Supplementary Information

Programmable bivalent peptide-DNA locks for pH-based control of antibody activity

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Contents

1. General

All oligonucleotides were purchased HPLC purified from Integrated DNA Technologies. See Table S1 for sequences of the oligonucleotides used in this study. Sulfo-SMCC and anti-HA epitope tag antibody (clone: 2-2.2.14) and Alexa647-labeled anti-HA epitope tag antibody (clone: 2-2.2.14) were purchased from ThermoFisher Scientific. To ensure that the ionic strength of PBS solutions at various pH are the same, PBS solutions of the required pH were obtained by mixing appropriate amounts of PBS made with mono-basic sodium phosphate and PBS made with di-basic sodium phosphate.

2. Safety Statement

No unexpected or unusually high safety hazards were encountered

2. Synthesis of HA peptide epitope and oligonucleotide-peptide conjugates.

The HA peptide epitope was synthesized by automated Fmoc peptide synthesis on Rink amide MBHA resin. After synthesis the peptide was cleaved from the resin by soaking the resin in a mixture of TFA/TIS/H_2O/EDT (92.5:2.5:2.5:2.5 v/v) for 3 hours at room temperature, followed by precipitation in ice cold diethylether. Subsequently, the peptide was purified via reversed phase HPLC-MS on a Shimadzu LC-9A HPLC system with a VYDAC protein & peptide C18 column. The correct mass and purity were confirmed by analytical reversed phase LCMS.

Oligonucleotides modified with a 5 or 3'-primary amine were dissolved in PBS (100 mM sodium phosphate, 150 mM NaCl, pH 7.2) and incubated for 2 hours at room temperature with 20 equivalents of Sulfo-SMCC (Thermo Fisher Scientific, 2 mg no-weigh format, freshly dissolved in DMSO), yielding a final oligonucleotide concentration of 1 mM in 50% DMSO. Subsequently, the oligonucleotides were purified from excess Sulfo-SMCC by ethanol precipitation. In short, 10% (v/v) 5 M NaCl and 300% (v/v) ice cold ethanol was added and incubated for 1 hour at -30 °C followed by centrifugation for 15 min. at 14,000 rpm at 4 °C. The resulting pellet was dried under vacuum. Next, the oligonucleotides were dissolved in phosphate buffer (100 mM sodium phosphate, pH 7.0) and incubated with 10 equivalents of HA peptide epitope for 2 hours at room temperature. The resulting oligonucleotide-peptide conjugates were purified by reversed phase HPLC on a

GraceAlpha C18 (250 x 4.6 mm) column with a gradient of 5 – 50% acetonitrile in 100 mM triethylammonium acetate (TEAA, pH 7.0) and subsequently lyophilized to obtain a white powder (Fig. S2). The correct mass and purity of the oligonucleotide-peptide conjugates were determined by mass spectrometry with flow injection analysis on a LCQ Fleet (Thermos Finnigan) ion-trap mass spectrometer in negative mode by injection of 5 µL oligonucleotide-peptide conjugate dissolved to 10 µM in 1:1 isopropanol/water + 1% triethylamine (pH 10). See Table S2 for calculated and observed masses of the synthesized oligonucleotide-peptide conjugates. The oligonucleotides and oligonucleotide-peptide conjugates were dissolved to approximately 100 µM in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and quantified by UV-VIS using their extinction coefficients at 260 nm. Finally, all oligonucleotides were further diluted to stock solutions of 50 µM and stored at -30 °C.

3. Labeling of Cetuximab with Cy3 and HA peptide epitope.

Before labeling the anti-EGFR antibody Cetuximab (C225, Erbitux) with NHS-Cy3 the antibody was buffer exchanged to 100 mM sodium phosphate at pH 7.0 using a PD-10 desalting column (GE Healthcare). Subsequently, 250 µL Cetuximab (30 µM) was mixed with 1500 µL 100 mM sodium phosphate pH 7.0 and 150 µL NHS-Cy3 (850 µM stock in DMSO) and incubated for 2 hours at room temperature under continuous shaking at 850 rpm. Finally, Cetuximab-Cy3 was purified from excess Cy3 by gel filtration using a PD-10 desalting column. The labeling efficiency was determined using UV-VIS spectroscopy, yielding on average 4.1 Cy3 labels per antibody.

To label Cetuximab with the HA peptide epitope, the antibody was buffer exchanged to 100 mM sodium phosphate pH 7.0 as described above. Subsequently, 32 µL Sulfo-SMCC (~115 µM in DMSO) was added to 2500 µL Cetuximab (30 µM), resulting in a 50-fold molar excess of crosslinker. This mixture was incubated for 2 hours at room temperature under continuous shaking at 850 rpm. Unreacted crosslinker was removed by gel filtration, after which 2500 µL of maleimide activated antibody (~20 µM) was mixed with 320 µL of the HA peptide epitope (9 mM in PBS) and incubated for 2 hours at room temperature under continuous shaking at 850 rpm. Finally, the Cetuximab-HA conjugate was purified from unreacted epitopes by gel filtration.

