Synthetically Modified Fc Domains as Building Blocks for Immunotherapy Applications

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Supporting Information

General experimental procedures and materials

Unless otherwise noted, all chemicals and solvents were of analytical grade and used as received from commercial sources. Water (dd-H_2O) used in biological procedures or as the reaction solvent was deionized using a NANOpure purification system (Barnstead, USA). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). All cell culture reagents were obtained from Gibco/Invitrogen Corp. (Carlsbad, CA) unless otherwise noted. Cell culture was conducted using standard techniques. Jurkat and Ramos cells were grown in T-25 culture flasks (Corning) in RPMI Medium 1640 supplemented with 10% (v/v) fetal bovine serum (FBS, Omega Scientific) and 1% penicillin/streptomycin (P/S, Sigma). U266 cells were grown in T-25 culture flasks (Corning) in RPMI Medium 1640 supplemented with 15% (v/v) fetal bovine serum (FBS, Omega Scientific) and 1% penicillin/streptomycin (P/S, Sigma).

Instrumentation and sample analysis

Liquid chromatography-mass spectrometry (LC-MS). Fc protein bioconjugates were analyzed using an Agilent 1200 liquid chromatograph (LC; Santa Clara, CA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA), located in the QB3/Chemistry Mass Spectrometry Facility at UC Berkeley. The LC was equipped with a reversed-phase C_8 column (100 mm × 1.0 mm, 5 μm particles, Restek, Bellefonte, PA). Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). For each sample, approximately 200 picomoles of protein analyte was injected onto the column. Following sample injection, analyte trapping was performed for 5 min with 99.5% A. The elution program consisted of a linear gradient from 30% to 95% B over 19.5 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 0.5 min, and then isocratic conditions at 0.5% B for 9.5 min. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection, to avoid cross-

contamination between samples. Mass spectra were recorded in the positive ion mode over the range m/z = 450-2000. Raw mass spectra were processed using Xcalibur software (version 2.0.7 SP1, Thermo) and protein charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ).

High Performance Liquid Chromatography (HPLC). HPLC was performed on an Agilent 1100 Series HPLC System (Agilent Technologies, USA). Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD). Anion exchange HPLC of Fc-DNA conjugates was accomplished using Biosep-DEAE-PEI column (Phenomenex).

Gel Analyses. For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad (Hercules, CA), following the protocol of Laemmli.[1] The reducing protein electrophoresis samples were heated for 10 minutes at 95 °C in the presence of β-mercaptoethanol to ensure reduction of any disulfide bonds. Gels were run for 40 minutes at 200 V to allow good separation of bands. Commercially available markers (Fisher) were applied to at least one lane of each gel for assignment of apparent molecular masses. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Bio-Rad). For fluorescent protein conjugates, visualization was accomplished on a Typhoon 9410 (Amersham Biosciences).

Construction of the plasmids for the expression of Fc domains

To express the Fc substrates, the pInFuse-hIgG1-Fc2 (InvivoGen, San Diego, CA) plasmid, which contained the human IgG1-Fc gene with its introns and an IL2 signal sequence, was used. A short intron was present between each region: one intron was located between the hinge and the CH2 domain and one intron was located between CH2 and CH3 (Figure S1b). The Fc protein expressed from this plasmid was comprised of the CH2 and CH3 domains of the IgG1 heavy chain. Intracellular cleavage of this sequence occurs after Ser20 and leads to the secretion of the protein to extracellular medium (Figure S1b). As in the sequence shown in Figure S1c, Ile-Ser-Ala remains at the N-terminus of the secreted Fc protein after the IL2 signal sequence is cleaved. The DNA sequence ATATCGGCC encoding Ile-Ser-Ala at the N-terminus was replaced with GCAAAGACC, encoding Ala-Lys-Thr (the optimized sequence for PLP-mediated transamination) using Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Moreover, the ATG following these 9 bases was also changed to ACG to eliminate the potential of having another translation initiation site. This was accomplished via two rounds of Quikchange site-directed mutagenesis: the first round mutated ISA to AKT and the second mutated M to T. The sequences of mutagenic and sequencing primers used in this study are listed in Table 1**.**

