Electronic Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Aptamer-based Switchable Nanovalves for Stimuli-Responsive Drug Delivery

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MATERIALS

MCM-41 type (hexagonal) mesoporous silica particles were obtained from Sigma-Aldrich (Sigma-Aldrich Quimica SA, Madrid, Spain). The properties of the particles were indicated by the supplier as follows; the unit cell size: 4.6-4.8 nm; pore volume: 0.98 cm^3/g; pore size: 2.3-2.7 nm; spec. surface area: ~1000 m^2/g (BET); bp: 2230 °C; mp: >1600 °C and bulk density: 0.34 g/mL.

Sulfo-GMBS (Sulfo-N-succinimidyl 4-maleimidobutyrate sodium salt, CAS number: 185332-92-7) was from Fisher Scientific (Madrid, Spain). (3-Mercaptopropyl)triethoxysilane (CAS number: 14814-09-6), Fluorescein sodium salt (CAS number: 518-47-8) and all other chemicals were purchased from Sigma-Aldrich. Nucleolin protein was obtained from Abcam (Cambridge, UK).

The oligonucleotides were synthesized by Biomers (Ulm/Donau, Germany). The DNA sequence used in this study is given below. ATP aptamer sequence was selected by Huizenga.[1] Red nucleotides were added for creating hairpin structures. The hairpin of ATP aptamer was previously used for gating function by us.[2]

Table SI. The sequences of the oligonucleotides used in this study:

Nucleolin-Binding Aptamer Hairpin	5′-NH_2-CCACCACGGTGGTGGTGGTTGTGGTGCGTGGTGG-3′
Complementary DNA	5′-FAM-CCACCACGCACCACAACCACCACCACCGTGGTGG-3′
Control (ATP aptamer Hairpin)	5′-CACCTGGGGGAGTATTGCGGAGGAAGGTTCCAGGTG-NH2-3′

NH_2: *Amino-C6-modification,* ***FAM:*** *6-Carboxyfluorescein*

Black: Nucleolin-binding aptamer sequence[1], **Red:** Added nucleotides for creating hairpin

Particle Size Distribution and TEM: The particles used in this study were characterized further by electron microscopy (TEM, Figure S1A). TEM samples were prepared by ultra-sonication of powders in ethanol for 5min and drying of a droplet of suspension on a standard holey carbon TEM grid. TEM analysis was carried out on Titan 60–300 electron microscope (FEI, Netherlands) operating at 300 kV in TEM mode. The average diameter of particles was determined by dynamic light scattering (DLS) with a Zetasizer Nano-S (Malvern Instruments, Worcestershire, UK). About 5 mg aptamer-functionalized MCM-41 particles were suspended in PBS buffer for DLS investigation. The average size was determined to be 190 nm.

SYNTHESIS OF DYE-LOADED APTAMER-CAPPED PARTICLES

The immobilization of aptamers on the surface of MCM-41 particles was described previously.[2] Figure S1C is a depiction for aptamer conjugation on the silica surfaces. The details of the procedure are summarized below.

Silanization: 50 mg of MCM-41 powder in 20 ml of 95% ethanol containing 1 mM acetic acid and 3% (w/w) of 3-Mercaptopropyl)triethoxysilane were stirred for 30 min at room temperature, followed by three-fold washing with ethanol during centrifugation (13000xg, 3 min).

Loading and Capping: The sulfhydryl-modified nanoparticles (1 mg) were loaded with fluorescein by incubation in 100 µM fluorescein solution in **PBS** buffer (0.01 M phosphate buffered saline; NaCl-0.138 M; KCl-0.0027 M; pH 7.4) overnight and used for coupling with amino-modified oligonucleotides. 2 mg/ml sulfo-GMBS (Sulfo-N-succinimidyl 4-maleimidobutyrate) was mixed with the sulfhydryl-modified nanoparticles in PBS buffer and mixed for 30 min, washed 3 times with buffer and followed by 1 hour incubation under mixing in 100 µM solution of amino-modified aptamer (50 nmol in 500 µl PBS buffer containing 100 µM fluorescein). The particles were then washed thoroughly 3 times with buffer. Loading of fluorescein was calculated from the fluorescence of the particles measured and by comparing with a calibration curve.