4. Characterization of antibody activation at high pH by pHAbs-1 and pHAbs-2.

Titrations to determine the efficacy of the base-triggered ligands shown in Fig. 1 were performed by mixing 100 nM anti-HA antibody with either 400 nM of the appropriate monovalent control or 200 nM of all ligand components in PBS+ (50 mM sodium phosphate, 500 mM NaCl at pH 5.0, supplemented with 1 mg mL^{-1} BSA) for 1 hour at room temperature. Subsequently, a dilution series of (blocked) antibody was obtained by serial two-fold dilutions in PBS+ at pH 5.0. Finally, 5 µL of the prepared antibody solution was added in a 384 well-plate to 45 µL PBS+ containing 2.22 nM FITC-labeled HA peptide epitope [45] at either pH 5.0 or pH 8.0 (yielding final pH of 5.0 and 7.3, respectively), resulting in a final antibody concentration ranging from 10 pM to 10 nM and a constant concentration of 2 nM fluorescently labeled HA peptide epitope. After incubating the mixtures for 1 hour at room temperature the fluorescence polarization of all the wells was measured using a platereader (Tecan, Saffire-II, ex/em = 470/520 nm).

To determine the pH-dependency of pHAbs-1 and pHAbs-2 the antibody-ligand complex was diluted in PBS+ at pH 5.0 to a stock concentration of 50 nM. In a 384 well-plate, 5 µL of the blocked antibody, antibody without ligand, or PBS+ only, was rapidly mixed in 45 µL PBS+ (containing 2.22 nM FITC-labeled peptide epitope) at a range in pH values. Directly after rapid mixing, the fluorescence polarization of all the wells was measured for 90 minutes at a 60 second interval. To derive the fraction of activated antibody at each pH value, the measured fluorescence polarization was normalized to the control with free antibody (1, representing fully activated antibody) and the control lacking antibody (0, representing fully inhibited antibody). For pHAbs-2, 100 nM displacer oligonucleotide or PBS at pH 5.0 was added to the samples.

5. Characterization of antibody activation at low pH by pHAbs-3.

To determine the efficacy and pH-dependence of pHAbs-3 the same experiments as for pHAbs-1 and pHAbs-2 were performed. However, the assembly of the antibody-ligand complexes was performed at pH 8.0 rather than pH 5.0.

6. Extraction of dissociation constant of anti-HA antibody binding to HA peptide epitope.

To calculate the dissociation constant (K_d) of the anti-HA antibody binding to the HA peptide epitope at various pH, non-linear least square optimization of equation 1 to the experimental data (Fig. S1) was performed. In

equation S1, A is the measured fluorescence polarization A_f the polarization coefficient of the unbound peptide, A_b the polarization coefficient of the antibody-bound peptide, [P] the concentration of fluorescently labeled HA peptide and Ab the concentration of antigen binding sites (i.e. 2 times the antibody concentration).

$$A = A_f + (A_b - A_f)\ \frac{([P]+K_d+[Ab])-\sqrt{([P]+K_d+[Ab])^2-4[P][Ab]}}{2[P]} \qquad \text{(S1)}$$

7. Extraction of p$K_{a,app}$ of pHAbs-1, pHAbs-2 and 20, 40 and 60% TAT pHAbs-3.

To calculate the apparent pKa of the pH-responsive bivalent ligands, non-linear least square optimization of equation 2 to the experimental data was performed. In equation S2, p$K_{a,app}$ the apparent pK_a of the ligand switch and *n* the Hill coefficient.

$$Active\ antibody\ fraction\ = \frac{pH^n}{pK_{a,app} + pH^n} \qquad \text{(S2)}$$

8. Yeast surface display.

The gene encoding for the HA-Citrine construct was cloned into the pCT-CON2 vector as described previously [32]. Subsequently, the plasmid was transformed into EBY100 yeast cells using the LiAc/PEG/ssDNA method and plated on SDCAA agar (15 g L^{-1} Bacto agar, 6.7 g L^{-1} Difco yeast nitrogen base without amino acids, 5 g L^{-1} Bacto casamino acids, 20 g L^{-1} D-(+)-glucose. After overnight incubation at 30 °C a single colony was cultured in SDCAA medium and frozen in 5% glycerol/10% DMSO. From the glycerol stock, 5 mL SCDAA medium (20 g L^{-1} dextrose, 6.7 g L^{-1} Difco yeast nitrogen base, 5 g L^{-1} Bacto casamino acids, 5.4 g L^{-1} Na_2HPO_4) was inoculated and grown overnight at 30 °C, 220 rpm. The next morning a fraction of the yeast cells was transferred to 5 mL fresh SDCAA medium to a final OD_{600} of 0.1 and allowed to grow to OD_{600} = 0.4. Next, the cells were spun down for 5 minutes at 2,500xg and resuspended in 5 mL SGCAA (20 g L^{-1} glucose, 6.7 g L^{-1} Difco yeast nitrogen base, 5 g L^{-1} Bacto casamino acids, 5.4 g L^{-1} Na_2HPO_4) and incubated for 48 hours at 20 °C, 220 rpm to induce protein expression.