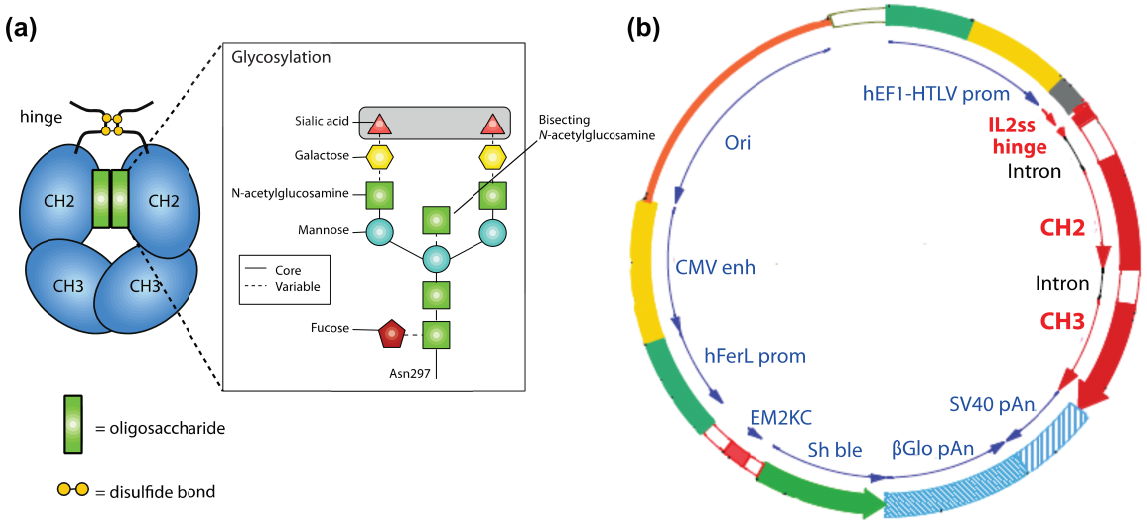
The polymerase chain reaction (PCR) mixture was composed of 1 μL of 50 ng/μL plasmid template, 5 μL of each primer (25 ng/μL), 1 μL of dNTP mixture, 5 μL of 10x buffer, 1μL of PfuTurbo DNA polymerase (2.5 units/μL) and 33 of μL of dH_2O. The reaction was started with 2 min at 95 °C to predenature the template, followed by 18 cycles of 30 sec at 95 °C, 1 min at 55 °C and 6 min at 70 °C. The PCR ended with final polymerization at 70 °C for 10 min and the reaction mixture was left at 10 °C until the next step. After the PCR reaction, 1 μL of DpnI (10 units/μL) was added and the mixture was incubated at 37 °C for 2 h to degrade the original unmodified plasmid templates. After DpnI digestion, 2 μL of the mixture was used to transform *E. coli* XL1-Blue competent cells by heat-shock following the manufacturer's protocol. The

transformed *E. coli* XL1-Blue was spread on LB plates containing Zeocin (Fast-Media Zeo Agar, InvivoGen) and incubated at 37 °C overnight (~16 h). Colonies were selected and grown in 5 mL terrific broth (TB) media containing Zeocin (Fast-Media Zeo TB, InvivoGen) at 37 °C overnight (12-16 h). Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen). The DNA sequences were confirmed by gene sequencing. The sequences of the primers used for sequencing are listed in Table 1.

Table 1. Primer sequences used for construction of plasmid expressing AKT-Fc and sequencing primers.

Purpose	Primers*
Mutating ISA to AKT	5'-GCACTAAGTCTTGCACTTGTCACGAATTCG**GCAAAGACC**ATGGTTAGATCTGACAAAACT-3'
	5'-ATGTGTGAGTTTTGTCAGATCTAACCAT**GGTCTTTGC**CGAATTCGTGACAAGTGCAAGAC-3'
Mutating M to T	5'-TGTCACGAATTCGGCAAAGACC**ACG**GTTAGATCTGA-3'
	5'-AGTTTTGTCAGATCTAAC**CGT**GGTCTTTGCCGAATTC-3'
Sequencing	5'-TGCTTGCTCAACTCTACGTC-3'
	5'-TTGCAGCTTATAATGGTTACAAA-3'

*The mutated sites are in bold.



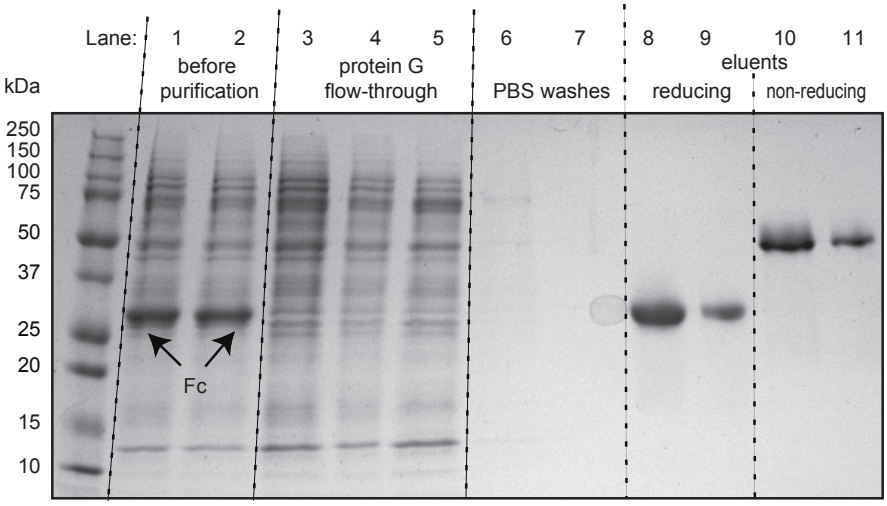
(c)

