Electronic Supplementary Information

Aptamer-Based Cell Imaging Reagents Capable of Fluorescence Switching

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Experimental Section

1. Materials

Recombinant human EpCAM protein (extracellular domain, amino acid residues 1-265, 34 kDa) was purchased from R&D Systems (Minneapolis, MN, USA). The other proteins and chemicals including streptavidin (SA), bovine serum albumin (BSA), immunoglobulin G (IgG), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. The random single-stranded (ss) library, primer blockers, unlabeled primers, Alexa 488-modified forward primers and biotinylated reverse primers were synthesized and purified by Integrated DNA Technologies (Coralville, IA, USA). Our starting ssDNA library was composed of a central random region of 60 bases flanked by two specific 20-base sequences (5‘-AGCAGCACAGAGGTCAGATG-[60N]-CCTATGCGTGC TACCGTGAA)

that act as primer-binding sites for PCR primers. HotStar Master Mix and PCR water were purchased from Qiagen (Venlo, Netherlands). Dynabeads MyOne SA C1, Dynabeads M-270 carboxylic acid and penicillin–streptomycin were purchased from Life Technologies (Carlsbad, CA, USA). Diacetylene monomers, 10,12-tricosadiynoic acid (TCDA) and 10,12-pentacosadiynoic acid (PCDA) were purchased from GFS Chemicals (Powell, OH, USA). HT-29 (human colon cell adenocarcinoma), WM-266-4 (human skin cell melanoma) and WI-38 (human lung cell normal) lines were obtained from the American Type Culture Collection (Rockland, MD, USA). RPMI medium was purchased from PAA Laboratories (Pasching, Austria). MEM medium and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Alexa Fluor 555-labeled anti-EpCAM antibody was obtained from Cell Signaling Technology (Danvers, MA, USA) and Dako mounting medium (Glostrup, Denmark) was used to mount the cells onto glass slides. Eight-chamber slides were obtained from Nunc (Roskilde, Denmark). All solvents used in this study were of analytical grade. Millipore Microcons (molecular weight cut off; MWCO 3 kDa and 10 kDa) were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Vivaspin centrifugal concentrators (MWCO 5 kDa) were purchased from Cole-Parmer (Vernon Hills, IL, USA).

2. EpCAM protein immobilization on beads.

100 μL of Dynabeads M-270 carboxylic acid-terminated magnetic beads were washed twice with 100 μL of 25 mM 2-(N-morpholino)ethane sulfonic acid (MES) (pH 5.0) for 10 minutes. 50 μL of 50 mg/mL EDC and 50 μL of 50 mg/mL NHS freshly prepared in cold 25 mM MES (pH 5.0) were added to the washed beads, which were mixed well and then incubated with slow rotation at room temperature for 30 minutes. After incubation, the activated beads were washed four times in a Dynal Magnetic Particle Concentrator (MPC)

(Life Technologies) and the supernatant was removed. Solution containing 10 μg of target protein EpCAM in 25 mM MES (pH 5.0) buffer was added to the beads to a final volume of 100 μL. This mixture was then incubated at room temperature for 2 hours with slow rotation, which allowed the EpCAM protein to be immobilized on the surface of the micron-sized magnetic beads by forming a stable amide bond between the NHS-activated magnetic beads and the amine group of EpCAM. After incubation, the unbound EpCAM was removed by MPC separator. The protein-coated beads were further incubated with 50 mM Tris (pH 7.4) for 15 minutes to quench the unreacted carboxylic acid groups, then washed 4 times with 100 μL PBS buffer containing 0.1% Tween-20 and finally stored at 4°C in the same buffer. To identify and quantify successful surface coverage of EpCAM on carboxylic acid beads, we conducted NanoOrange protein quantification (Life Technologies) and EpCAM ELISA (R&D Systems) assays. For the protein quantification, a sample volume of 50 μL/well was analyzed using a 485 nm excitation filter and 590 nm emission filter in a microplate reader (T ecan, San Jose, CA, USA).