Directly before yeast binding experiments the cells were washed with PBS+ (by pelleting and resuspending) and again spun down for 5 minutes at 2,500xg and resuspended in PBS+ at the desired pH to OD_{600} = 1 (~10^7 cells mL^{-1}), yielding the yeast stock suspension. For yeast binding experiments the anti-HA

antibody (alexa-647 conjugate) was pre-incubated with the ligands and the proper controls (PBS+ for free antibody control, a single peptide-DNA conjugate for monovalent control and a pH-insensitive bivalent ligand for bivalent control) resulting in a stock concentration of 100 nM antibody with 400 nM total peptide-DNA conjugate. Next, 232.5 µL PBS+ (pH 5.0 and pH 8.0) was mixed with 12.5 µL yeast cell stock solution and 5 µL of the (blocked) anti-HA antibody. After incubating for 1 hour at room temperature the samples were directly analyzed by flow-cytometry on a FACS Aria III equipped with a 70 µm nozzle. Citrine positive cells were selected by exciting the Citrine with a 488 nm laser and detected via a 530/30 bandpass filter. Of the Citrine positive cells the alexa-647 fluorescence intensity was measured by excitation at 633 nm and detected via a 660/30 bandpass filter. Single cells were selected by standard doublet discrimination using forward- and side-scatter height versus width scatter plots (Fig. S5).

9. pH-controlled mammalian cell targeting.

Human skin epidermoid carcinoma cells (A431) were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin / Streptomycin (P/S, Gibco) at 37 °C with 5% CO_2. Cells were harvested at 80% confluency by trypsin treatment for 5 minutes. Subsequently, the trypsin was inactivated by adding a 10-fold excess of culture medium, after which the cells were pelleted by centrifugation for 5 minutes at 100xg and washed once with PBS+ at pH 7.5 and diluted to a stock suspension of OD_{600} = 3.0. To covalently label the A431 cells with the HA peptide epitope, the cells were resuspended in PBS (without BSA) to an OD_{600} of 0.4 (~$2.5x10^6$ cells mL^{-1}). Next, 2000 µL of the cell suspension was mixed with 1980 µL PBS pH 7.5 and 20 µL Sulfo-SMCC (1 mg dissolved in 20 µL DMSO). After incubating for 45 minutes at room temperature on a rotating wheel, the excess of Sulfo-SMCC was removed by washing the cells three times by pelleting and resuspending. Next, the cells were resuspended in 1 mL PBS pH 7.5 and mixed with 1 mL of freshly dissolved HA peptide (1 mg mL^{-1} in PBS pH 7.5). After incubating for 45 minutes at room temperature on a rotating wheel the cells were washed three times by pelleting and resuspending in PBS+ at pH 7.5. Finally, the cells were resuspended to OD_{600} = 3.0. The (blocked) anti-HA antibody solutions at a stock concentration of 100 nM were prepared as described above. 232.5 µL PBS+ at pH 7.5 or 5.0 was mixed with 5 µL of the (blocked) anti-HA antibody and 12.5 µL of the A431 cell stock suspension, yielding a final concentration of 2 nM anti-HA antibody and ~10^6 cells per mL. After incubating for 30 minutes at room temperature on a rotating wheel the cells were pelleted by centrifugation for

10 minutes at 100xg and prior to flow-cytometry analysis resuspended in PBS+ at pH 7.5. Doublet discrimination was performed using forward- and side-scatter height versus width scatter plots (Fig. S6).

10. Primary antibody mediated pH-controlled mammalian cell targeting.

A431 cells were harvested as described above and resuspended in PBS+ at pH 7.5 to a stock suspension of OD_{600} = 3.0. 150 µL of this cell suspension was mixed with 3 mL of Cetuximab-Cy3/Cetuximab-HA (2 nM of both) and incubated for 20 minutes at room temperature on a rotating wheel, after which the cells were washed with PBS+ and resuspended in 150 µL PBS+ at pH 7.5. The (blocked) anti-HA antibody solutions at a stock concentration of 100 nM were prepared and binding studies were performed as described above for the covalently HA-peptide decorated A431 cells.

11. Fabrication of microfluidic chip.

The polydimethylsiloxane (PDMS) microfluidic chips were composed of a 10:1 (w/w) mixture of PDMS base : PDMS curing agent (Dow Corning). 40 g of PDMS base and 4 g of PDMS curing agent were placed in a conditioning mixer (Thinky Mixer ARE-250) to mix for 2 min at 2,000 rpm followed by de-foaming for 2 min at 2,000 rpm. The mixture was poured on a SU-8 master (diameter 100 mm) and placed in a desiccator for 20 min to degas. The pre-cured PDMS mixture was then cured for 3 hours at 65 °C. Subsequently, the cured PDMS was peeled from the SU-8 master. The PDMS layer was cut into separate devices and 1.2 mm holes were punched at the inlets and outlets using a biopsy punch. Dust or residual pieces of PDMS were removed with scotch tape. Next, the glass slides were prepared by cleaning them with soap-water and isopropanol, followed by blow-drying with nitrogen. Subsequently, a clean PDMS device and glass slide were placed in the plasma asher Emitech K1050X for the plasma treatment. After the treatment, the PDMS device was carefully pressed onto the glass slide to enable bonding. Finally, the microfluidic chips were silanized by injecting a solution of 5% 1H,1H,2H,2H-Perfluorooctyltriethoxysilane (Sigma) in HFE 7500 (3M) into the device channels using a 1 mL syringe and Teflon tubing (Sigma, 58696-U) and followed by incubation at 65 °C for 30 minutes. Subsequently, the excess silane solution was flushed out of the channels by injecting HFE 7500 into the PDMS device and incubated overnight

