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Modifying cellular properties using artificial aptamer-lipid receptors

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Correspondence and requests for materials should be addressed to
W.H.T. (tan@chem.ufl.edu)

* These authors contributed equally to this work.

Meghan O. Altman^{1*}, Yun Min Chang^{1*}, Xiangling Xiong^{1,2} & Weihong Tan^{1,2}

¹Department of Chemistry and Department of Physiology and Functional Genomics, Center for Research at the Bio/Nano Interface, Shands Cancer Center and UF Genetics Institute, University of Florida, Gainesville, Florida, 32611-7200, United States, ²Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Biology, College of Chemistry and Chemical Engineering, Collaborative Innovation Center for Chemistry and Molecular Medicine, Hunan University, Changsha, 410082, China.

We demonstrate that artificial aptamer-lipid receptors (AR), which anchor on the surface of cells, can modify important cellular functions, including protein binding, enzymatic activity, and intercellular interactions. Streptavidin (SA)-AR-modified CEM cells captured the tetravalent SA with one biotin binding site. The remaining biotin sites captured biotinylated TDO5 aptamers, which target IgM on Ramos cells, to form CEM-Ramos cell assemblies. In another design, thrombin, an enzyme involved in blood clotting, was captured by thrombin-AR-modified cells and clot formation was visualized. Lastly, hematopoietic stem cell (HSC) mimics were modified with a tenascin-C-AR to improve the homing of HSC after an autologous bone marrow transplant. Tenascin-C-AR modified cells aggregated to cells in a tenascin-C expressing stem cell niche model better than library-AR modified cells. Modification of cellular properties using ARs is a one-step, dosable, nontoxic, and reversible method, which can be applied to any cell-type with any protein that has a known aptamer.

The ability to choose which proteins are present on a cell's surface can be an important tool for modulating cell behavior. Methods have been designed to artificially add proteins, such as recombinant proteins¹, glycosyl-phosphatidyl-inositol-anchored proteins²⁻⁴, NHS-functionalized poly(ethylene glycol) oleyl derivatives⁵, and palmitated protein A complexes⁶, to cell membranes. However, recombinant strategies are time-consuming, and the use of lipid-functionalized proteins cannot be modulated. In addition to proteins, several groups have linked cDNA to the surface of cells via lipid attachment⁷ or used broad chemical modification of the cell surface⁸ in order to attach cells.

Building on these attempts, we sought to use aptamers as artificial receptors to capture proteins onto cell surfaces in a rapid, reversible, and dose-controllable manner. Aptamers are short DNA or RNA sequences that bind to specific targets, including proteins. Previously, our group synthesized a diacyllipid phosphoramidite⁹ nucleoside building block that has two long-saturated fatty acid chains held together with a 1,3-diamino-2-propanol connector (Figure 1). Micelles added to cells actively disassemble and intercalate into or attach onto the cell membrane by hydrophobic interactions^{10,11}. This diacyllipid phosphoramidite can easily be attached to the 5' end of any synthesized oligonucleotide. Thus, in theory, any aptamer can be functionalized with the lipid, which anchors the aptamer in the membrane, where it will protrude from the cell, ready to bind its target. Aptamer-micelles have previously been used to deliver dye-loaded micelles specifically to leukemia cells expressing the aptamer's target protein¹¹.

Results

In our first test to capture proteins onto the cell surface, cells were modified with streptavidin (SA) artificial receptors (ARs), enabling them to capture fluorescently labeled SA in a dose-controllable manner. SA is a tetravalent protein that binds the small molecule biotin with a very high affinity $K_d \approx 10^{-14}$ M, making it a useful tool in cell biology. SA-ARs were made by attaching the lipid tail to a 29-nucleotide (nt) aptamer that binds SA (40 nM K_d)¹². To confirm that SA-ARs retained their binding ability to SA, FITC-labelled SA-aptamers were competitively removed from SA-coated magnetic beads by SA-AR (Figure S1 in Supporting Information). All cell lines tested were able to capture Alexa-488-labeled SA (SA-488) on their cell membranes after insertion of SA-ARs (Figure 2A). On CEM cells (T-cell leukemia), aptamer insertion is detectable after 5 min and reaches saturation after 1 h (Figure S2 in Supporting Information).

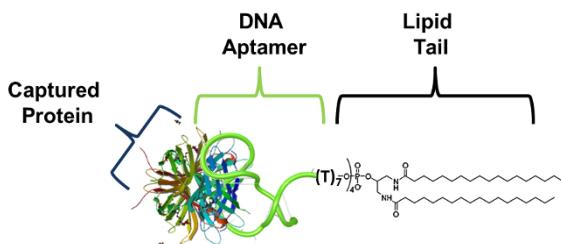


Figure 1 | Schematic of streptavidin-artificial receptors. Diacyllipid phosphoramidite is attached to the 5' end of the streptavidin-aptamer, which we call a streptavidin (SA) artificial receptors (AR). This AR can be added to cells to capture streptavidin and thereby modify their properties.