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACGAATTCG**ATATCGGCCATG**GTTAG
M Y R M Q L L S C I A L S L A L V T N S **I S A M** V R
ATCTGACAAAACTCACACATGCCCACCGTGCCCAGgtaagccagcccaggcctcgccctccagctcaaggcgggacaggtgccctag
S D K T H T C P P C P A
agtagcctgcatccagggacaggccccagccgggtgctgacacgtccacctccatctcttcctcagCACCTGAACTCCTGGGGGGACCGTC
P E L L G G P S
AGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTG
V F L F P P K P K D T L M I S R T P E V T C V V V
GACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGAC
D V S H E D P E V K F N W Y V D G V E V H N A K T
AAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCT
K P R E E Q Y N S T Y R V V S V L T V L H Q D W L
GAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGC
N G K E Y K C K V S N K A L P A P I E K T I S K A
CAAAGgtgggacccgtggggtgcgagggccacatggacagaggccggctcggcccaccctctgccctgagagtgactgctgtaccaacctctgtcc
K G
ctacagGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAG
Q P R E P Q V Y T L P P S R D E L T K N Q V S
CCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGA
L T C L V K G F Y P S D I A V E W E S N G Q P E N
ACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAA
N Y K T T P P V L D S D G S F F L Y S K L T V D K
GAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAA
S R W Q Q G N V F S C S V M H E A L H N H Y T Q K
GAGCCTCTCCCTGTCTCCGGGTAAATGA
S L S L S P G K •••

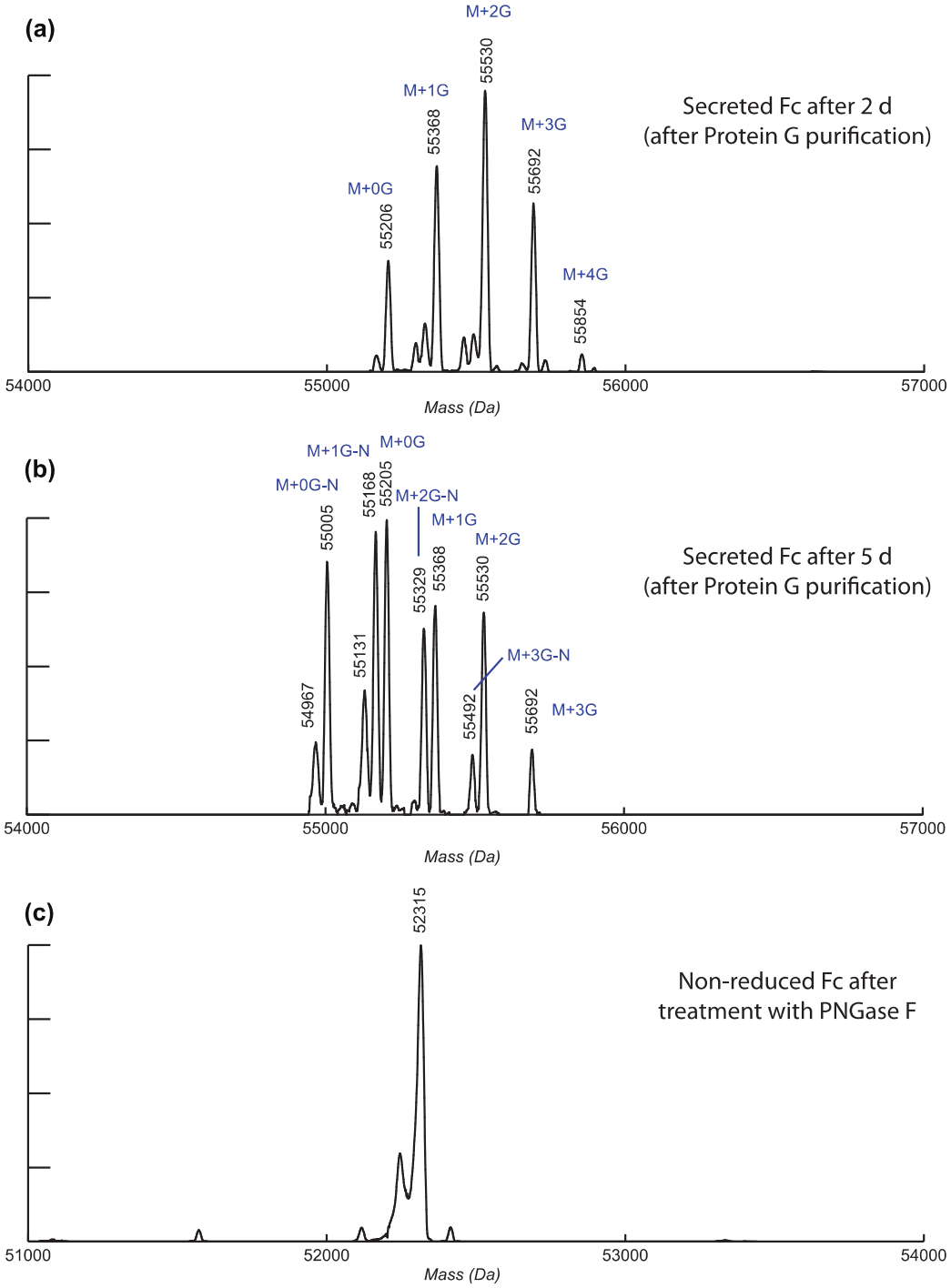
Supplementary Figure S1. Structure and expression vector for Fc of human IgG1. (a) Structure of Fc region of human immunoglobulin G1 (IgG1) is comprised of two monomers, each containing two domains (CH2 and CH3), with glycosylation at Asn297. The hinge region, which contains two disulfide bonds, serves as a flexible spacer between Fc and Fab. The sialic acids highlighted in the gray shaded box were not observed using our expression system. (b) A plasmid expressing human IgG1 Fc protein with introns and IL2 signal sequence (IL2ss) was constructed and used for these experiments. The introns helped to increase the expression level of the IgG1 Fc, and IL2ss signaled the secretion of the Fc protein to the extracellular medium. (c) The sequence of the IL2ss (grey) and human IgG1 Fc (upper case letters) is shown with introns (lower case letters) inserted between hinge and CH2 and between CH2 and CH3 (red). The mutated region is in bold.