at 65 °C to complete the bonding process. The fabricated PDMS devices were covered with scotch tape and stored at room temperature until further use.

12. Encapsulation of single cells in water-in-oil microdroplets.

A431 cells were harvested and pre-labeled with Cetuximab-Cy3/Cetuximab-HA as described above and resuspended to a stock concentration of 10^6 cells per mL in Droplet-Buffer (2 mM Sodium Phosphate, 100 mM NaCl at pH 6.8 supplemented with 1 mg mL^{-1} BSA, 2 g L^{-1} glucose and 0.1 mg mL^{-1} dsDNA from salmon testes (Sigma Aldrich). The (blocked) anti-HA antibody was prepared as described above in Droplet-Buffer and diluted to a concentration of 4 nM. The cells and anti-HA antibody were injected in separate channels in the microfluidic chip with a flow rate of 300 µL h^{-1}. A solution of 3% Picosurf in HFE 7500 was injected in the carrier oil channel with a flow rate of 1800 µL h^{-1} to produce cell-encapsulating droplets. The produced droplets were collected at the outlet in a tube chilled on ice. After collecting droplets for 15 minutes the droplets were incubated on ice or in a water bath at room temperature. Subsequently, excess fluorinated oil was removed and 200 µL PBS+ with high buffer capacity (50 mM sodium phosphate, 500 mM NaCl, 1 mg mL^{-1} BSA at pH 7.5) was added to the emulsion. To de-emulsify the water-in-oil droplets, 100 µL of 10% (v/v) perfluorooctanol in HFE 7500 (3M) was added. After incubating for 2 minutes at room temperature the water-phase was extracted and transferred to fresh tubes. Finally, the cells were pelleted by centrifugation for 10 minutes at 100xg. The pelleted cells were resuspended in PBS+ at pH 7.5 and analyzed by flow-cytometry. To correct for the correlation between primary (cetuximab) and secondary (anti-HA) antibody labeling, linear least squares regression was performed on the cetuximab vs anti-HA data per individual measurement. The obtained linear model was subtracted from the data after which the average anti-HA labeling intensity was added.