3. SELEX assay.

We mixed 10 μM ssDNA library (0.1 nmol, 6×10^{13} different molecules) with two blocking oligos (20 μM each) to block both primer sequences during binding: forward primer-blocker (FP-B) 5'-CATCTGACCTCTGTGCTGCT-3' and reverse primer-blocker (RP-B) 5'-TTCACGGTAGCACGCATAGG-3'. This mixture was heated to 95°C for 10 minutes, slowly cooled down to room temperature and then incubated with 3×10^{13} EpCAM molecules immobilized on magnetic beads (~2:1 molar ratio between random library and EpCAM protein) in binding buffer (3.0 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 155 mM NaCl, 2.7 mM KCl, 30 mM $MgCl_2$, pH 7.2) for 2 hours at room temperature. To increase the stringency

of the selection process, we decreased the concentration of EpCAM to 6×10^{11} and 3×10^{11} for the second and third rounds, respectively, and performed the binding reactions at 37°C for 2 hours and 1 hour, respectively. After incubation, the aptamer-bound EpCAM magnetic beads were sequentially separated from unbound free oligos through two stringent washing steps, using the MPC separator and the previously-described micromagnetic separation (MMS) chip, a separation method that is quite advantageous for precisely manipulating small numbers of beads.[9a]

More specifically, the mixture was washed five times with high-salt buffer (10 mM Tris, 1 mM EDTA, 1 M NaCl, 0.01% Tween-20, pH 7.4) and 10 times with washing buffer (binding buffer with 0.05% Tween-20, pH 7.2) using the MPC. The collected bead pellets were subsequently flowed at 5 mL/hr through the MMS chip to capture the pre-washed aptamer-bound EpCAM beads. Washing buffer was simultaneously run through the bottom inlet at 0.5 mL/hr. We performed rigorous cleaning of trapped beads at a flow-rate of 50 mL/hr to remove weakly bound molecules. The external magnets were then removed, and the captured beads were eluted with 500 μL binding buffer into the collection tube, resulting in a total volume of about 650 μL.

We generated an amplified pool of ssDNA using a biotinylated reverse primer (5'-TTCACGGTAGCACGCATAGG-3') and unmodified forward primer (5'-AGCAGCACAG AGGTCAGATG-3') for both a pilot PCR and full PCR. The pilot PCR was a small-scale preliminary PCR with the aim of determining the ideal number of cycles to optimize correct amplification without non-specifically amplified products. Full PCR represents a large-scale PCR using a predetermined cycle number. The pilot PCR was performed with a mixture containing 50 μL HotStar Taq Master Mix, 0.25 μL of 100 μM unmodified forward primer, 0.25 μL of 100 μM biotinylated reverse primer, 39.5 μL of H_2O and 10 μL of eluate sample. After preheating at 95°C for 15 minutes, we subjected the samples to 35 thermal cycles of

95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. We collected 5 μL of PCR reaction sample during the elongation step at specific cycles and stored on ice until all samples were collected, and determined the optimal cycle number for PCR amplification via electrophoresis on a 10% PAGE-TBE gel. The collected aptamer pool was then amplified for the full PCR reaction at this optimized cycle number. We incubated the PCR solution with Dynabeads MyOne Streptavidin C_1 (Life Technologies) for 2 hours at room temperature to isolate only biotinylated double-stranded (ds) DNA from the PCR reaction mixture. ssDNA pools were then generated in solution for the next round of selection by incubating the beads in 15 mM NaOH for 4 minutes at room temperature. The supernatant was collected and neutralized with 1 M HCl. The amount of ssDNA in solution was determined at 260 nm via UV-visible absorption. The purity of the ssDNA was examined on a 10% PAGE–TBE urea gel. To confirm the efficiency of our SELEX process, we incubated 1 μL of the starting ssDNA library and the enriched aptamer pool obtained after one round of selection (final 100 nM) with EpCAM-coated magnetic beads or BSA-coated magnetic beads (1.5×10^8 beads) for 2 hours at room temperature, washed 5 times with 100 μL of PBS buffer, eluted bound DNA from the beads, and then added 5 μL of 1×SYBR Green dye to each fraction (Figure S1) to determine the amount of DNA present by measuring the fluorescence of the solution under a red filter (λ_{ex} = 488 nm, λ_{em} = 522 nm) with a TECAN microplate reader. To examine the nonspecific binding affinity of the selected pool, we performed the same procedure employing magnetic beads coated with BSA (5.13 μg/mL) instead of EpCAM (5.33 μg/mL). This selection process for EpCAM was repeated three times, with stringency gradually increased by decreasing the concentration of EpCAM, reducing incubation time or elevating reaction temperature. This results in increased aptamer affinity at later rounds. To improve the specificity of the aptamers selected after three rounds, we performed an additional round of counter-SELEX against BSA-coupled M-270 beads before isolating the final pool.