Incubation of CEM cells with different concentrations of SA-AR resulted in the dose-dependent capture of SA on the cell surface (Figure 2B). Specifically, incubation with as little as 31 nM of SA-AR, which is below the K_d for the SA aptamer, was sufficient for detection of SA-488 on the cell surface by flow cytometry. Increasing the SA-AR concentration increased the fluorescence signal from SA-488 in a concentration-dependent manner until it reached a plateau at around 5 μ M (Figure 2B).

SA-ARs persisted on the cell membrane for an extended time, but the amount of aptamer slowly decreased over the 2 days after incubation (Figure 2C). After 2 days, fluorescence became undetectable, indicating that SA-AR modification is temporary, and that cells returned to normal after being cultured for 2 days. In fact, SA-AR insertion had no effect on cell proliferation as measured by the standard assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Figure 3). These results indicate that SA-AR treatment does not negatively affect cell growth, an important criterion for future applications.

Because this SA aptamer does not bind at 37°C, the SA-488 signal on CEM cells dropped to background levels when the temperature was increased from 4°C to 37°C (data not shown). This result demonstrates that the binding of SA-AR to its target SA-488 can be modulated by altering environmental conditions such as temperature. Further, we confirmed that the biotin binding sites on SA were still available for binding (Figure 4A,B).

To demonstrate that we could isolate SA-AR-modified cells, we captured SA-AR-functionalized cells with SA-coated DynaBeads (Figure 4C). In this experiment, CEM cells were modified with either the control, PDGF-AR, or the target, SA-AR, artificial receptors. When they were mixed with streptavidin-coated magnetic beads