Quantification of Immobilized Aptamer: The amount of aptamer immobilized on the surface of silica particles was measured by using complementary DNA (Table S1) labeled with FAM. The unloaded aptamer-particles were prepared as above and 0.01 mg of particles mixed with 5 µM FAM-labeled complementary DNA, followed by an incubation of 30 min. The particles were washed 5 times and re-suspended in 2 ml buffer for fluorescence reading. Bare particles were used to normalize the readings and the amount of immobilized aptamers was calculated from a calibration. For a typical preparation, 9.1 ± 1.1 pmol aptamer were immobilized per mg of particles. Coverage was estimated to be 40,500 aptamer molecules on average per NP particles.

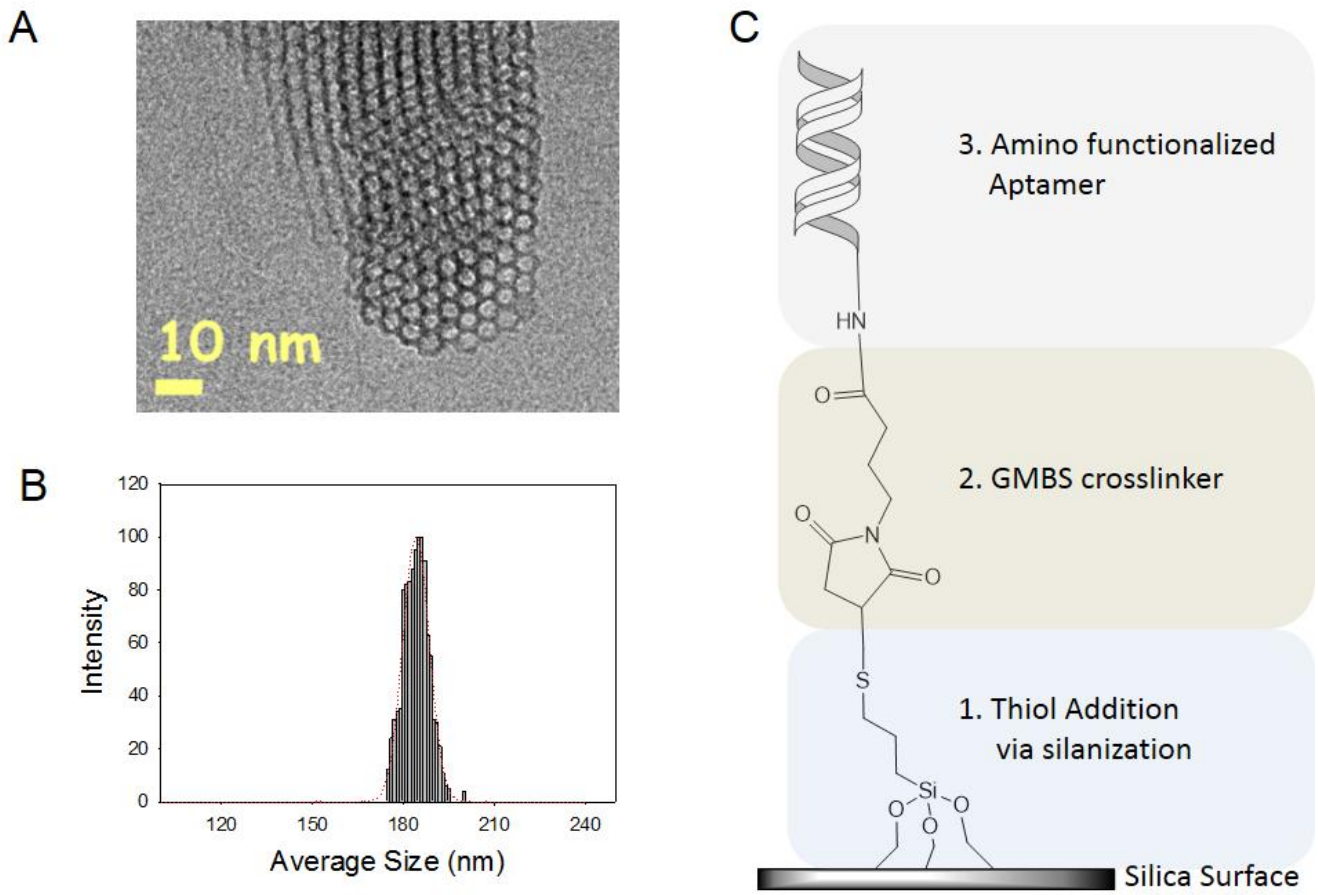

Figure S1. Characterization of aptamer-silica nanoparticles; A) Transmission Electron Microscopy (TEM) image shows the size of nanopores and B) Particle size distribution of aptamer-functionalized MCM-41 nanoparticles as determined by dynamic light scattering. C) Surface immobilization of aptamers on the silica surface via GMBS linker.

