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Supporting Information

Polyclonal aptamer libraries as binding entities on a graphene FET based biosensor for the discrimination of apo- and holo- retinol binding protein 4

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Abstract: Oligonucleotide DNA aptamers represent an emergently important class of binding entities towards as different analytes as small molecules or even whole cells. Without the canonical isolation of individual aptamers following the SELEX process already the focused polyclonal libraries prepared by this *in vitro* evolution and selection can directly be used to label their dedicated analytes and to serve as binding molecules on surfaces. Here we report the first instance of a sensor able to discriminate between loaded and unloaded retinol binding protein 4 (RBP4), an important biomarker for the prediction of diabetes and kidney disease. The sensor relies purely on two aptamer libraries tuned such that they discriminate between the protein isoforms, requiring no further sample labelling to detect RBP4 in both states. The evolution, binding properties of the libraries and the functionalization of graphene FET sensor chips are presented as well as the functionality of the resulting biosensor.

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Experimental Procedures

Materials.

Acetic acid, agarose, ampicillin, boric acid, bromophenol blue, $CaCl_2$, isobutanol, K_2HPO_4, KCl, KOH, KH_2PO_4, LB medium, HCl, NaCl, Span® 80, Tris, triton X-100, tween® 80 and urea were ordered from Roth (Carl Roth GmbH and Co. KG, Karlsruhe, Germany). All*trans*-retinal, bovine serum albumin, coomassie brillant blue G-250, ethanol, N-hydroxysuccinimide, sodiumdodecyl sulfate (SDS) and tRNA were purchased from Sigma Aldrich (St. Louis, Missouri, USA). EDTA, Dulbecco's Phosphate-Buffered Saline (1x), Dynabeads® M 280 tosyl-activated were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). BioMag® Streptavidin was ordered from QIAGEN (Venlo, Netherlands) and human serum was obtained from Merck KgaA (Darmstadt, Germany) (Product Number H4522-20ML),

Recombinant RBP4 production in *E. coli* and purification from inclusion bodies.

Chemical competent *E. coli* BL21 (DE3) Tuner™ (200 µL) (Merck Millipore, Darmstadt, Germany) were transformed using 1 µL pET22b-rRBP4 (191 ng/µL). The RBP4 gene was gained from pET302-rBBP4 (Thermo Fisher Scientific, Darmstadt, Germany) and it was cloned into a pET22b vector, previously. Cells were incubated at 4 °C for 45 min, followed by heat shock treatment for 90 sec at 42 °C and subsequent incubation for 2 min at 4 °C. Then, 800 µL pre-warmed lysogeny broth (LB) medium was added and incubated at 37 °C for 1 h to regenerate the cells. After regeneration cells were plated on selective LB-amp (100 µg/mL) agar plates and incubated overnight at 37 °C. To produce RBP4, one clone of freshly transformed *E. coli* BL21 (DE3) was used to inoculate 25 mL selective LB-amp (100 µg/mL) medium containing 0.4 % glucose and cultivated overnight at 37 °C and 150 rpm. Expression cultures were inoculated with an optical density (OD) of 0.1 at 600 nm in 250 mL LB medium (0.4 % glucose, 100 µg/mL ampicillin) in 1 L Erlenmeyer flasks without chicanes. Induction of cultures was performed using 1 mM isopropyl-β-D-thiogalactoside (IPTG) after growth to an OD_{600} of 0.6 and as control, a non-induced culture was cultivated similarly. After induction, the expression cultures were further cultivated for 1 h at 37 °C, followed by a temperature shift to 30 °C for 4 h. After cultivation, the cells were harvested by transferring them to 50 mL tubes followed by centrifugation (4000 xg, 20 min, 4 °C). Then, the supernatants were discarded and the cell pellets were washed with 5 mL 1x PBS, combined, and centrifuged (4000 xg, 20 min, 4 °C). Afterwards, supernatants were discarded, and the cell pellets were frozen at -80 °C overnight. SDS-polyacrylamide gel electrophoresis was performed to verify the production process.[1]

Frozen cell pellets were thawed on ice, suspended in 15 mL lysis buffer (50 mM Tris, 2 mM EDTA, 0.1 % Triton X-100, pH 7.5) and sonicated on ice for 12 min in 6 cycles at 40 % intensity. The *E. coli* lysate was then centrifuged (14 000 xg, 45 min, 4 °C) and the pellets containing the insoluble inclusion bodies were dissolved in 15 mL denaturation buffer (50 mM Tris-HCl, pH 8, 2 mM EDTA, 0.1 % (v/v) Triton X-100, 8 M Urea). The protein solution was centrifuged (13 000 rpm, 30 min, 4 °C) and the collected supernatant was diluted 1:14 with renaturation buffer (50 mM Tris-HCl, pH 8, 5 mM EDTA, 1 % (v/v) Triton X-100, 0.8 M Urea) and incubated at 4 °C overnight. Afterwards, the protein solution was concentrated using Vivaspin 20 centrifugal concentrators (molecular weight

cut-off 10 kDa) (Sartorius, Göttingen, Germany) at 4000 xg in 1 h intervals at 4 °C, buffer exchange was performed by washing three times with 20 mL 1x PBS.