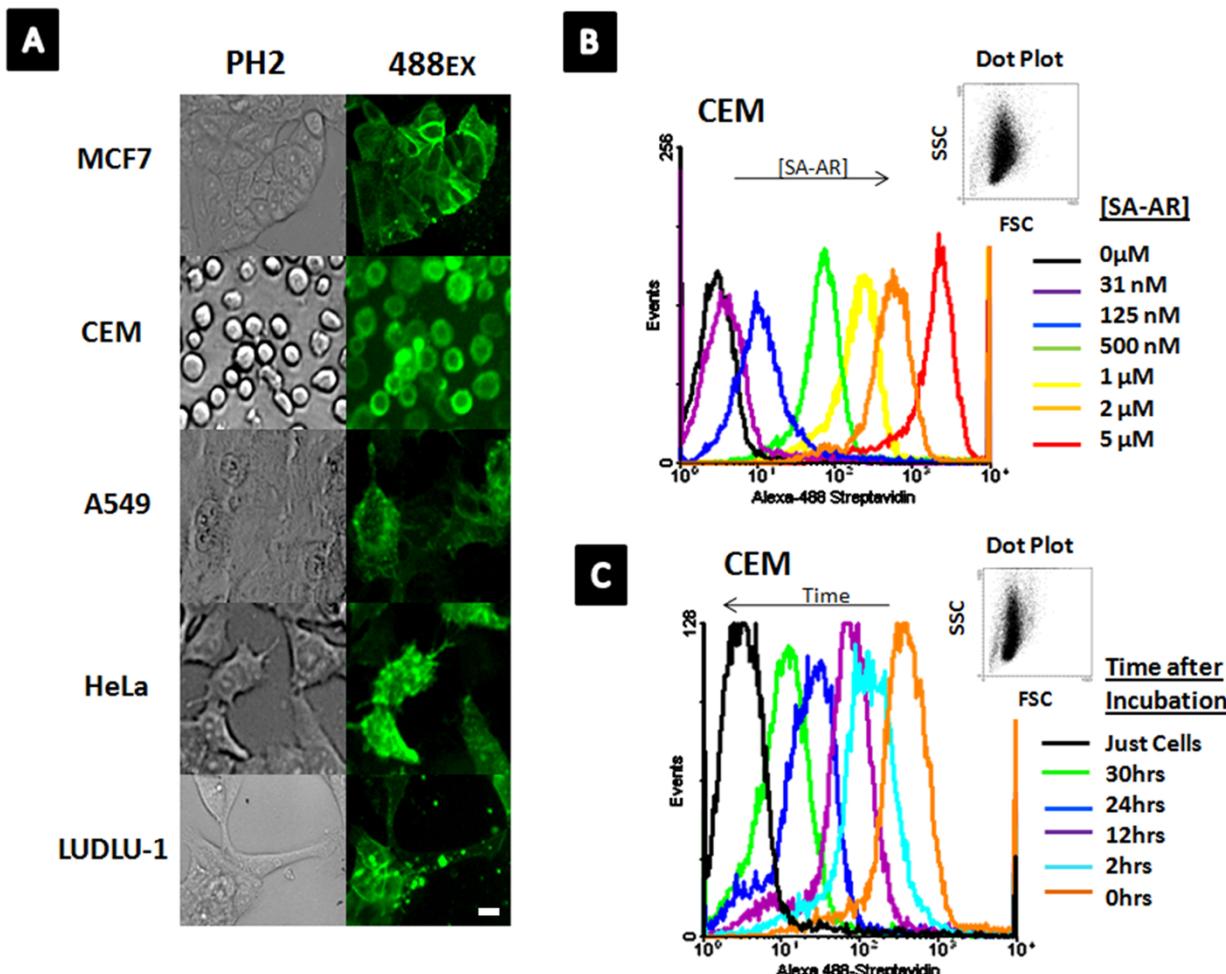


Figure 2 | Characteristics of streptavidin-artificial receptors (SA-AR). (A) Artificial receptors can be inserted in many different cell types. Cells were incubated with 2 μ M SA-ARs for 2 h at 37°C. After adding SA-488, cells were imaged with bright field and fluorescence (488 ex) microscopy. (B) CEM cells were incubated with different concentrations from 31 nM to 5 μ M SA-ARs for 2 h at 37°C, followed by addition of SA-488. SA-488 on the cell surface was analyzed by flow cytometry using channel 1 for SA-488. (C) CEM cells were incubated with 2 μ M SA-AR for 2 h at 37°C, followed by washes and incubation at various times from 0 to 30 h before addition of SA-488. Following treatment, the cells were analyzed by flow cytometry. All histograms are un gated.

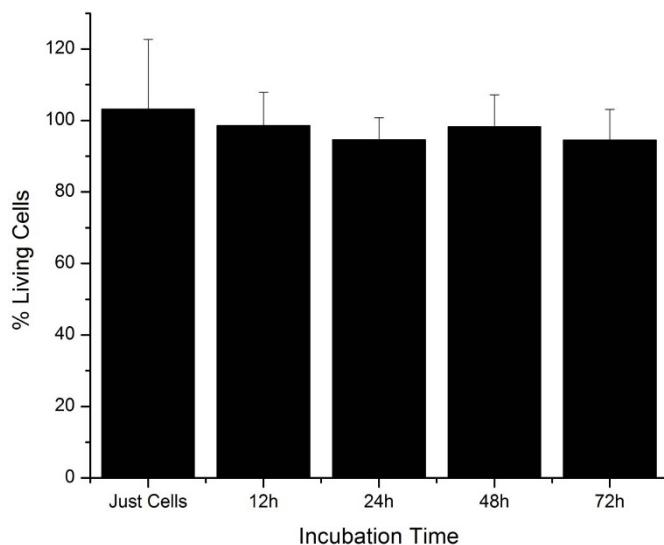


Figure 3 | SA-AR does not inhibit cell growth. CEM cells were incubated with 2 μ M SA-AR in 100 μ L media over 72 h, after which proliferation was determined by MTS.

in a buffer, the cells were enriched on the beads. After lysing the cells and probing them *via* Western blot for β -actin, only the 2 positive controls (the sgc8-biotin-bound cells in Lane 3 and the pure cell lysate in Lane 7) and the SA-AR- modified cells (Lane 5) were able

to enrich the cells in buffer, indicating the presence of β -actin. The untreated cells (Lane 1), the TD05-biotin (Lane 2), which does not bind CEM, and the PDGF-AR (Lane 4), which does not bind streptavidin, showed no evidence of β -actin. This assay shows that SA-AR can be used to modify cells and then recapture them with SA-coated beads.

In addition to SA-AR, we also prepared a thrombin aptamer-receptor (T27-AR) that captures thrombin, allowing modified cells to cause clotting. Thrombin is an enzyme important in the clotting of blood. When activated, it cleaves its substrate, soluble fibrinogen, into insoluble fibrin. Thrombin has two very well studied aptamers: a 15 nt aptamer (T-15), which binds the fibrinogen cleavage site on thrombin with relatively low affinity ($K_d = 450$ nM), and a 27 nt aptamer (T-27), important for heparin binding, which has a lower K_d and higher binding affinity ($K_d = 0.7$ nM). By attaching a lipid tail to T-27, we made a high affinity thrombin-aptamer artificial receptor (T27-AR). The T27-AR binds thrombin through the heparin binding site, leaving the fibrinogen cleavage active site free for binding to fibrinogen or the low affinity T-15 aptamer. We confirmed thrombin bound to the T27-AR modified cell surface by staining the cells with SA-488 tagged T-15 aptamer and measuring their signal by flow (Figure S3 in Supporting Information).