4. Cloning, sequencing and structure analysis of selected aptamers.

The final aptamer pool was amplified with unlabeled forward and reverse primers at the optimized cycle number determined by the pilot PCR, purified using a MiniElute PCR Purification Kit (Qiagen), and cloned with a TOPO TA cloning kit (Life Technologies). Fifty colonies were randomly picked and sequenced at Genewiz (South Plainfield, NJ, USA). The sequences were analyzed and divided into three groups based on sequence similarity using Vector NTI Explorer (Life Technologies). Secondary structure analysis was performed with *mfold* software (http://www.idtdna.com/Scitools/Applications/mFold/).

5. Binding affinity measurements of the final aptamer pool and the top selected aptamer.

To measure aptamer binding affinity and selectivity, we PCR-amplified either the final aptamer pool or individual aptamers selected based on sequencing via the procedure described above, using Alexa Fluor 488-labeled forward primer instead of unmodified forward primer and alkaline denaturation. Solutions of Alexa Fluor 488-labeled ssDNA (in a range of 0-80 nM) were heated to 95°C for 10 minutes and cooled down to room temperature for 10 minutes, and then incubated with 1.5×10^8 EpCAM-, BSA- or COOH-coated magnetic beads for 2 hours at room temperature with gentle rotation. Each sample was then washed five times with 1× PBS buffer containing 0.05 % Tween-20 using a MPC, and the remaining bound DNA was eluted with 100 μL of 1× PBS buffer via incubation at 95°C for 10 minutes with vigorous shaking. The fluorescence intensity of each supernatant was measured with a TECAN microplate reader (λ_{ex} = 488 nm, λ_{em} = 522 nm) and fitted to a saturation binding curve using nonlinear regression (assuming 1:1 binding) with Origin software (OriginLab) to

determine the K_d according to Michaelis-Menten kinetics (Figure 1A and B, Figure S2B).

6. Fabrication of aptamer-conjugated PDA liposomes.

An excess amount of TCDA-NHS (450 nmol) dissolved in 20 μL of DMSO was incubated with either of two aminated top binding clones (**JYK-01** and **JYK-02**) or a random control sequence (50 nmol) in 10 μL of deionized (di)-water for 4 hours at 37°C, yielding DNA-conjugated diacetylene monomers. Unreacted ssDNA and TCDA-NHS were removed by 10 kDa MWCO centrifugal filters with excess di-water. Mixed lipid constituents containing PCDA (400 nmol) and DMPE (300 nmol) were dissolved in $CHCl_3$, and the solvent was evaporated by nitrogen purging. After adding 1 mL of di-water, the resulting suspension was sonicated using a Branson 2510 sonicator (Danbury, CT, USA) for 5 minutes at 80°C. These lipid dispersions were immediately mixed with diacetylene filtrate suspension conjugated with an aptamer or a random sequence, yielding a final liposome concentration of 1.15 mM. As described previously,[6] the optimal PDA liposome composition was 6.7% aptamer-conjugated TCDA, 53.3% PCDA and 40% DMPE. The resulting solution was sonicated for 5 minutes at 80°C and then stored at 4°C for at least 4 hours to induce crystallization of lipid membranes. The aligned diacetylene monomer-phospholipid mixtures were polymerized by UV irradiation (400 $\mu W/cm^2$; Spectroline ENF-260C/FE UV lamp, Spectronics Corp., Westbury, NY, USA) at 254 nm for 45 minutes, producing blue-colored PDA liposomes.