Purification of *E. coli*-derived recombinant RBP4 isolated from inclusion bodies[2] was performed by size-exclusion chromatography using the NGC™ chromatography system (Bio-Rad Laboratories, Inc., Hercules, California, USA) with ChromLab Software and SuperdexTM 200 Increase 10/300 GL (GE Healthcare Bio-Sciences AB, Danderyd, Sweden). First, the column and the chromatography system were rinsed with 36 mL (1.5 column volumes) filtered dH_2O at a flow rate of 0.5 mL/min and then equilibrated with 36 mL filtered 1x PBS using a flow rate of 0.75 mL/min, a 1 mL loop was rinsed with 3 mL 1x PBS prior to sample injection. After the sample was applied, UV detection was performed at 280 nm and fractionation was started after a retention volume of ~7.5 mL, 200 µL fractions were collected stepwise in a 96-well-microplate. Afterwards, the system and column were rinsed with 36 mL filtered dH_2O using a flow rate of 0.75 mL/min and then with 36 mL 20% ethanol at a flow rate of 0.5 mL/min. Fractions of purified RBP4 were analyzed by SDS PAGE and stored at -20 °C. Concentrations were calculated by the ChromLab Software for fractions obtained from SEC.

All-*trans*-retinal binding properties of apo-RBP4.

Quenching of intrinsic protein fluorescence was analyzed to determine RBP4 activity by adding increasing all-*trans*-retinal amounts. Working solutions were prepared by diluting retinal and apo-RBP4 in DPBS. After the addition of increasing amounts of retinal (0 - 2.125 µM) to a constant apo-RBP4 amount of 0.425 µM, the mixture was incubated at 25 °C for 90 min under exclusion of light in a Lumox® multiwell 384 well-plate. Then, the fluorescence emission was measured using a Spark® multimode microplate reader, the emission was monitored at 340 nm with an excitation wavelength of 280 nm.

***In vitro* selection of aptamer libraries against apo- and holo-RBP4.**

The selection of aptamer libraries was based on the FluMag-SELEX procedure using tosyl-activated magnetic Dynabeads M-280.[3] Immobilization of proteins was performed by covalent coupling of the p-toluenesulphonyl (tosyl) groups on the surface of the magnetic beads with primary amino groups of RBP4. The magnetic beads were coated as described by the manufacturer and aliquoted into 200 µL aliquots containing 0.68 mg beads, respectively. To gain holo-RBP4-coated beads, beads coated with apo-RBP4 were incubated with 3x excess all-*trans*-retinal for 90 min at 25 °C with the exclusion of light on a rotator. Thereafter, the beads were washed twice with 1 mL 1x DPBS, pH 7.4, containing 0.1 % (w/v) BSA, resuspended, and aliquoted into 200 µL samples (0.68 mg beads per aliquot) and stored at 4 °C. In counter selection steps naked beads were used which were treated similarly prior to usage.

The commercial library (TriLink BioTechnologies, Inc, San Diego, California, USA) with approximately $6x10^{14}$ individual aptamers contained a central random 40-nucleotide (nt) region flanked on both sides by 23 nt primer binding sites. The selection of aptamers was carried out using primers with the following sequences: Cyanine 5-labeled forward primer (Cy5 FW): 5'-[Cy 5]T AGG GAA GAG AAG GAC ATA TGA T-3' and biotin-labelled reverse primer (Bio RW): 5'-[BI O]T CAA GTG GTC ATG TAC TAG TCA A-3' (Eurofins Genomics, Ebersberg, Germany). Prior to each SELEX round, coated magnetic beads were washed three times with 500 µL binding buffer (100 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02 % (v/v) Tween 20, pH 7.6). For aptamer activation, the ssDNA library was heated to 90 °C for 10 min, immediately cooled, and kept at 4 °C for 15 min, followed by incubation at 25 °C for 7 min. Counter selections against naked beads were performed prior to selection rounds 1 – 6 and twofold counter selections were conducted prior to selection rounds 7 and 8. In the first selection round, naked beads were suspended in 500 µL DPBS containing 1 nmol of random ssDNA library and incubated at 25 °C for 30 min under rotation in the dark. Afterwards, the supernatant containing unbound aptamers was mixed with 600 pmol BSA (100 mg/mL) and 600 pmol tRNA (10 mg/mL), transferred to either washed apo-RBP4-coated beads or holo-RBP4-coated beads and incubated again. Unbound aptamers were removed by washing with 500 µL binding buffer and elution of bound aptamers from apo- or holo-RBP4-coated magnetic beads was performed by incubating the beads in 200 µL DPBS at 95 °C for 5 min, followed by magnetic separation for 2 min. After elution 40 µL of the aptamer solutions were analyzed by monitoring the fluorescence intensity using a Tecan infinite F200 Microplate Reader (Tecan Group AG, Männedorf, Suisse) at an excitation wavelength of 635 nm and an emission wavelength of 670 nm. After each SELEX round, the eluted ssDNA aptamers were amplified by emulsion polymerase chain reaction (ePCR).[4] Therefore, 200 µL aqueous phase (40 µL 5x Herculase II reaction buffer, 5 µL dNTPs (10 mM, 2.5 mM each), 0.5 µL Cy5 FW and 0.5 µL Bio RW, 1 µL Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, California, USA), 20 µL BSA (100 mg/mL), 113 µL Water HPLC Plus and 20 µL ssDNA aptamers) were mixed with 400 µL of the oil-surfactant mixture (4.5 % (vol/vol) Span® 80, 0.4 % (vol/vol) Tween® 80, 0.05 % (vol/vol) Triton X-100, 95.05 % (vol/vol) mineral oil) and incubated for 10 min on a vortexer.