DYE RELEASE EXPERIMENTS

Release of fluorescein dye from pores was monitored in a FS920 steady-state spectrofluorimeter from Edinburgh Instruments (Edinburgh, Scotland, UK) equipped with a 450-W xenon arc lamp. Fluorescein was excited at 480/3 nm and emission was measured at 520/6 nm over time. The aptamer-capped mesoporous silica particles were placed in a cell holder, such that the particles were not

exposed to excitation light. The particles were kept from mixing by trapping them in a compartment created by a dialysis membrane (cellulose membrane with molecular cut of 12,000 Da) at the top of spectroscopy cuvette. Silica particles were large enough to be completely retained by dialysis membrane. A design of a two-compartment cuvette by using dialysis membrane was previously described in detail.[2]

The maximum loading of the particles with fluorescein was determined by preparing a mixture of 1 mg aptamer capped silica particle mixture in PBS buffer and measuring the fluorescence in a well-mixed solution. The amount of fluorescein released into the buffer was quantified by using a fluorescein calibration curve. For a typical experiment, the maximum loading of fluorescein dye was calculated to be 12.17 ± 1.42 pmol/mg particle.

Cell Culture and Flow Cytometry. MDA-MB-231 cells were maintained in Leibovitz's L-15 Medium supplemented with 2mM L-glutamine and 10% FBS. MCF 10A cells were cultured and maintained in MEBM complete growth medium (ATCC, USA) supplemented with 100 ng/ml cholera toxin. Cells were cultured in 60 mm dishes and harvested with 0.25% trypsin-EDTAas previously described.[3] Cells were blocked with 10% FBS in Dulbecco's Phosphate Buffered Saline (DPBS) for 10 min at room temperature. 25µg/ml of either Apta-NP-Fl or NP-FL was added to $2x10^5$ cells of MDA-MB-231 or MCF10A and incubated for various time ponts (1, 2, 3, 4, and 5 h) at 37ºC. Cells were washed twice with 10% FBS, once with DPBS and further resuspended in DPBS for flow cytometry analysis. Samples were analyzed using FACScan instrument and CellQuest Pro software.

Fluorescence microscopy: MDA-MB-231 and MCF 10A cells were grown in MatTek 35-mm glass bottom culture dishes one day prior to the experiment. On the day of the experiment, cells were washed with pre-warmed DPBS buffer and blocked in 10% FBS in DPBS for 20 min at room temperature. Next, cells were washed once with DPBS and incubated for 60 min at 37°C with 200 µL of Apta-NP-Fl (2.5µg/ml). Next, cells were washed twice with DPBS and nuclei were stained with DAPI (300 nM) for 30 min at room temperature. Images of the bound/internalized Apta-NP-FL were acquired with a 40 x oil objective of an Olympus IX71 inverted equipped with a CCD camera and filters for FITC (excitation BP450–490, emission BP515–565) and DAPI (excitation D360/40, emission D460/50). The fluorescence images reported here are representative of at least three captured images per dish/per condition. Fluorescence signal is given by the bound or internalized Apta-NP-Fl FITC channels were overlapped with the DAPI channels and subsequently, with the phase/contrast images using ImageJ 1.46 d software.