7. Fluorescent detection of EpCAM with JYK-01 aptamer-conjugated PDA probes.

JYK-01 aptamer-decorated PDA probes (1.15 mM) were incubated with 4 ng/mL of

EpCAM, BSA and IgG at room temperature for 1 hour with gentle shaking. As a negative control, we prepared PDA liposomes modified with a random DNA sequence. The fluorescence intensity of the mixed PDA probes was measured (Fig. 1C) with a TECAN microplate reader (λ_{ex} = 485 nm, λ_{em} = 563 nm).[9b,9c] Additionally, the quantitative titration curve for fluorescence intensity (a.u.) of **JYK-01** aptamer-decorated PDA probes vs. EpCAM concentration (ng/mL) was plotted (Fig. 1D) with Origin software to determine the limit of detection (LOD). The **JYK-01** aptamer-modified PDA probes were incubated with various concentrations of EpCAM (0, 1, 2, 5, 10, 20, or 40 ng/mL) at room temperature for 1 hour.

8. Epithelial cancer cell imaging by aptamer-decorated PDA liposomes.

HT-29 and WI-38 cells were cultured in RPMI medium supplemented with 10% FBS and 100 units/mL penicillin–streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. WM-266-4 cells were grown in the same environment in MEM medium. To detect EpCAM protein overexpression, we seeded 3×10^4 cells of each cell line (2×10^6 cells/ml) on each well of an eight-chamber slide and allowed them to attach to the well surface. After growing in media for 24 hours, the cells were washed with 1× PBS solution and fixed with 100% ice-cold methanol at -20°C for 10 minutes. The fixative agent was removed by washing twice with PBS for 5 minutes, and then the cells were incubated with 3% BSA in PBS at room temperature for 1 hour. After this blocking step, excess BSA was removed via three washes with PBS solution for 10 minutes. Randomized control PDA, selected aptamer-incorporating PDA (**JYK-01** or **JYK-02** (Fig. S3†)) and Alexa Fluor 555-labeled anti-EpCAM antibody (200 μL) were applied to the cells and incubated at room temperature under shaking for 2 hours. To wash out unbound PDA or antibodies, the cells were stringently washed five times for 15 minutes in PBS containing 0.3% Triton X-100. After

performing nuclear staining with 1μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, MO, USA) at room temperature for 10 min, the cells were washed with PBS solution for 5 min and rinsed in running tap water for 1 min. They were gradually dehydrated using 50%, 80%, and 100% ethanol and left to dry overnight. Cover slips were mounted with mounting medium, after which we imaged aptamer and antibody fluorescence labeling (Fig. 2 and S3) with a confocal laser scanning microscope (Zeiss LSM 510 META, Jena, Germany) using two filters (λ_{ex} = 405 nm and 543 nm, λ_{em} = 420-480 nm and **560** nm) with 1700× magnification. The images were then processed with the aid of Zeiss LSM software.