Afterwards, the emulsion was transferred into PCR reaction tubes as aliquots of 50 µL and then overlaid with 50 µL mineral oil. The amplification conditions were 2 min at 80 °C, 2 min at 85 °C, 2 min at 90 °C, 3 min at 94 °C and 25 cycles of 30 sec at 94 °, 30 sec at 49.1 °C, 30 sec at 72 °C, then 2 min at 72 °C after the last cycle. After ePCR run, the emulsified PCR reactions were pooled in a microcentrifuge tube (2 mL), mixed with 1 mL isobutanol, centrifuged at 13 000 rpm for 1 min at 25 °C, and then the upper phase was discarded. Afterwards, the PCR products were cleaned using the QIAquick PCR Purification Kit (QIAGEN, Venlo, Netherlands) and analyzed on a 2 % agarose gel by agarose gel electrophoresis. In order to separate the relevant DNA strands from the double-stranded PCR products, streptavidin-coated magnetic beads were used in alkaline strand separation. Therefore, 50 µL streptavidin beads were washed three times with 1 mL DPBS by magnetic separation, resuspended in the PCR product, and then incubated for 16 h at 25 °C under rotation without light exposure. Afterwards, unbound DNA was separated and removed, and the streptavidin beads were washed with 1 mL DPBS. Then, 50 µL NaOH (100 mM, pH 13) was added, incubated for 4 min in total and after 2 min the microcentrifuge tubes were placed in a magnetic separator. In the next step, 45 µL of the NaOH solution were added to 126 µL DPBS in a new microcentrifuge tube, followed by subsequent addition of 34.4 µL 100 mM NaH_2PO_4. The remaining 5 µL of NaOH solution were analyzed on a 2% agarose gel to monitor successful separation and the correct size of the ssDNA fragments, and the concentration of the ssDNA was measured using a NanoPhotometer (IMPLEN, Munich, Germany). After the first selection round, 30 pmol ssDNA from the previous round was used in the SELEX rounds 2 – 6, 10 pmol ssDNA was used in rounds 7 and 8. All rounds started with binding to naked magnetic beads with increasing incubation times, followed by selection with increasing amounts of tRNA and BSA and washing steps with binding buffer. In selection round 6 a reciprocal washing step containing RBP4 was performed in addition. There, apo-RBP4 coated beads were washed with 13.6 µg holo-RBP4 diluted in binding buffer, and the holo-RBP4 magnetic beads were washed with 13.6 µg apo-RBP4 bevor elution.

Specificity analysis of RBP4 aptamer libraries.

The enrichment of specific ssDNA aptamers over the FluMag-SELEX process was verified in a binding assay. Therefore, the aptamer libraries from each round were bound to either apo- or holo RBP4-coated magnetic beads, incubated and quantified by fluorescence measurement afterwards. In the first step, 10 pmol of each aptamer library were activated as described earlier and then the libraries against apo-RBP4 were incubated for 30 min at 25 °C with apo-RBP4-coated magnetic beads and the holo-RBP4 libraries with holo-RBP4-coated beads. The supernatants were separated by magnetic separation, the beads were washed with 500 µL binding buffer and resuspended in 200 µL DPBS. After elution (95 °C, 5 min), 40 µL of the aptamer solutions were analyzed by measuring the fluorescence emission using a Tecan infinite F200 microplate reader, the emission was monitored at 670 nm with an excitation wavelength of 635 nm. In addition, the selectivity of the aptamer libraries was analyzed in a characterization assay. Similar to the binding assay, 10 pmol of activated aptamer libraries were used and incubated for 30 min at 25 °C with apo- and holo-RBP4-coated magnetic beads as well as with naked magnetic beads. Then, the supernatants were separated by magnetic separation and the beads were washed with 500 µL binding buffer and resuspended in 200 µL DPBS. After elution (95 °C, 5 min), 40 µL of the aptamer solutions were analyzed by monitoring the fluorescence emission at 670 nm using a Tecan infinite F200 microplate reader with an excitation wavelength of 635 nm. Experiments were conducted in triplicates.

