL of 10x reaction buffer were combined and the solution was brought to a 100 µL volume with H_2O. The free F-Sub solution was prepared similarly, but with a 10 nM F-Sub concentration. Both solutions were calculated to have equal F-Sub concentrations, in a process described earlier[1]. The respective solutions were pipetted into separate wells of a 96-well plate (Greiner Bio, Kremsmünster, Austria) and fluorescence was monitored with respect to time using the SpectraMax i3x Microplate Reader (Molecular Devices, LLC., CA, USA).

Fluorescence measurements. The final MaB concentration was 25 µg particles per well (12.5 µg each of DZa- and Hook-modified nanoparticles) or 83 µg/mL. Neodymium N-52 magnets (K & J Magnetics, Inc., Pipersville, PA, USA) measuring 3/8" × 3/16" × 1/16" were glued to 48-well plates (Nunc™ Cell-Culture Treated Multidishes, ThermoFisher Scientific). The pre-incubated Twist, DZb and DZa-bound MaB conjugates were added to respective wells followed by the F-sub-Hook-MaB conjugates. Fluorescence measurements were taken using the SpectraMax i3x Microplate Reader (Molecular Devices, LLC., CA, USA).

Confocal laser scanning microscopy. For intracellular monitoring we used a Leica TCS SP5 II Laser Scanning Confocal Microscope. For magnet-controls, we placed NdFeB, grade 52, magnets measuring 5/16" diameter × 1/8" thickness (K & J Magnetics, Inc.) under each cell dish. Cells were seeded at 60-70% confluence 3 days prior to measurements in 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA), USA. Hoechst Stain (Santa Cruz Biotechnology, Dallas, TX,

USA) and EpCAM surface antibody stain (BioLegend, San Diego, CA, USA) were added at 0.5% and 1%, respectively, and allowed to incubate for 45 minutes. LysoTracker Green DND-26 (ThermoFisher Scientific) was used to stain endosomal compartments according to package instructions. MaBiDZ probe was then added at a 90 pM concentration (40 μg/mL). Prior to addition, DZb, and the prepared DZa-bound and F-Sub-Hook-bound MaB conjugates were pre-incubated in a thermostated water bath at 30^{o} C for 20 min. Confocal images were taken every hour for four hours, using the 405 nm, 488 nm, and 635 nm lasers to visualize Hoechst staining; Lysotracker staining and MaBiDZ probe fluorescence; and EpCAM staining, respectively.

Flow cytometry. Cells were seeded in 35 mm glass bottom dishes at 80% confluence. Cells were incubated with MaBiDZ for 2 hours. For magnet controls, a NdFeB, grade 52, magnet measuring 5/16" diameter × 1/8" thickness (K & J Magnetics, Inc.) was placed under each dish for 4 hours. Cells were then rinsed with Dulbecco's phosphate buffered saline (Gibco) and trypsinized with 500 μL trypsin for 20 minutes. Trypsin was neutralized with 500 μL media containing 10% FBS. Cells were centrifuged at 2000 r.p.m. for 3 min. Supernatant was removed and cells were re-suspended in a 1% solution of Alexa Fluor 647-conjugated EpCAM antibody (clone 9C4, Biolegend, San Diego, CA) in the dark at 4^{o} C. Cells were centrifuged at 2000 r.p.m. for 3 min and re-suspended in 500 mL 4% paraformaldehyde in PBS, pH 7, for 20 min. Cells were centrifuged at 2000 r.p.m. for 3 min. Supernatant was removed and replaced with 300 μL of 2% FBS.

References

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3 A. Bumb, M. W. Brechbiel, P. L. Choyke, L. Fugger, A. Eggeman, D. Prabhakaran, J. Hutchinson and P. J. Dobson, *Nanotechnology* 2008, **19**, art. No. 335601.