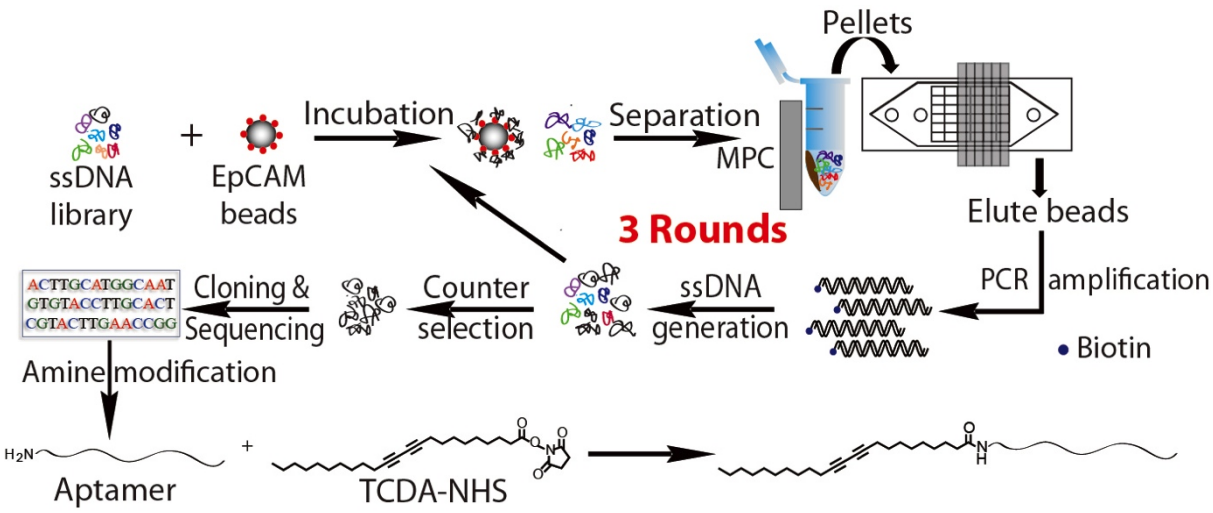
8. Liposome structural analysis using SEM and DLS.

We performed structural characterization of the aptamer-conjugated PDA vesicle suspensions before and after interaction with EpCAM or IgG using scanning electron microscopy (SEM) and dynamic light scattering (DLS).[6] SEM images (1000 X) of dried PDA vesicle samples were obtained with a field emission-SEM (Hitachi S-4800, Hitachi High-technologies Corp., Tokyo, Japan**).** A PDA vesicle solution dispersed in distilled water was applied to a silicon wafer, and then dried at room temperature for 2 hr. The sample was coated with pure platinum nanoparticles for 1 min and examined in the SEM at an accelerating voltage of 10 kV.

The average liposome size was determined using Brookhaven BIC DLS software, with instrumentation consisting of a BI-200SM goniometer, a 637-nm vertically polarized He–Ne laser, BI-9000AT correlator, and a BI-APD avalanche photodiode detector (Brookhaven Instruments Corp., Holtsville, NY, USA). We used a photon-counting photomultiplier detector with a fixed angle of 90°with respect to the direction of the incident beam. PDA vesicle solutions were prepared in a glass tube.

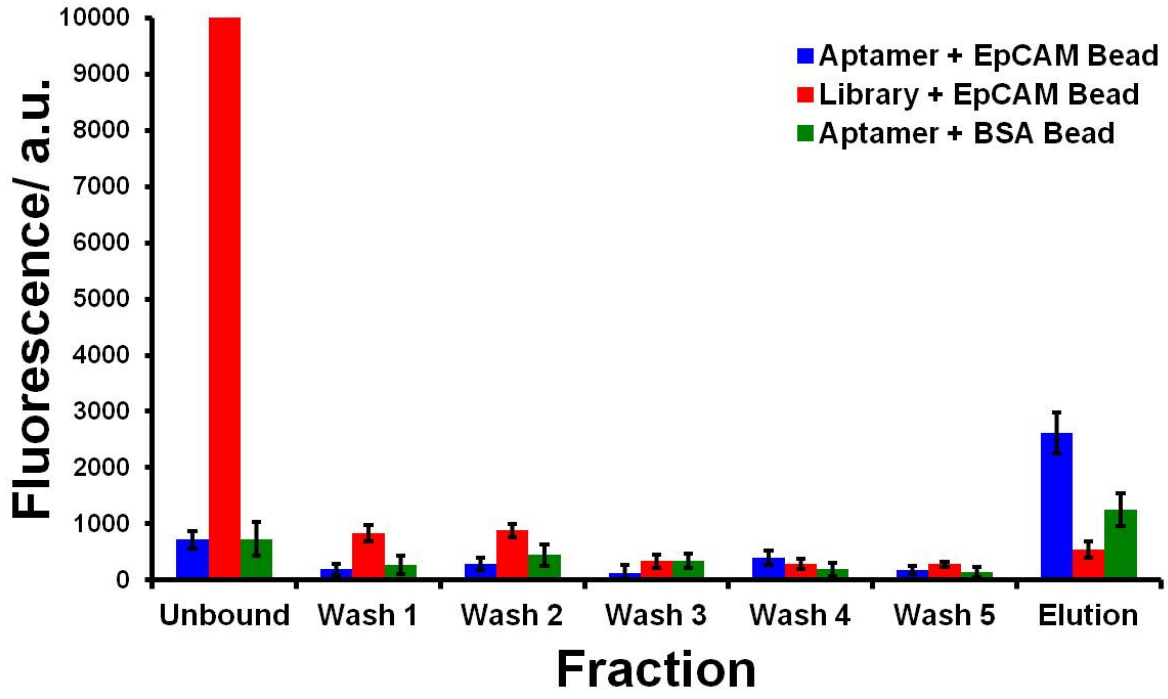
Electronic Supplementary Material (ESI) for Chemical Science.