MEMBRANE-BOUND NUCLEOLIN PREPARATION

Since Apta-NPFl should be targeted to the nucleolin proteins on the cell plasma membrane surface for an efficient drug delivery application, we tested the functionality of our aptamer nanovalve by isolating membrane-bound nucleolin. Surface nucleolin was reported previously to be expressed constantly in confluent MDA-MB-231 cells by Hovanessian et. al.[4] The plasma membrane fragments were isolated and used in AS1411 aptamer characterization asSays according to a similar approach reported previously by Soundrarajan et. al.[5] We obtained membrane-bound fractions of MDA-MB-231 and MCF10A cells by centrifugation. A crude membrane fragment from each cell line was prepared according to Weeks et. al.[6] Cells were collected by scraping from confluent plates and suspended in ice-cold hypotonic lysis buffer (25 mM imidazole at pH=7 with protein inhibitor coctail of 1 mM PMSF and 0.1 mM aprotinin and 1.5 mM pepstatin A). The cells were disrupted by a loose-fitting teflon-glass homogenizer. The mixture was centrifuged at 2800 g for 10 min at 4 °C to remove nuclei and large fragments. The supernatant was subsequently centrifuged at 40,000 g for 30 min at 4 °C. The crude membrane fragments in the pellet were washed once with cold PBS. The preparation was solubilized in PBS and sonicated briefly before use. The total protein content was determined with Bradford protein assay (Pierce Biotechnology, Radford, IL). The same amount of total protein equal to 1 µg was used in the release experiments.

Figure S2 shows that nucleolin aptamer capped NPs retained fluorescein cargo compared to rapid release of cargo from uncapped NPs. Figure S3 is a comparison of nucleolin aptamer capped NPs and ATP aptamer capped NPs after addition of crude membrane preparation from nucleolin positive MDA-MB-231 cell line. The ATP binding aptamer was used as control sequence that is non-responsive to nucleolin. The nucleolin on the membrane fragments caused a rapid increase in the release of fluorescein cargo from 23 % to 70 % in 6 hours. The control sequence did not respond significantly. The same release experiments were repeated with crude membrane fractions from nucleolin negative MFC10A cells (Fig. S4). In this case, the control aptamer capped NPs did not respond to addition of 10 µl of membrane fragments similar to MDA-MB-231 cell fragments. The nucleolin aptamer capped NPs release some more fluorescein upon membrane fragment addition, but the level was not significant compared to that of MDA-MB-231 cell fragments.

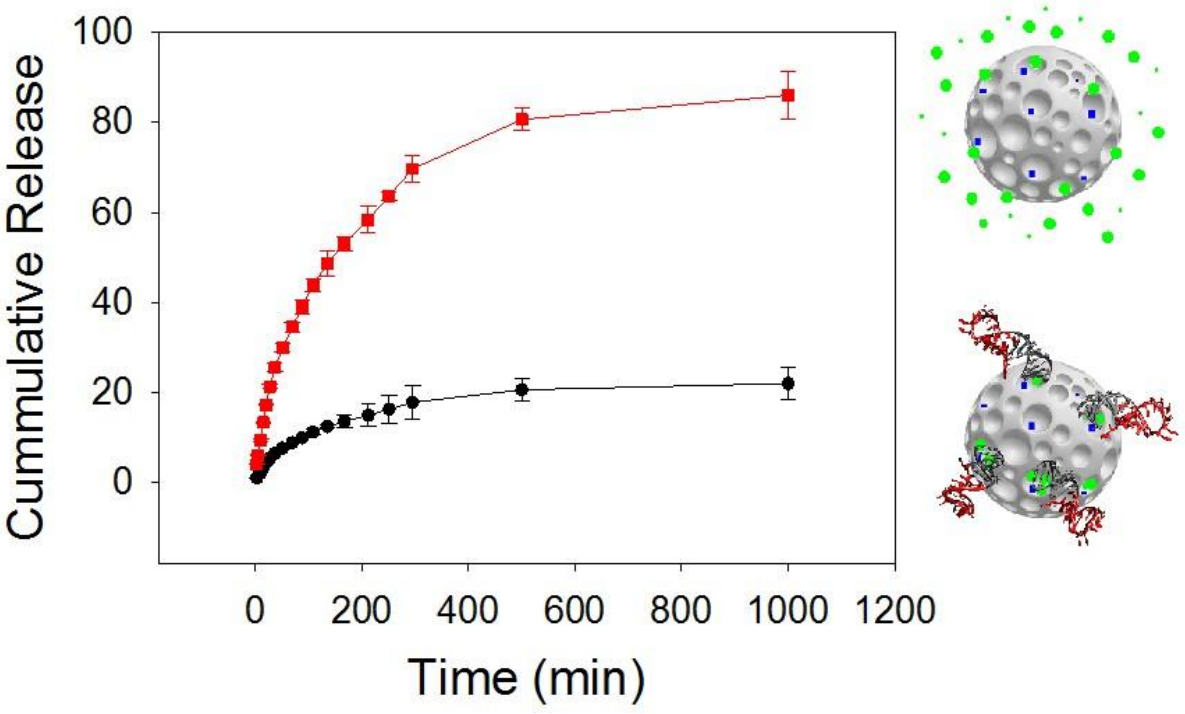

Figure S2. In vitro time dependent release of fluorescein molecules from loaded silica nanoparticles. Significant release of cargo molecule was observed for uncapped nanoparticle (red line). Aptamer-capped nanoparticles substantially retained fluorescein cargo molecules (black line).