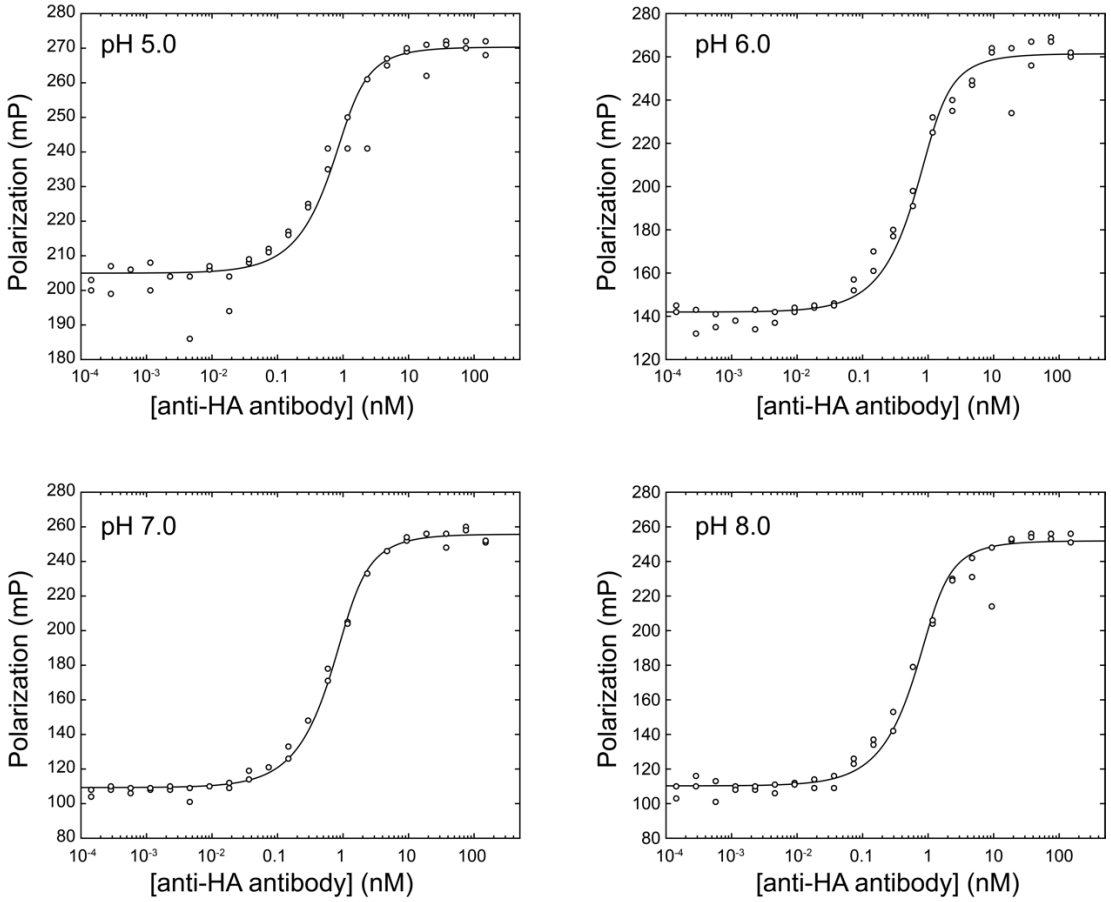

Fig. S1. Fluorescence polarization assay to determine the dissociation constant of anti-HA antibody binding to HA peptide epitope at various pH. Titration of anti-HA antibody to 2 nM of fluorescently labeled HA peptide epitope at pH 5.0, 6.0, 7.0 and 8.0. The dissociation constant was obtained by fitting Equation 2 to the mean of duplicate measurements, yielding $K_{d,pH5.0}$ = 0.46 ± 0.16 nM, $K_{d,pH6.0}$ = 0.42 ± 0.11 nM, $K_{d,pH7.0}$ = 0.49 ± 0.05 nM and $K_{d,pH8.0}$ = 0.41 ± 0.07 nM. The measurements were performed in PBS (50 mM sodium phosphate, 500 mM NaCl) supplemented with 1 mg mL^{-1} BSA.

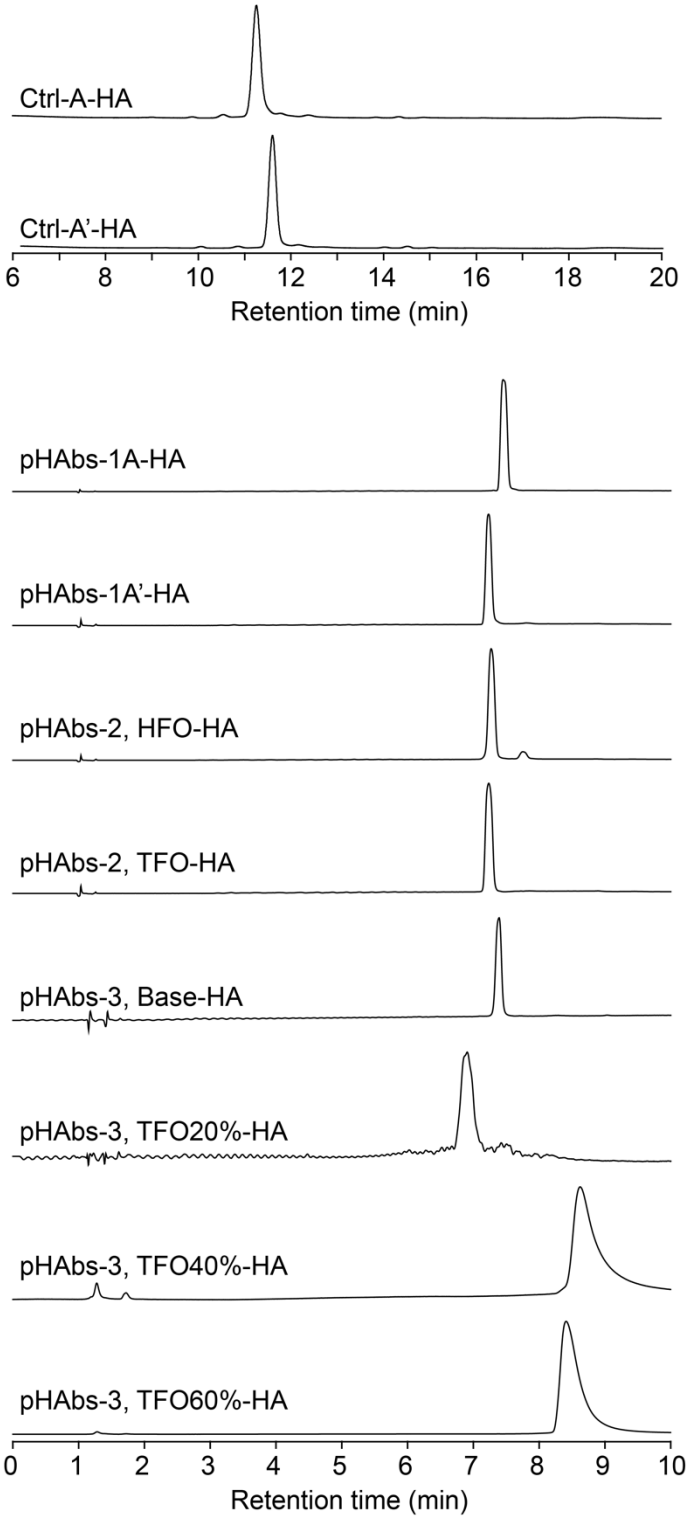

Fig. S2. HPLC traces of purified oligonucleotide-peptide conjugates.