Scheme S1. Overview of aptamer selection via magnetic particle concentrator (MPC) and microfluidic separation and conjugation of aptamer with activated diacetylene monomer (TCDA-NHS).



Electronic Supplementary Material (ESI) for Chemical Science.

Figure S1. Comparative EpCAM binding analysis of the aptamer pool obtained after one selection round. We incubated the aptamer pool with EpCAM- (blue) or BSA-conjugated (green) magnetic beads, washed the beads five times and finally eluted the bound DNA, with quantitation achieved via SYBR Green. In parallel, we incubated the starting library with EpCAM beads and subjected it to the same washing and elution procedures (red).



As shown in **Figure S1**, most of the starting library remained in the unbound fraction after incubation with EpCAM, while a relatively small amount of DNA from the selected aptamer pool was observed in the unbound fraction. Only a small amount of DNA was eluted during the following washing steps for both the starting library and the selected aptamer pool. Most importantly, the amount of DNA eluted from the selected aptamer pool was about five times greater than that obtained from the starting library, confirming the efficacy of our selection process. It is noteworthy that the sample eluted after incubating the aptamer pool with BSA-coated beads also displayed moderate fluorescence, implying that the selected aptamer pool also possesses undesired nonspecific binding affinity toward BSA. This clearly indicates the need to subject the enriched aptamer pool to negative selection with BSA-coated magnetic beads in order to remove nonspecific DNA molecules from the aptamer pool, thereby making the final aptamer pool more specific for the target EpCAM.

Electronic Supplementary Material (ESI) for Chemical Science.

Figure S2. (A) Sequences of three representative aptamers from Groups 2 and 3 (JYK-02, JYK-03, and JYK-04). **(B)** Fluorescence bead assay to determine binding affinity of these three representative aptamers for EpCAM-coated beads.

A

	Aptamer name	Sequences (60-mer)
Group 2	JYK-02	CGT TAC AGT TGG GTA ACG GGG TTA TGG GGT GGG TTG GGG CCA TAC CTC ACA TTG CGT TCA
	JYK-03	AGC CGG CTC GTC GGG GTT GGG CTG TCC GGC GGT TAG AAG TAC CCA ATC GCA TCT GGT AGT
Group 3	JYK-04	GAA CTG AAT GTA GGG TGT AGA AGG GGC CGG GAG CTA ATC GAG TGT TGA CCT GTA GAC GAC