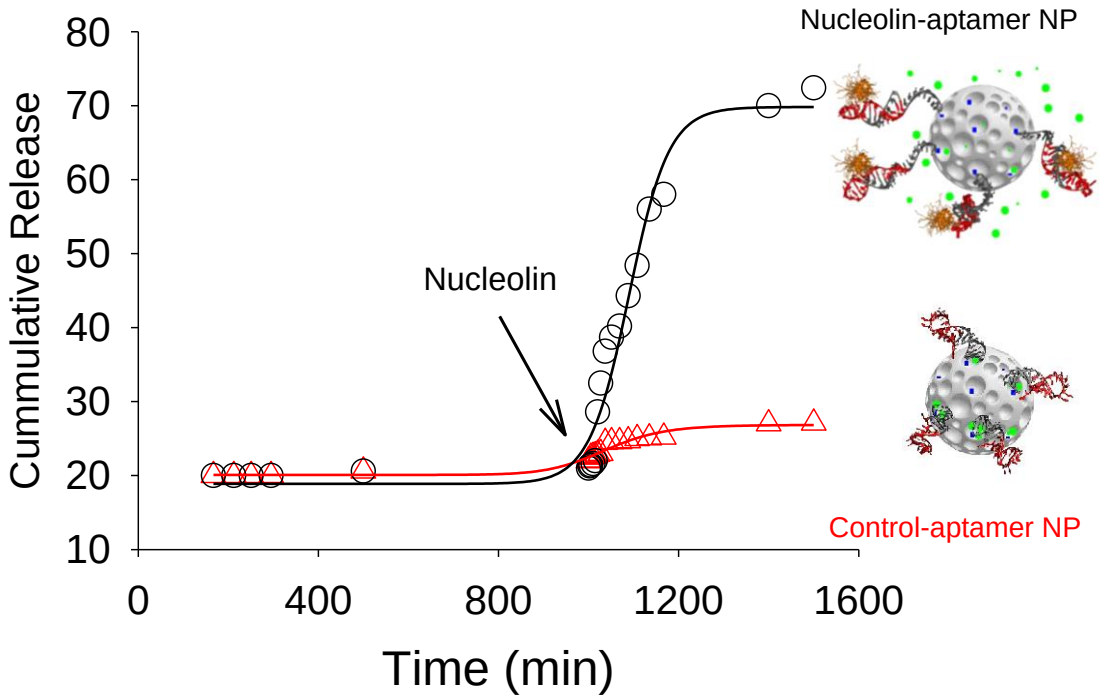

Figure S3. In vitro time dependent release of fluorescein molecules from loaded silica nanoparticles capped with either nucleolin aptamer hairpin or ATP aptamer hairpin (used as a control sequence). The arrow indicates the point of addition of nucleolin protein from MDA-MD-231 cells prepared as explained in section above. Significant release of cargo molecule was observed from nucleolin aptamer hairpin capped nanoparticle (black line). ATP aptamer-capped nanoparticles did not respond to nucleolin addition and substantially retained fluorescein cargo molecules (red line).