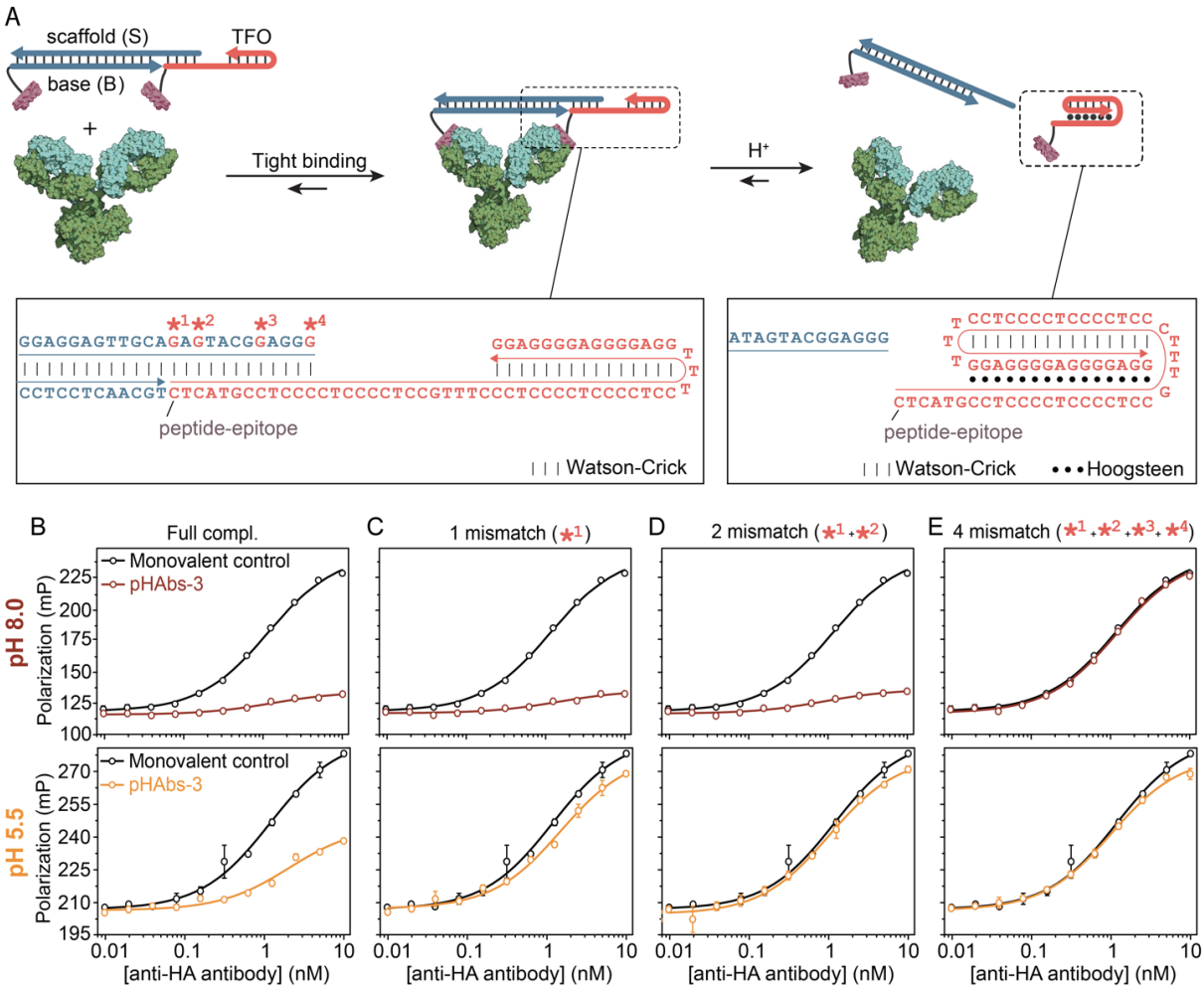

Fig. S3. Mismatch screening for optimal pH induced ligand switching of pHAbs-3. (A) Schematic representation of antibody activation upon a decrease in pH. Optimization of ligand switching induced by a decrease in pH is performed via systematic introduction of mismatches in the duplex between the scaffold and TFO strands. The asterisks indicate the positions of the introduced mismatches. (**B-E**) Fluorescence polarization competition assay to evaluate the efficiency of blocking and subsequent activation of the antibody by the bivalent pHAbs-3 ligand at pH 8.0 (top) and pH 5.5 (bottom). pH ligands contained a 12 nucleotide toehold on the scaffold strand that is either **(B)** fully complementary to the 12 5'-bases of TFO, **(C)** containing 1 mismatch, **(D)** containing 2 mismatches or **(E)** containing 4 mismatches. Titrations were performed with a monovalent control where the anti-HA antibody is incubated with TFO only and with the fully assembled pHAbs-3 ligand harboring a 20% TAT triple helix. Error bars represent standard error of the mean of duplicate measurements.