Fluorescence microscopy.

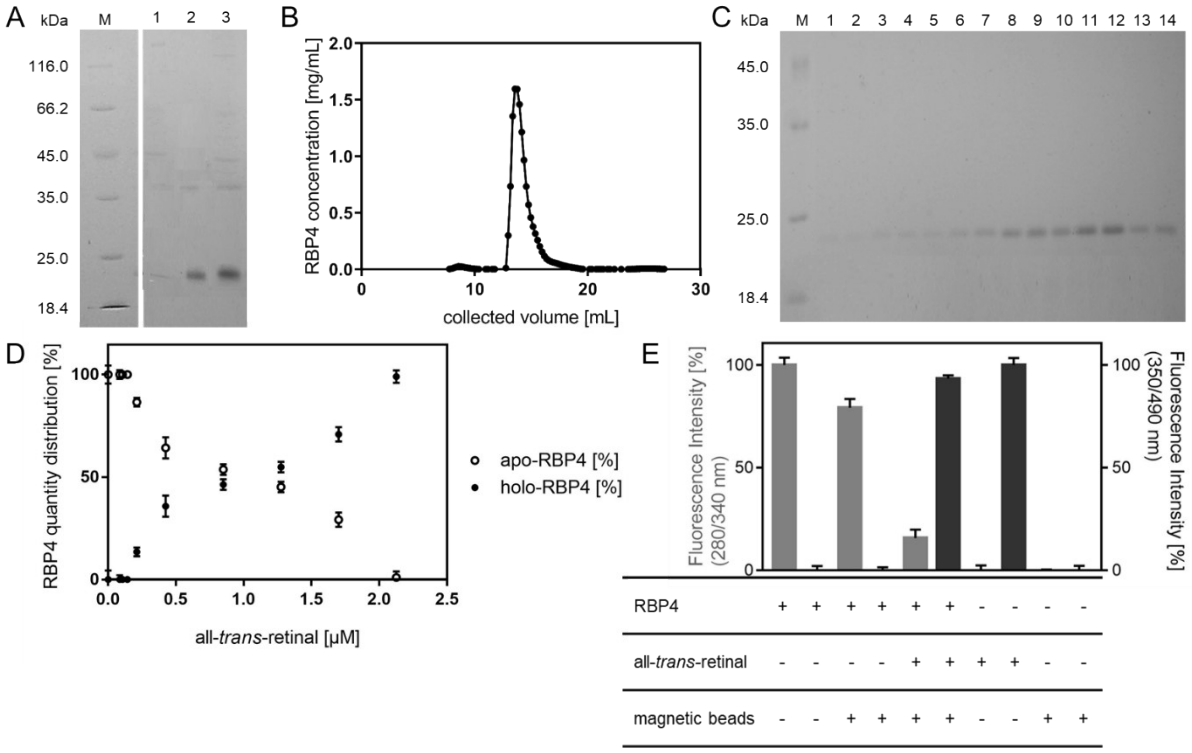
In order to visualize the specific target binding of both final aptamer libraries they were submitted to fluorescence microscopic analysis. Therefore, 30 pmol of each aptamer library were activated as described earlier and then the libraries were incubated for 30 min at 25 °C with apo- and holo-RBP4-coated magnetic beads as well as with naked beads. Afterwards, 100 µL of each reaction mixture were transferred to a 96-well microplate and fluorescence microscopy was performed using a Leica DMi8 coded (Leica Microsystems CMS GmbH, Wetzlar, Germany) at x40 magnitude under transmitted light and using the Y5 filter (excitation: 590-650 nm and emission: 662-738 nm) for fluorescence imaging.

Immobilization of aptamer libraries on rGO-FETs.