T-15 binds the fibrinogen cleavage site on thrombin and inhibits the ability to cleave fibrinogen^{13,14}. As the T-15 binding site was clearly exposed on cells modified with thrombin, we asked whether these cells could induce clotting if fibrinogen was added to them. In this assay, we captured thrombin on the surface of CEM cells using the T27-AR, followed by addition of fibrinogen. Cells modified with

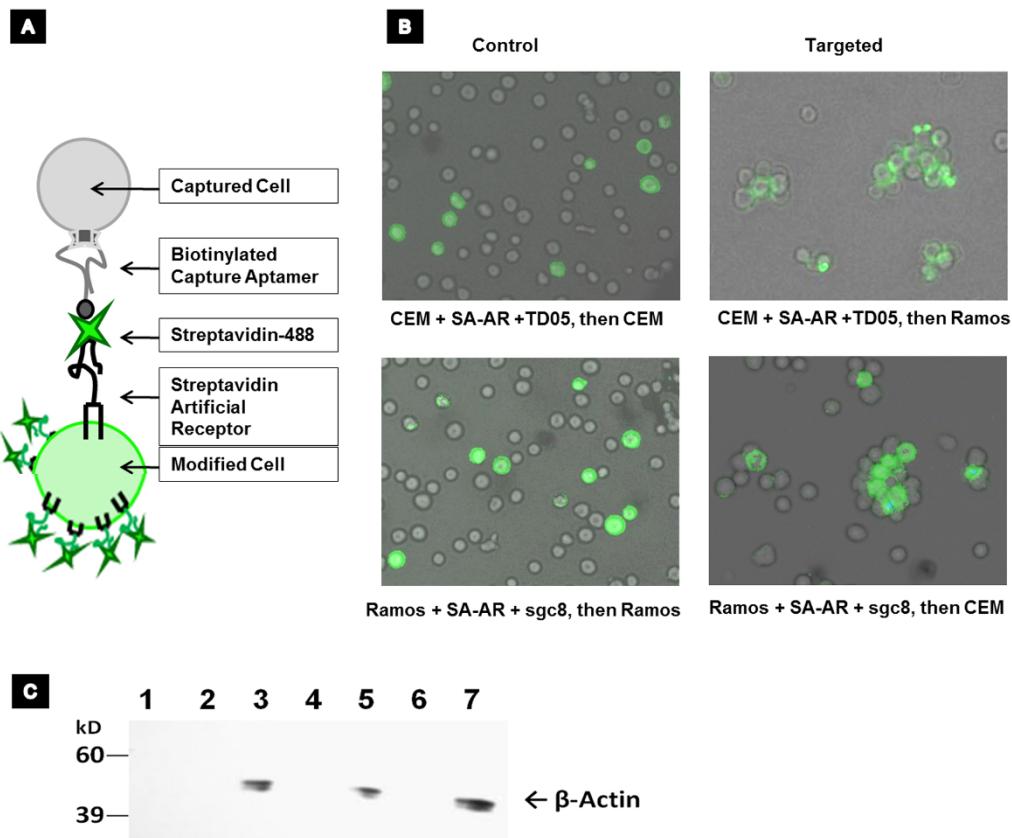


Figure 4 | Streptavidin-Modified Cells Bind Biotin to Make Cell Assemblies. (A) Scheme showing the cellular assembly. (B) Top: CEM cells modified with streptavidin-488 via SA-AR incubated with TD05 aptamer that binds Ramos cells and then either CEM (control, left) or Ramos (target, right). Bottom: Ramos cells modified with streptavidin-488 via SA-AR incubated with sgc8 aptamer that binds CEM and then either Ramos (control, left) or CEM (target, right). (C) SA-AR modified cells are collected with streptavidin coated magnetic beads. CEM cells modified with SA-AR or a non-specific PDGF-AR were collected with streptavidin coated DynaBeads. Cells were lysed, and the lysates were probed by Western blot for β -Actin. (1) Unmodified cells. (2) TD05-biotin (3) Sgc8-biotin. (4) PDGF AR. (5) SA-AR. (6) Empty. (7) CEM lysate.