B

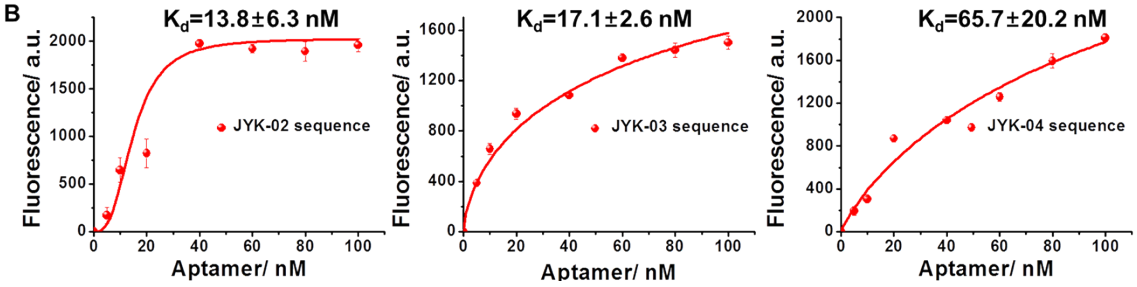
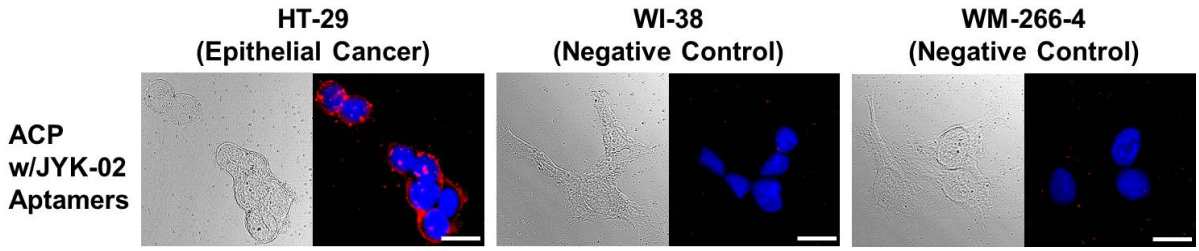
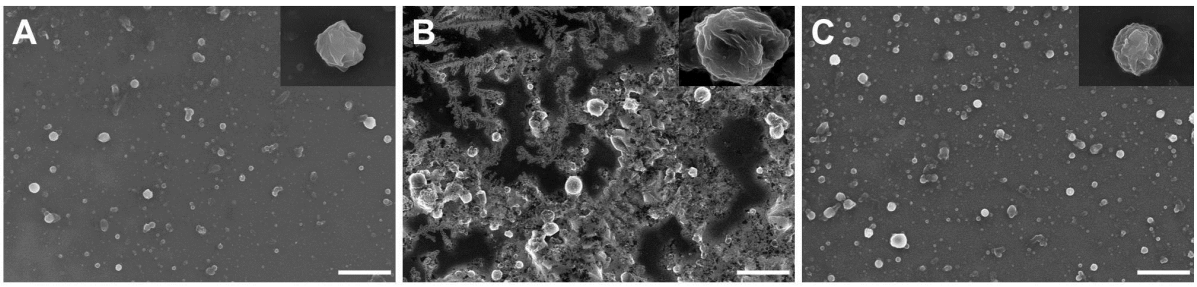


Figure S3. JYK-02 ACP targeting of EpCAM expressed on epithelial cancer cell membranes. Bright-field (**left**) and confocal fluorescence (**right**) microscopy images of HT-29 (epithelial cancer cell), WI-38 (normal epithelial cell; negative control), and WM-266-4 (human skin melanoma; negative control) after 2 hour incubation. This ACP exclusively generated a red fluorescence signal with HT-29 cancer cells, with no significant fluorescence associated with membranes of WI-38 or WM-266-4 cells. This aptamer thus achieves similar targeting to the JYK-01 ACP. DAPI was used for nuclear staining (blue). Scale bar = 20 μm.



Electronic Supplementary Material (ESI) for Chemical Science.

Figure S4. SEM images show the morphology of the JYK-01 ACP probes (A) in the absence of proteins and in the presence of (B) EpCAM and (C) IgG at magnifications of 1,500 X. Insets show magnified images of ACPs (30,000 X). Scale bar = 10 μm. (D) Particle size measurements (nm) based on dynamic light scattering before and after protein addition.



D

Without proteins	EpCAM treated	IgG treated
364 ± 37	933 ± 525	392 ± 57