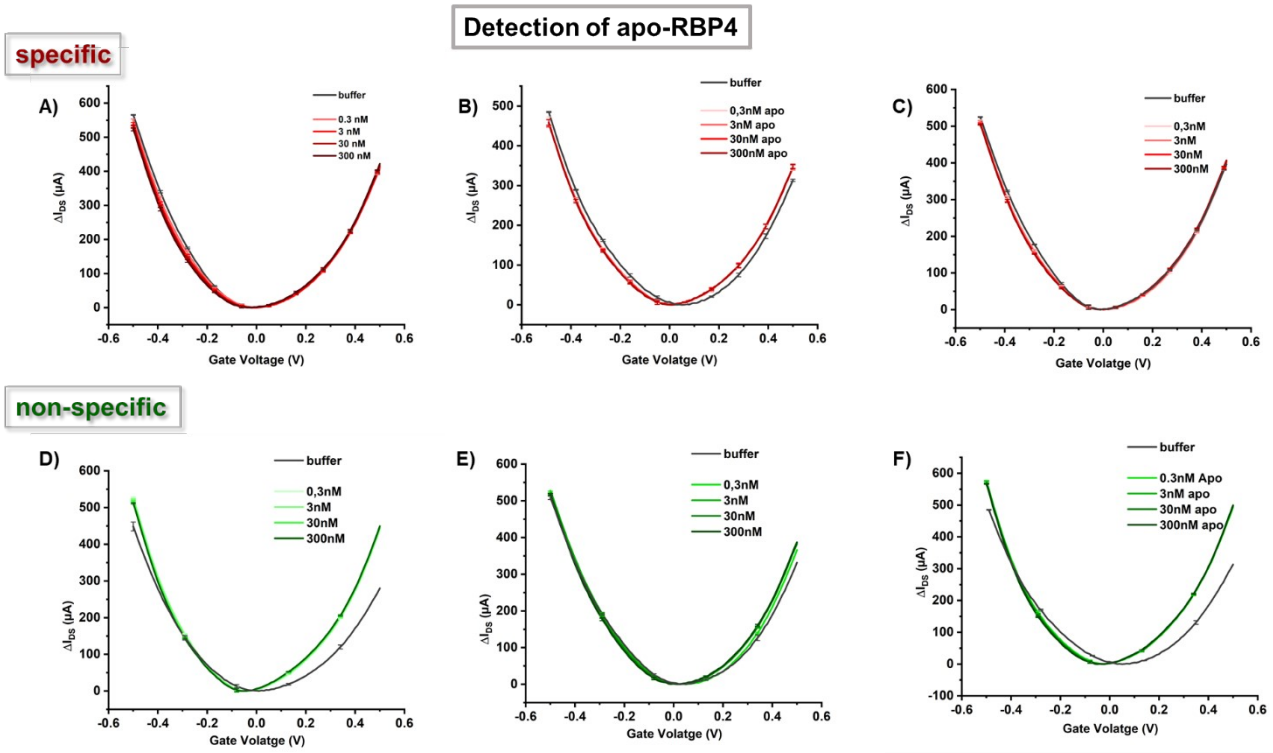
Prior to aptamer immobilization on the rGO-FETs a PCR, was performed to introduce an amine at the 5'-end of the relevant strands. Therefore, a special amino forward primer (Amino-FW) was used: 5'-[AC 6]T AGG GAA GAG AAG GAC ATA TGA T-3' (Eurofins Genomics, Ebersberg, Germany). The rGO-FETs (fabrication method as reported previously[5]) were immersed into a 2.5 mL mixture of 500 µM PyPEG (blocking agent polyethylene glycol was pre-conjugated with PBSE linker) containing 50 µM 1-pyrenecarboxylic acid (PCA) in DMSO and incubated for 12 h at 25 °C. Afterwards, they were rinsed thoroughly with DMSO and then with dH_2O. In the following step, the carboxyl groups were activated by immersion of the rGO-FETs into a solution of 15 mM EDC/15 mM NHS in

0.1 M PBS (pH 7.4) for 30 min at 25 °C, followed by covalent coupling of the 5′-NH_2-modified apo- or holo-RBP4 aptamer libraries (15 µL, 100 nM in DNase-free water) by incubation for 40 min at 25 °C and subsequent washing with PBS for three times.