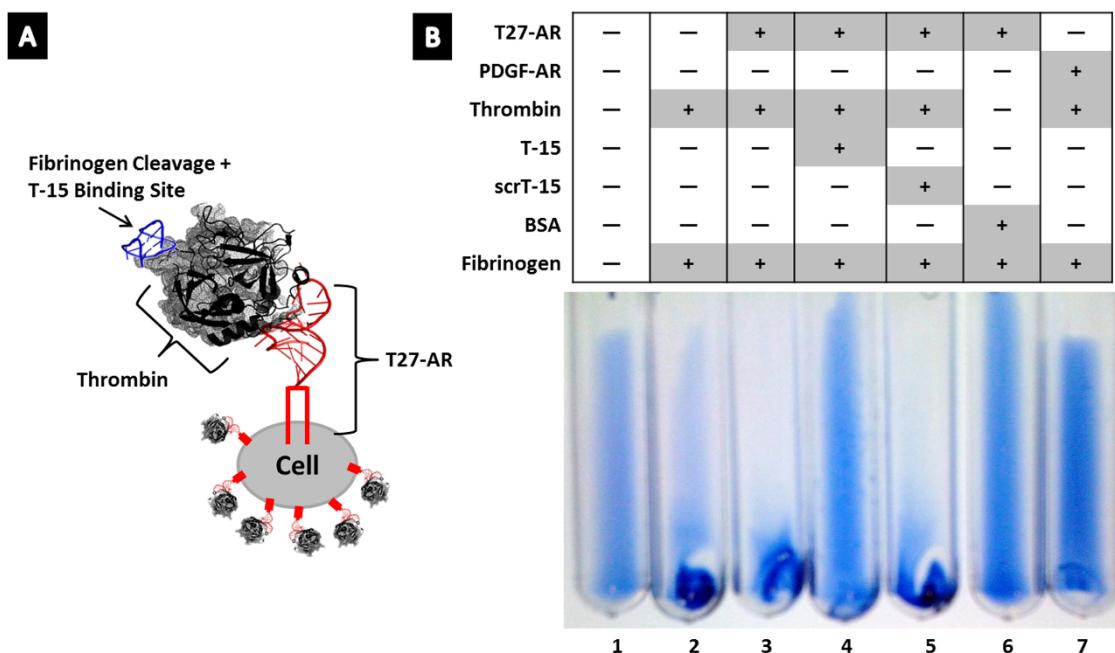


Figure 5 | Thrombin-Modified cells cause clotting. 500 K healthy CEM cells were washed and incubated for 2 h at 37°C in 50 μ L media (Tubes 1&2), 5 μ M T27-AR (Tubes 3–6), or PDGF-AR (Tube 7). After washing, cells were incubated with buffer (Tube 1), 500 nM thrombin (Tubes 2,3,7), 500 nM thrombin pre-incubated with 1 μ M T-15 (Tube 4), 1 μ M scrambled T-15 (Tube 5), or 200 nM BSA (Tube 6) for 30 min at RT. After 5 washes, fibrinogen was added to Tubes 2–7. Clots formed and were visualized by adding 50 μ L trypan blue stain.

thrombin clotted fibrinogen within 15 sec (Figure 5, Tube 3). The clots had a gel-like consistency visualized by trypan blue. As expected, addition of T-15 to the mixture competed with fibrinogen for access to thrombin's active site, and no clotting was seen (Tube 4). By contrast, when a scrambled T-15 (scrT-15) was added, clotting was recovered (Tube 5). Likewise, use of a control protein, BSA (Tube 6) or a control aptamer-receptor, PDGF-AR (Tube 7), did not cause clotting.

Next, we modified CEM cells with tenascin-C (TNC) aptamer¹⁵ AR to simulate the phenomenon of stem cell homing after an autologous bone marrow transplant. Stem cells rely on their local environment or “niche” to survive and differentiate¹⁶. Outside this niche, hematopoietic stem cells (HSC) cannot function, and do not produce the different lineages necessary to re-establish the blood and immune system. During bone marrow transplants, bone marrow stem cells are injected into a patient's bloodstream where they follow chemo-attractive signals to find their stem cell niche. This homing process is not perfect, however, and many cells do not arrive at their proper locations, causing transplant failure. Increasing the homing ability of HSC would relieve this problem.

Tenascin-C is found in the matrix of the stem cell niche and plays a key role in homing hematopoietic cells to their niche¹⁷. We hypothesized modifying stem cells with TNC-AR would allow the cells to better find and remain in their niche after bone marrow transplants. As a proof of concept, we modified a T-cell leukemia cell-line, CEM, with TNC-AR. Monolayers of MDA-MB-231, a human breast adenocarcinoma cell line high levels of surface tenascin-C, provided our model stem cell niche. To this model niche we added TNC-AR-modified T-cell leukemia CEM cells (Figure S4 in Supporting Information)¹⁸ and calculated binding.

One million CEM cells were labelled with Tracker Green (1.0 μ M) for 10 min at 37°C then modified with TNC-AR labelled with FITC, which also excites at 490 nm, for 20 min at 37°C. MDA-MB-231 cells were not modified with any artificial receptors but were labelled with Far Red (1.0 μ M), which excites at 640 nm. Tracker Green-labelled CEM cells modified with library-AR-FITC were used as a

control. The TNC-AR modified and control CEM cells were allowed to interact with the labelled MDA-MB-231 at 4°C for 1 h. Interactions were carried out at two different CEM to MDA-MB-231 ratios: 10 to 1 and 5 to 1. The intersection of the two fluorescence signals, indicating binding, was seen on the dot plot of the flow cytometry analysis (Figure 6A). The number of cells in the upper right (UR) quadrant over the total number of cells in both the lower right (LR) and upper right quadrants yielded the percentage of aggregation between MDA-MB-231 and CEM cells. CEM cells modified with TNC-AR bound MDA-MB-231 cells twice as efficiently as library-artificial receptor modified CEM cells (Figure 6B).