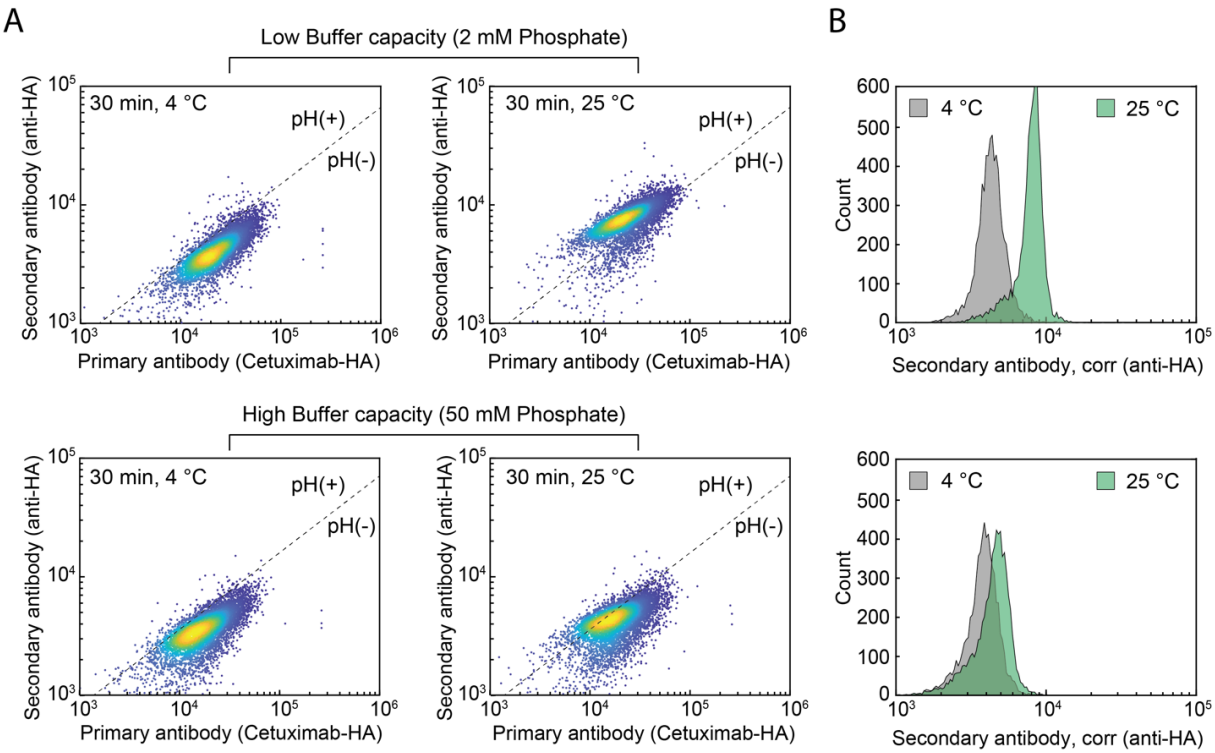

Fig. S4. Antibody activation in microfluidic droplets with high buffer capacity. (**A**) A431 human epidermoid carcinoma cells were encapsulated in droplets together with the 40% TAT pHAbs-3 ligand for 30 minutes at either 4 °C to inhibit cell metabolism or 25 °C to promote cell metabolism. Experiments were performed in buffer containing 2 mM phosphate, identical to the conditions in Fig. 5, as well as in a control buffer with high buffering capacity (50 mM phosphate). After incubation the droplets were demulsified and the degree of (pH-responsive) secondary antibody labeling versus primary antibody labeling via Cetuximab of the retrieved cells were analyzed by flow-cytometry. **(B)** Histograms showing the degree of pH-responsive secondary antibody derived from the scatterplots shown in (**A**) corrected for primary antibody labeling efficiency. Scatterplots and histograms were constructed from ~7,000 individual cells per experiment.

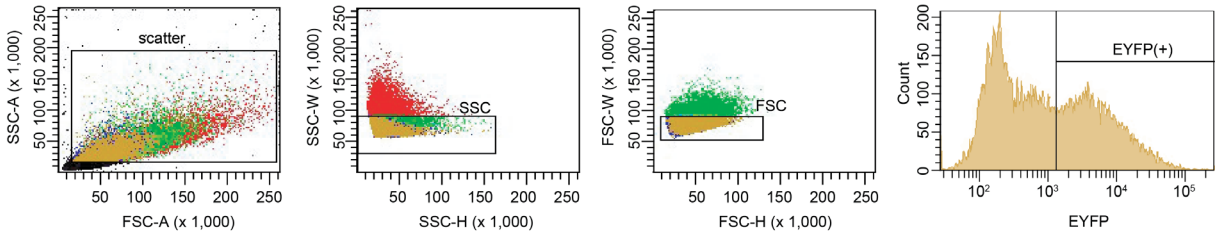

Fig. S5. Gating strategy for doublet discrimination and selection of Citrine(+) yeast cells. Flow-cytometry scatter plots showing the gating strategy to select single cells based on forward- and side-scattering. After selecting single cells, only yeast cells that show high Citrine expression were selected.