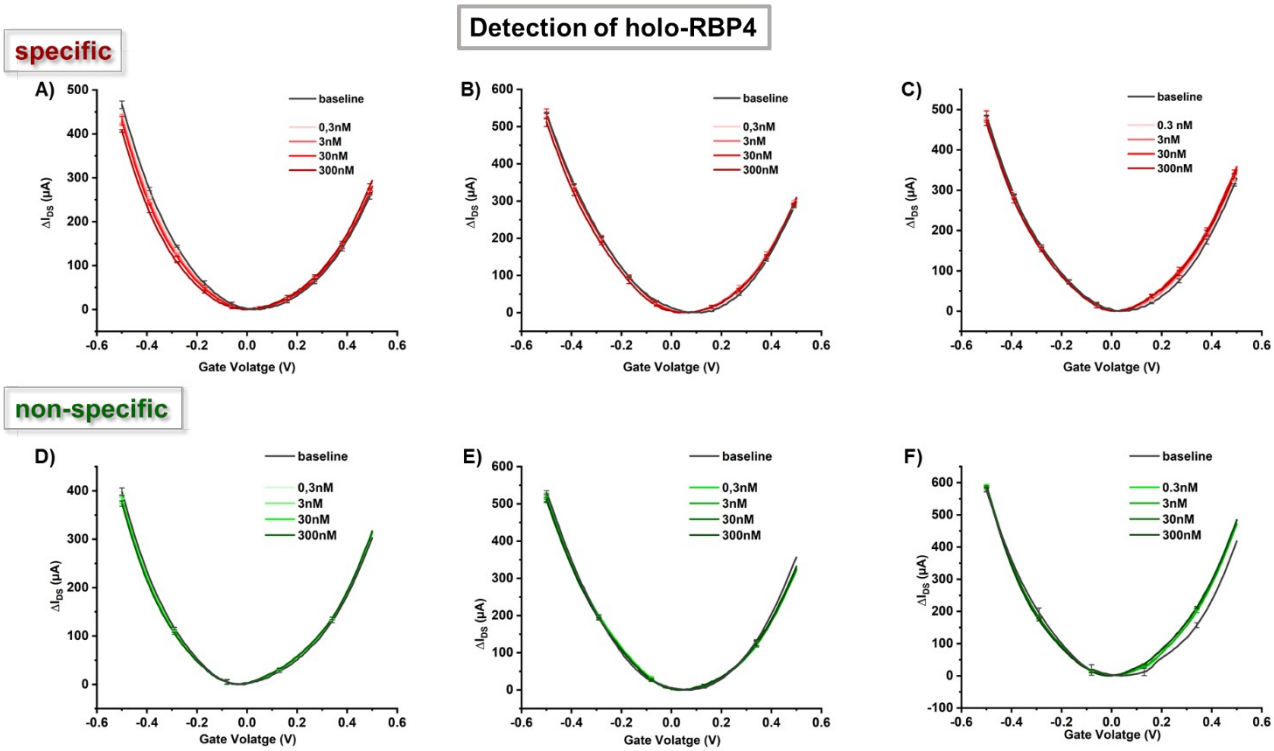
Results and Discussion



Supplementary Figure S1. Expression, purification and characterization of *E. coli* BL21 (DE3)-derived RBP4. A) SDS-PAGE analysis of renaturation of *E. coli* BL21 (DE3)-produced RBP4. Separation through a polyacrylamide gel containing 0.1 % SDS, the gel was stained using Coomassie Brillant Blue G-250. Lane M, Pierce™ unstained protein molecular weight marker; lane 1, harvested cell lysate supernatant; lane 2, insoluble protein fraction after addition of 8 M urea; lane 3, harvested RBP4 from inclusion bodies after renaturation through incubation with buffer containing 0.8 M urea in excess. B) Concentration determination of purified RBP4. Concentrations were calculated by the ChromLab Software for fractions obtained from size exclusion chromatography using a NGC™ Chromatography System (Bio-Rad Laboratories, Inc., Hercules, California, USA) and Superdex 200 Increase 10/300 GL (GE Healthcare, Chicago, Illinois, USA) with the extinction coefficient 77400 M^{-1} cm^{-1} and the molecular weight of 21 kDa for RBP4. C) Fractions obtained from size exclusion chromatography were analyzed by electrophoresis through a polyacrylamide gel containing 0.1 % SDS. Lane M, Pierce™ unstained protein molecular weight marker; lanes 1-14, Superdex 200 Increase 10/300 GL fractions containing purified apo-RBP4. The gel was stained with Coomassie Brillant Blue G-250. D) Binding of all-*trans*-retinal to *E. coli*-derived apo-RBP4 and formation of holo-RBP4. After addition of increasing amounts of all-*trans*-retinal to apo-RBP4, the fluorescence emission was measured using a Spark® multimode microplate reader (Tecan Trading AG, Männedorf, Switzerland). Emission was monitored at 340 nm with an excitation wavelength of 280 nm. The amount of *E. coli*-derived apo-RBP4 was kept constant at 0.425 µM, while the all-*trans*-retinal concentration was varied between 0 and 2.125 µM. Error bars symbolize standard deviations of measurements conducted in triplicates. E) Verification of coating of Dynabeads M-280 tosyl-activated with apo-RBP4 and holo-RBP4. Left y-axis: Intrinsic protein fluorescence measurement. Signals of single apo-RBP4, apo-RBP4 and holo-RBP4 coated beads (grey bars). Quenching of holo-RBP4 fluorescence after complexation of apo-RBP4 with all-trans-retinal. Emission was monitored at 340 nm with an excitation wavelength of 280 nm. Error bars symbolize standard deviations of measurements conducted in triplicates. Right y-axis: Fluorescence measurement of all-*trans*-retinal, signals of retinal and holo-RBP4 coated beads (black bars). Emission was monitored at 490 nm with an excitation wavelength of **350** nm. Error bars symbolize standard deviations of measurements conducted in triplicates.