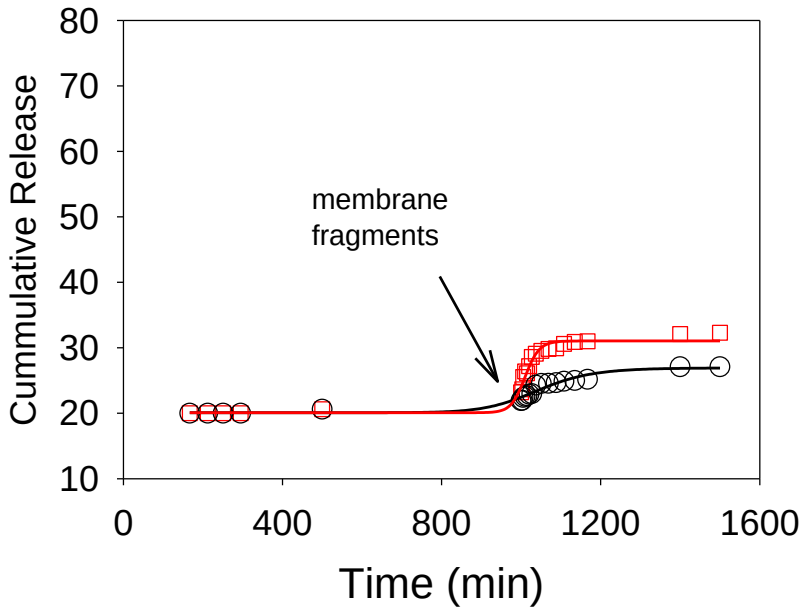

Figure S4. In vitro time dependent release of fluorescein molecules from loaded silica nanoparticles capped with either nucleolin aptamer hairpin or ATP aptamer hairpin (used as a control sequence). The arrow indicates the point of addition of cell membrane fragments from MCF10A cells prepared as explained in section above. A slightly more release of cargo molecule was observed from nucleolin aptamer hairpin capped nanoparticle (red line). ATP aptamer-capped nanoparticles did not respond to membrane fragment addition and substantially retained fluorescein cargo molecules (black line).

Surface Plasmon Resonance Analysis: Nucleolin aptamer hairpin sequences were analyzed by surface plasmon resonance (SPR) to verify binding to nucleolin proteins. A gold chip (Bionavis Ltd, Ylöjärvi, Finland) was treated with Piranha solution at room temperature, then rinsed with water and dried with nitrogen. Subsequently, the chip was mounted in SPR using PBS at 20 µL/min as running buffer. Streptavidin (1 mg/ml) in PBS was injected twice in both channels for 4 min to promote physical adsorption on the gold surface. Biotinylated ATP binding aptamer hairpin (200 nM) was injected for 4 minutes in channel 1. Similarly, biotinylated AS1411mb (200nM) was injected for 4 minutes in channel 2. Nucleolin protein at 50 nM in PBS buffer was injected in both channels and the response signal was recorded as seen in Fig. S5.

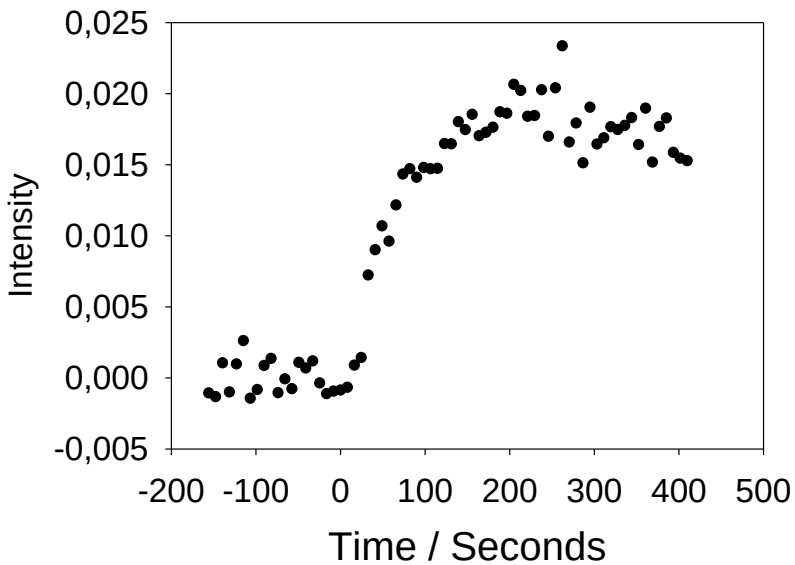

Figure S5. SPR response curve of nucleolin binding aptamer hairpin after injection of 50 nM purified nucleolin protein in PBS buffer. The presented figure is the difference graph of channel 2 (Nucleolin aptamer hairpin) and channel 1 (ATP aptamer hairpin). About 0.017 changes in intensity shows that nucleolin binds to nucleolin aptamer hairpin, but not to ATP aptamer hairpin.

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