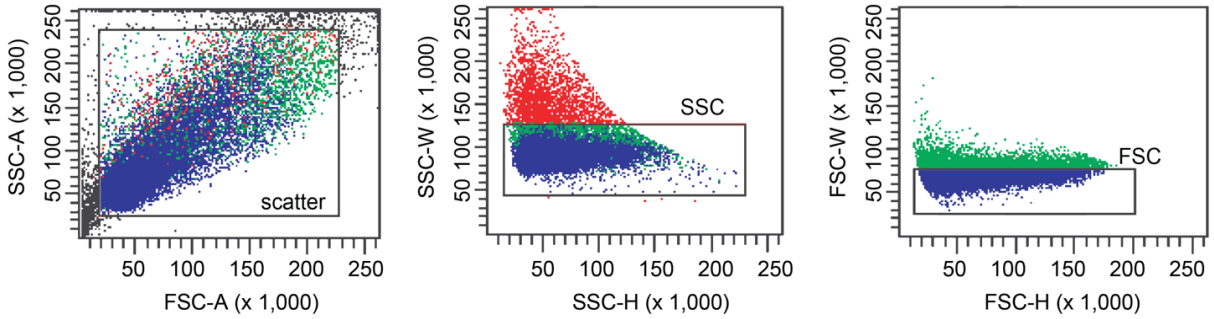

Fig. S6. Gating strategy for doublet discrimination of mammalian A431 cells. Flow-cytometry scatter plots showing the gating strategy to select single cells based on forward- and side-scattering.

Table S1. Oligonucleotide sequences.

Name	Sequence [a,b]	ligand
Ctrl-A	5'-TGGAGA CGTAGGGTATTGAATGTGCTGTAG-NH_2-3'	Bivalent control
Ctrl-A'	5'-CTACAGCACATTCAATACCCTACG-NH_2-3'	
pHAbs-1-A	5'-H_2N-AGGAGAGAAGCATGAAAGAC-3'	pHAbs-1
pHAbs-1-A'	5'-H_2N-GTCTTTCATGCTTCTCTCCT GTTTG TCCTCTCTTC-3'	
Displacer (ODN)	5'-GGACAAACAGGAGAGAAGCATGAAAGAC-3'	
HFO	5'-GGAGAAAGGAAGAGAGGAAG TTT CTTCCTCTCTTCCTTTCTCC-NH_2-3'	pHAbs-2
TFO	5'-CCTCTTTCCTTCTCTCCTTC-NH_2-3'	
base	5'-H_2N-ACCTCCTCCCTCCTCAACGT-3'	
TFO 20%	5'-H_2N -CTCATGCCTCCCCTCCCCTCC GTTTC CCTCCCCTCCCCTCC TTT GGAGGGGAGGGGAGG-3'	20% TAT pHAbs-3
Scaffold 20% Full	5'-GGGAGGCATGAGACGTTGAGGAGGGAGGAGGT -3'	
Scaffold 20% 1M	5'-GGGAGGCATGATACGTTGAGGAGGGAGGAGGT -3'	
Scaffold 20% 2M	5'-GGGAGGCATTATACGTTGAGGAGGGAGGAGGT -3'	
Scaffold 20% 4M	5'-AGGAAGCATTATACGTTGAGGAGGGAGGAGGT -3'	
TFO 40%	5'-H_2N -CTCATGCCTCCTCTCTTCTCC GTTTC CCTCTTCTCTCCTCC TTT GGAGGAGAGAAGAGG-3'	40% TAT pHAbs-3
Scaffold 40%	5'-AGGAGGCATGATACGTTGAGGAGGGAGGAGGT-3'	
TFO 60%	5'-H_2N -CTCATGTCTTCTCTCTTCTCT GTTTC TCTCTTCTCTCTTCT TTT AGAAGAGAGAAGAGA-3'	60% TAT pHAbs-3
Scaffold 60%	5'-AGAAGACATGATACGTTGAGGAGGGAGGAGGT-3'	

[a.] Colors represent individual oligonucleotide domains.
[b.] Underlined sequence denote the triplex forming portion of the oligonucleotide.

Table S2. MS analysis of synthesized oligonucleotide-peptide conjugates.

Name	Calculated mass (Da)	Observed mass (Da)
Ctrl-A-HA	11202.29	11202.26
Ctrl-A'-HA	9030.96	9030.79
pHAbs-1A-HA	8031.0	8032.0
pHAbs-1A'-HA	12287.7	12287.5
pHAbs-2, HFO-HA	15014.4	15015.0
pHAbs-2, TFO-HA	7677.7	7678.3
pHAbs-3, Base-HA	7682.7	7682.0
pHAbs-3, TFO20%-HA	19640.4	19639.2
pHAbs-3, TFO40%-HA	19682.4	19683.8
pHAbs-3, TFO60%-HA	19724.5	19721.2