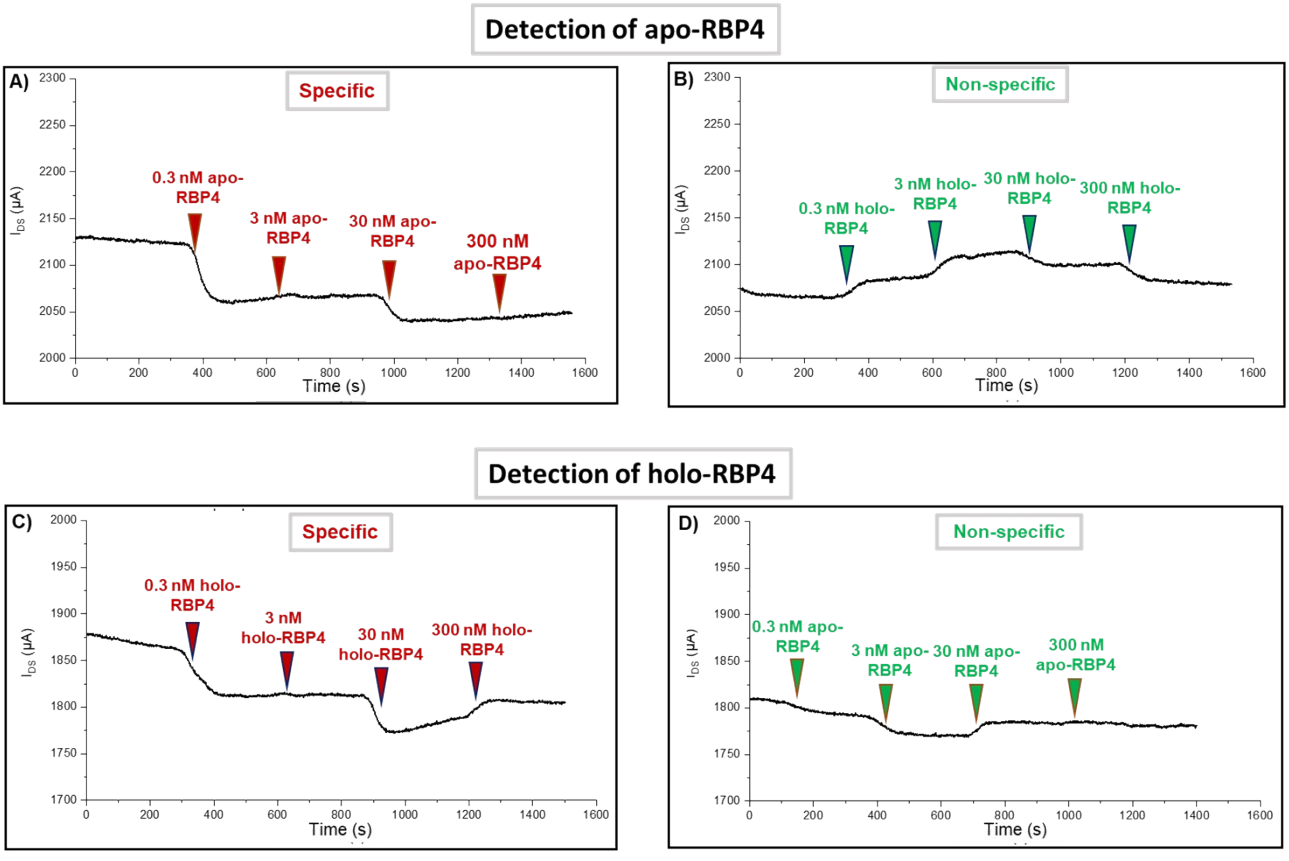
Supplementary Figure S2. I_DV_G characterization of sensing devices obtained by sweeping the gate voltage from -0.5V to 0.5V. A, B and C show 3 specific interactions between apo-RBP4 and the corresponding aptamer library immobilized on the channel. D, E and F show the holo-RBP4 interaction with the same aptamer library that should have no affinity for holo-RBP4. Conditions and parameters were identical in each experiment. Each individual curve is an average of three measurements. Error bars indicate the standard deviation. Incubation time for each concentration was 20 minutes followed by rinsing with 1X PBS buffer (pH 7.4).