Discussion

Artificial aptamer-lipid receptors offer a simple, biomimetic, non-toxic, reversible, and dose-controllable strategy for modifying cell membranes with any protein for which there is a known aptamer. This new strategy is rapid, one-step, reversible, and it does not alter the captured protein, making it an improvement over current methods. In this work we show the ease and flexibility of this approach by modifying cell surfaces for three separate purposes: (1) to label or capture cells with streptavidin; (2) to attach thrombin onto T-cells giving the cells a *de novo* ability to cleave fibrinogen and cause clotting; and (3) to facilitate binding between two cell types, one modified with TNC-AR and one expressing high levels of TNC, which may be helpful for increasing stem-cell homing after autologous bone marrow transplants.

The cell membrane is the main site for cellular interactions with its environment. By placing temporary receptors on the surface of cells, we have constructed self-assemblies combining several proteins, cell types, or aptamers on the membrane. These complexes imbue the modified cells with new abilities that can be tailored to specific therapeutic or diagnostic needs just by altering the choice of aptamer. With the rise of stem-cell based and other whole-cell based therapies we envision this technology being used to encourage engraftment and make these cellular delivery systems more robust.

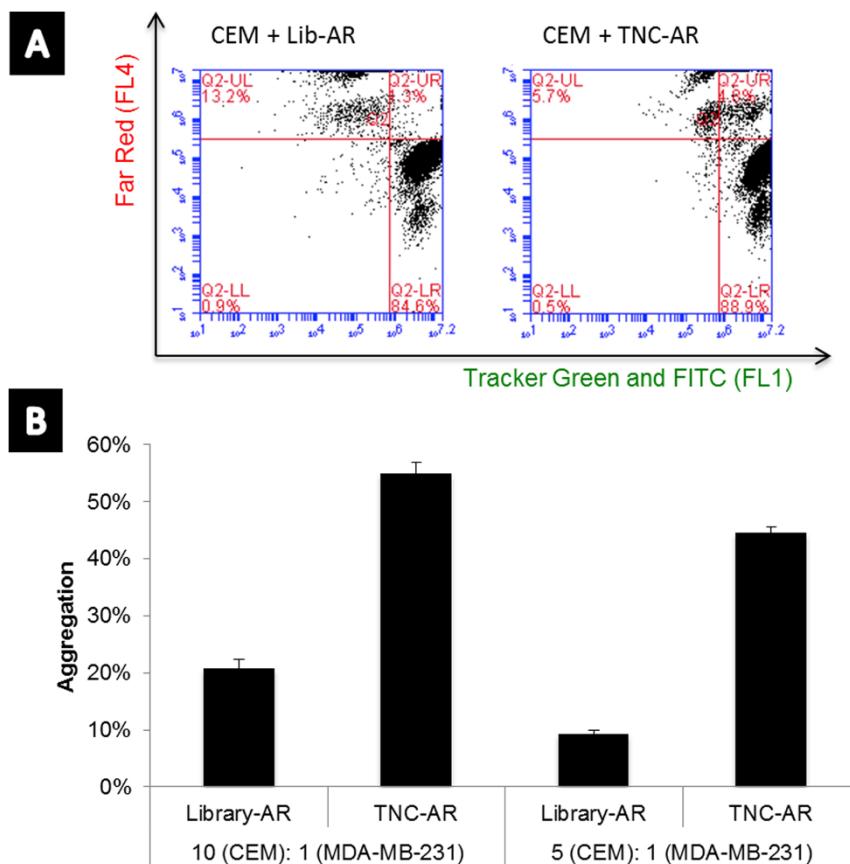


Figure 6 | Tenascin-C modified cells are better able to aggregate to tenascin-C containing cells. (A) Flow cytometry results show aggregation of TNC-AR modified CEM with MDA-MB-231 cells. A population of cells exhibiting both FL1 and FL4 signals can be seen when TNC AALR-modified CEM cells were allowed to aggregate with MDA-MB-231 at 4°C. (B) 55% aggregation was observed for TNC-AR modified cells compared to 21% for Library-AR modified cells at 10:1 (CEM:MDA-MB-231) ratio. 45% aggregation was observed for TNC-AR modified cells at a 5:1 ratio and 9.2% aggregation for Library-AR modified cells.

Methods

Materials. Unless otherwise noted, all chemicals and buffers came from Sigma-Aldrich and were not further purified. All DNA bases, except for the lipid-phosphoramidite, which was synthesized in house, were purchased from Glen Research. Oligonucleotides were synthesized in house on an automated ABI 3400 DNA synthesizer from Applied Biosystems. HPLC of the DNA sequences was performed with a Varian Prostar Instrument. UV/Vis measurements for purity and concentration determination were carried out on a Varian Cary 100 spectrophotometer.