4 Y.-H. Deng, C.-C. Wang, J.-H. Hu, W.-L. Yang and S.-K. Fu, *Colloids Surf. A* 2005, **262**, 87–93.

Additional figures

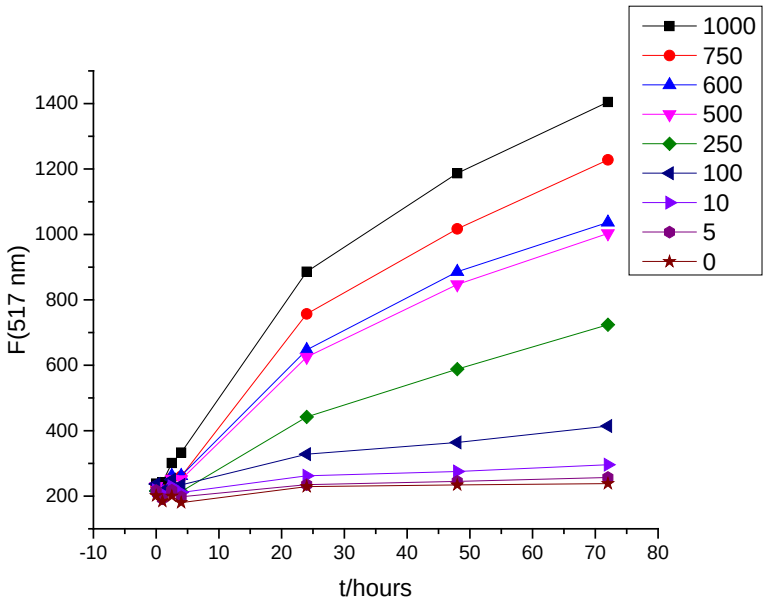

Fig. SI1. Kinetic plots of BiDz reaction for various Twist concentrations (in the picomolar range) measured by the fluorescence intensity at λ = 517 nm as a function of the reaction time. For 1 nM Twist, the reaction does not reach saturation even after 72 hours.

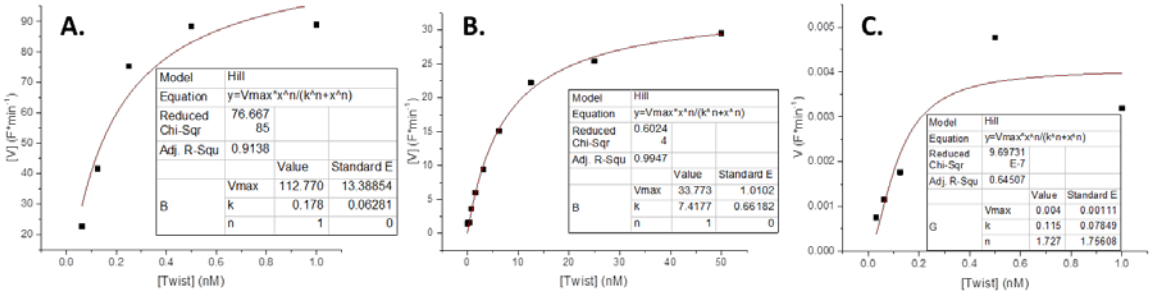

Fig. SI2. F-Sub cleavage kinetic studies with MaBiDz with a magnet applied (A), BiDz (B), and MaBiDz with no magnet applied.