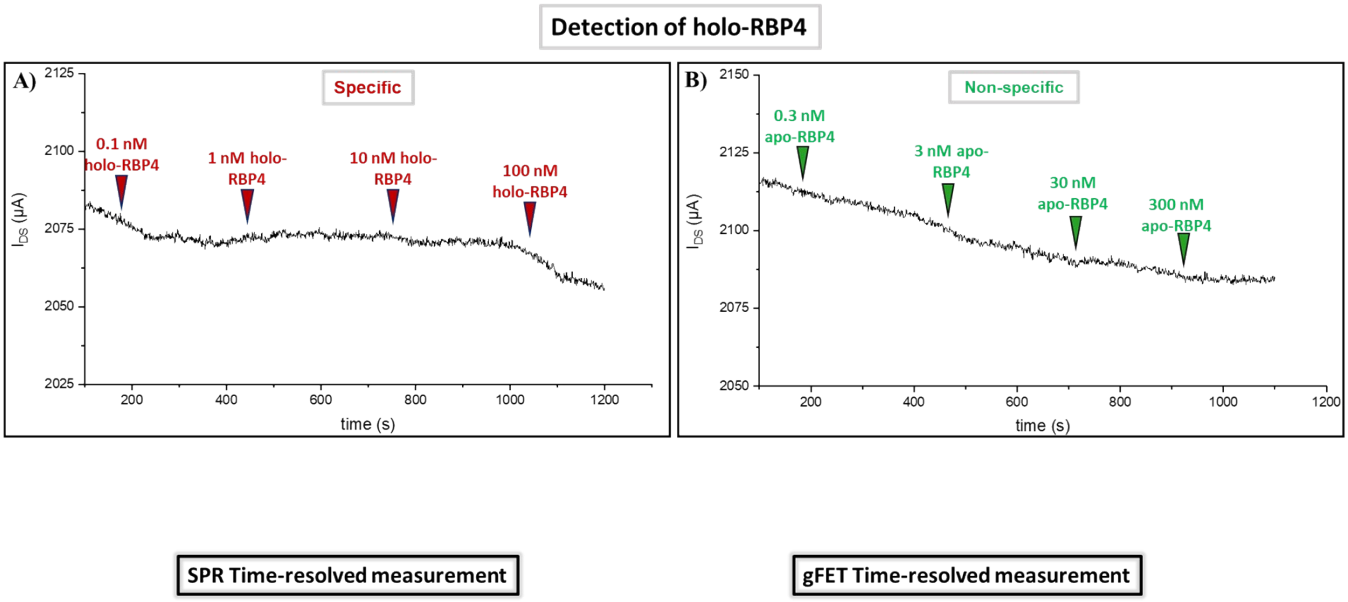


Supplementary Figure S3. I_DV_G characterization of sensing devices obtained by sweeping the gate voltage from -0.5V to 0.5V. A, B and C show 3 specific interactions between holo-RBP4 and the corresponding aptamer library immobilized on the channel. D, E and F show the apo-RBP4 interaction with the same aptamer library that should have no affinity for apo-RBP4. Conditions and parameters were identical in each experiment. Each individual curve is an average of three measurements. Error bars

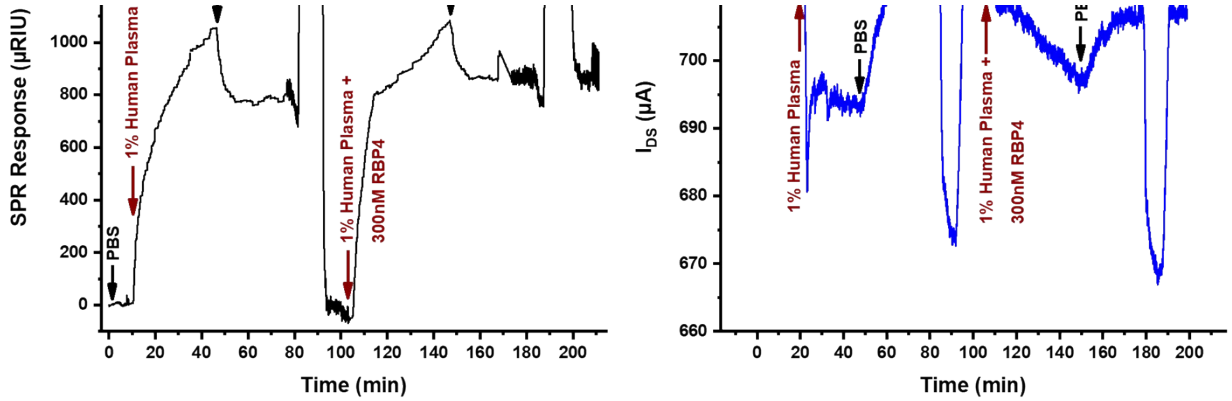
indicate the standard deviation. Incubation time for each concentration was 20 minutes followed by rinsing with 1X PBS buffer (pH 7.4).

Supplementary Figure S4. Real-time sensing of RBP4 on gFET-devices recording I_{DS} at a gate voltage of 400 mV. A) Titration with holo-RBP4 (control), B) titration with apo-RBP4 in real time. C) Titration with apo-RBP4 (control) and D) titration with holo-RBP4.





Supplementary Figure S5. It was possible to detect 100 pM holo-RBP4 with some devices, but further optimization is required to reliably reach this sensitivity. A) specific detection of holo-RBP4, B), non-specific interactions with apo-RBP4



Supplementary Figure S6. Real-time kinetic measurement of holo RBP4 specific aptamer library fuctionalisied surface. A and B are the time resolved SPR and gFET read-out, respectively upon addition of 1% HBP and 1% HBP spiked with 300 nM holo-RBP4.

Passivation against non-specific interactions was tested with human blood plasma (HBP). Figure S7 shows SPR and gFET measurements with 1% human plasma and 300nM RBP4 spiked 1% human plasma to demonstrate that the presence of blood plasma does not prevent RBP4 binding to the sensor surface. For SPR measurements, a glass slide coated with 2 nm chromium and 50 nm gold was functionalised with the holo-aptamer library *via* amine coupling. As shown in Figure 6A, 1% human plasma resulted in mass binding to the surface in the SPR experiment and a decrease in I_{DS} on the gFET, but in both cases the signal returned to baseline after regeneration with 2M NaCl solution. Upon addition of 1% HBP spiked with 300 nM RBP4, the signal did not return to baseline after regeneration which indicates that in the presence of HBP, RBP4 still preferentially binds to the aptamer library.

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A.-K. Kissmann, J. Andersson and A. Bozdogan contributed equally.

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