DNA synthesis. All DNA sequences were synthesized with an ABI 3400 synthesizer on a 1.0 micromolar scale. Biotinylated CPG lipid phosphoramidite was dissolved in 0.4 mL dichloromethane for coupling. For the lipid-DNA after synthesis, the DNA was cut from the CPG beads and deprotected in ammonium hydroxide at 55°C for 14 h. Next, the DNA was dissolved in 100 mM triethylamine-acetic acid buffer (TEAA, pH 7.5) and purified by reverse-phase HPLC using a C4 column with an acetonitrile gradient (0–30 min, 10–100%) as an eluent. For other DNA sequences

with no biotin or dye modifications, the sequences were deprotected for 20 min in AMA (1:1 ammonia hydroxide: 40% methylamine) and purified using Gel-Pak Purification Columns (Glen Research) followed by desalting on a Nap-5 column (GE Healthcare). The sequences used can be found in Table 1.

Cell culture. CCRF-CEM cells (T-cell, human acute lymphoblastic leukemia), Ramos (B-cell, human Burkitt's lymphoma), HeLa (human cervical adenocarcinoma), and A549 (human lung adenocarcinoma) were obtained from ATCC (American Type Culture Association). Ludlu-1 cells were obtained from the European Collection of Cell Cultures (ECACC). CEM, Ludlu-1, and Ramos cells were grown in RPMI-1640 media (GIBCO). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO). MCF7 cells were grown in Minimal Essential Media (MEM, GIBCO), and A549 cells were grown in F-12k media (GIBCO). All media were supplemented with 10% fetal bovine serum (Invitrogen), and cells were incubated at 37°C in 5% CO₂.

Flow cytometry. Five-hundred thousand cells were washed with 2 mL washing buffer (WB; 1 × PBS, 5 mM MgCl₂, 4.5 g/L glucose) and spun down at 1200 g for 3 min.

Table 1 | DNA sequences used in this work

Name ^[a]	Sequence
SA-AR	5' Lipid TT TTT TTA TTG ACC GCT GTG TGA CGC AAC ACT CAA T-3'
T27-AR	5' Lipid TT TTT TTG TCC GTG GTC CAG CTT GGG GTG AC-3'
PDGF-AR	5' Lipid TT TTT TTC AGG CTA CGG CAC GTC GAG CAT CAC CAT GAT CCT G-3'
TNC-AR	5' Lipid TT TTT TTC CCA GAG GGA AGA CTT TAG GTT CGG TTC ACG TCC-3'
T-15 FITC	5'-GGT TGG TGT GGT TGG FITC-3'
T-15 comp	5'-CCA ACC ACA CCA ACC-3'
T-15 scr	5'-CAC CAC CAA CAC CAC-3'
TNC	5'-CCC AGA GGG AAG ACT TTA GGT TCG GTT CAC GTC C FITC-3'

^[a]**SA:** streptavidin; **AR:** aptamer receptor; **TA:** thrombin; **T-15:** 15 nt thrombin aptamer; **T-15 comp:** complement of T-15; **T-15 scr:** scrambled T-15; **PDGF:** platelet derived growth factor; **TNC:** tenascin-C.



CEM cells were incubated with different concentrations of SA-ARs, ranging from 31 nM to 5 μ M, for 2 h at 37°C and washed 3 \times . This was followed by addition of 1:400 streptavidin-488 (SA-488) (Invitrogen) or streptavidin-phycocerythrin (SA-PE) (Invitrogen) and 30 min incubation at 4°C in WB. Cells were then washed once in WB and analyzed by flow cytometry on a FACScan (Becton Dickinson) using FL-1 for SA-488 and FL-2 for SA-PE. Data were analyzed using either Win-MDI or FCS Express 2.0. All data shown are ungated. To study aptamer-receptor permanence on the cell surface, CEM cells were incubated with 2 μ M of SA-AR for 2 h at 37°C, followed by washes and incubation at various times from 0 to 30 h before adding 1:400 SA-488, washing, and performing flow analysis.

Microscopy. Cells were plated at low confluence on Lab-Tek four-chambered slides and allowed to grow for 24 h before washing with WB and incubating with 2 μ M of SA-ARs for 2 h at 37°C. Afterwards, the cells were washed again 3 \times and incubated with 1:400 SA-488 for 30 min at 4°C. After washing, cells were imaged at 40 \times magnification using incubation and imaged by bright field and fluorescence (488 ex) microscopy using a Leica DM6000B microscope.