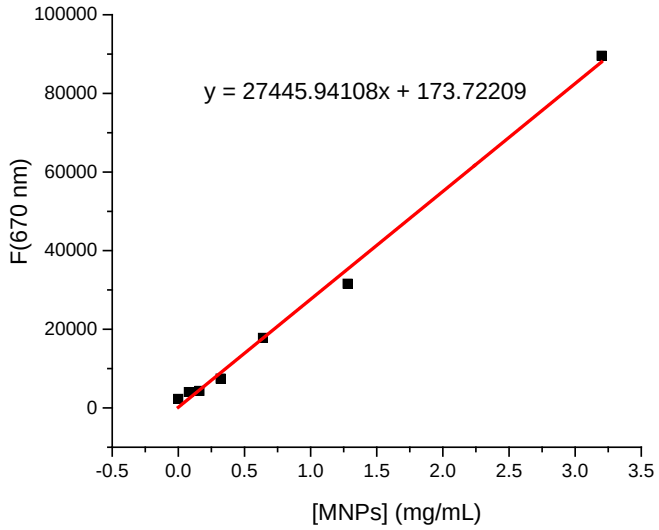

Fig. SI3. To calculate percent uptake of Quasar670-tagged oligo-modified magnetic nanoparticles (MNPs) into the cell, we first developed a calibration plot to relate Quasar670 fluorescence (F, measured at λ = 670 nm) to MNPs concentration. Using the resulting calibration equation, we could relate the fluorescence of MNPs internalized into cells with MNP concentration. Thus, we incubated MCF-7 cells (~4 x 10^5 cells/ well as counted by a hemocytometer) seeded in a Screenstar 96 well plate (Greiner Bio) with 320 µg of Quasar670-tagged oligo-modified MNPs for 30 min with a magnet applied underneath the cell and rinsed the cells to remove un-internalized MNPs. We then measured the fluorescence at 670 nm, subtracting the fluorescence of cells only: Fluorescence of cells + MNPs (avg of 3 measurements) - Fluorescence of cells only = 87793 – 4178 = 27445.94x + 173.72

This gave us a value of x = 3.04 mg/mL or 304 µg in 100 µL

We then calculated the uptake percentage as follows: 304 µg/320 µg = 95.00% uptake.

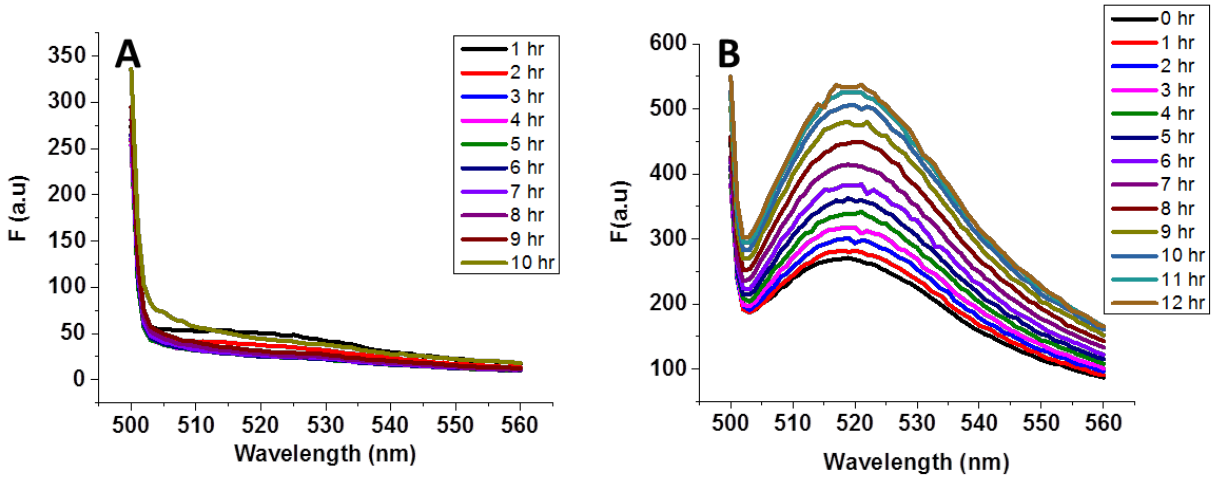

Fig. SI4. To study the specificity of the DNAzyme response, we prepared two sets of MCF-7 lysate from 600,000 cells (see Experimental section in the main paper) and incubated the respective lysed cells with DNAzyme machinery (100 nM DZa and DZb, 200 nM F-sub) A) specific to the analyte M.smeg, a mRNA sequence not found in mammalian cells and B) specific to Twist mRNA. A significant signal was not produced over a 10 hour period in A, indicating specificity of the DNAzyme response. Any increase in signal can be attributed to nuclease-induced cleavage of F-Sub, producing a fluorescent signal.