MTS assay. MTS works on the principle of a cell's ability to reduce the tetrazolium reagent (Owen's Reagent) via NADH or NADPH when it is alive. The reduced product absorbs at 490 nm and can be read at that wavelength; the higher the absorbance, the more viable are the cells. CEM cells (250 K) were washed and placed into sterile flow tubes with 1 mL of RPMI media. For each time point, each tube was treated in the same manner. Two mL of PBS was added, and cells were washed by spinning at 1300RPM for three minutes. The cell medium was removed by pouring out the supernatant, and cells were incubated with 50 μ L 500 nM streptavidin-Lipid #3 for 1-2 h at 37°C. Two mL PBS was used to wash the cells. Then the cells in each tube were resuspended in 1 mL RPMI. Finally cells were washed, resuspended in 100 μ L RPMI, and split into three 96-well plates to which 20 μ L MTS reagent CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (Promega) was added. Cells were measured at 490 nm after 4 h. The wells were all bright purple.

Cell capture with streptavidin DynaBeads. Healthy CEM-CCRF cells (12×10^6) were washed in 10 mL washing buffer. Cells were resuspended in 6 flow tubes with the following treatments: (1) Just Cells. Incubated 1 hour RT, washed 2 \times with 2 mL WB. (- control). (2) TD05-biotin 500 nM in 200 μ L BB. Incubated 30 min @ 4°C, washed 1 \times with WB(negative control). (3) Sgc8-Biotin 500 nM in 200 μ L BB. Incubated 30 min @ 4°C, washed 1 \times with WB. (+ control). (4) PDGF-lipid aptamer 2 uM in RPMI media. Incubated 1 h, washed 2 \times with 2 mL WB. (- control). (5) Streptavidin-lipid aptamer 2 uM in RPMI media. Incubated 1 h at RT, washed 2 \times with 2 mL WB. (6) Just Cells no DynaBead extraction (+ control). Tubes 1-5 were resuspended in 500 μ L BB, and 400 μ L of each sample was added to a fresh tube with 50 μ L DynaBeads (Invitrogen). Cells were placed on a rotator at 4°C for 30 min. Then, 150 μ L RIPA buffer with protease inhibitor was added to each tube. Cells were shaken at 4°C for 30 min and sonicated briefly. Then, 150 μ L 2 \times SDS Lammeli Sample Buffer was added, and the samples were boiled for 5 min. A 20 μ L sample was added to each lane of a 4-12% Bis-Tris Nupage NOVEX Gel (Invitrogen) and run at 200 V in MOPs running buffer for 1 h. Blot was transferred at 30 V for 1 h on a PVDF membrane. Blots were blocked with 5% milk for 1 h then incubated with 1:1000 1° anti-rabbit β -actin (Cell Signaling #4967S) overnight at 4°C. The next day, blots were washed and probed with 2° goat anti-rabbit HRP (Pierce #1858414, 1:2000). Blots were visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and imaged on Kodak film.

Clotting assay. Healthy CEM cells (500 K) were washed and incubated for 2 h at 37°C in 50 μ L RPMI media (Tubes 1 + 2), 5 μ M T27-AR (Tubes 3-6), or PDGF-AR (Tube 7). After washing 3 \times , cells were incubated with 100 μ L WB alone (Tube 1), 500 nM thrombin (human α -thrombin; Haematologic Technologies, HCT-0020) (Tube 1 + 7), 500 nM thrombin pre-incubated with 1 μ M T-15 (Tube 4), 1 μ M scrambled T-15 (Tube 5), or 200 nM bovine serum albumin (BSA, Invitrogen) for 30 min at RT. After five washes, cells were resuspended in 200 μ L clotting buffer (25 mM Tris-HCl, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, pH 7.5). Then, 4 μ L 20 mg/mL fibrinogen (fraction I, type I from human plasma; Sigma F3879) was added to Tubes 2-7. Clots formed and were visualized by adding 50 μ L trypan blue stain (GIBCO). Pictures were taken by placing the tubes on their sides and imaging against a white background with a digital camera.

TNC-AR binding assay. CEM cells were washed and dyed with 1 μ M Tracker Green (Invitrogen) for 20 min at 37°C. During the dying, MDA-MB-231 cells were washed. After CEM had been incubated with the dye, the cells were incubated with 2 μ M TNC-AR, and the MDA-MB-231 cells were dyed with 1 μ M Far Red (Invitrogen) for 20 min at 37°C. Four replicates of 5:1 and 10:1 (MDA-MB-231: CEM) ratios were prepared in a flow cytometry tube. The cells were allowed to bind in the tubes at 4°C for 20 min while lightly shaking. Each tube was read with an Accuri C6 flow cytometer using FL1 and FL4 channels. The dot plot was divided into four parts: Upper Left (UL), Upper Right (UR), Lower Left (LL), and Lower Right (LR). The equation UR/(UR + UL) was used to quantify the binding of the two cells.

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Author contributions

M.O.A. carried out the initial optimization studies, streptavidin-AR, and thrombin-AR studies. Y.M.C. carried out the TNC-AR studies with the help of X.X. All the results were reviewed and discussed with W.T. M.O.A. and Y.M.C. made all the figures and wrote the paper. All authors reviewed the manuscript.

Additional information

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