General procedure for expression of AKT-Fc

The plasmids expressing AKT-Fc constructed above were transiently transfected into human embryonic kidney (HEK) 293T cells using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium following the protocol from Invitrogen. The cells were incubated at 37 ˚C in 5% CO_2. After 2 days, media were collected and secreted antibodies were purified using protein G affinity chromatography, according to the procedure from the manufacturer (Pierce). Fresh media were added and cultures were grown for additional 3 days, after which additional antibodies were harvested and purified as above. Purified protein was buffer exchanged into PBS using Amicon Ultra 4 mL 10,000 MWCO (Millipore) centrifugal ultrafiltration membranes. Purity was evaluated by SDS-PAGE with Coomassie staining (Figure S2).



Supplementary Figure S2. SDS-PAGE analysis of the Fc protein collected and purified using a protein G column. Lanes 1 and 2 represent all secreted proteins collected before protein G purification. Lanes 3-7 are the flow-through and PBS wash fractions from the protein G column. These fractions contained all other proteins except the Fc (~30 kDa). Lanes 8 and 9 were the eluent from protein G column, analyzed under reducing conditions. The reduced Fc monomers appear at ~30 kDa. Lanes 10 and 1 1 were the same samples as lanes 8 and 9, but run under non-reducing conditions. Fc dimers are evident at ~ 55 kDa. Samples in all lanes except 10 and 11 were prepared with a reducing loading buf fer (β-mercaptoethanol).



Supplementary Figure S3. LCMS analysis of (a) the Fc protein collected 2 d after transfection; (b) the Fc protein collected 5 d after transfection (fresh Opti-MEM media was replaced after 2 d); and (c) the Fc protein after treatment with PNGase F (both 2 d and 5 d samples were identical). The heterogeneity was a result of differences in the number of galactose (G, 162 Da) and N-acetylglucosamine (N, 203 Da) residues that were incorporated. Because each monomer of the Fc protein could contain up to two galactose residues, there are five possibilities of glycosylation patterns for the Fc dimer, corresponding to a normal distribution of M+0G (55206 Da), M+1G (55368 Da), M+2G (55530 Da), M+3G (55692 Da), and M+4G (55854 Da). Additionally, peaks at 55005, 55166, 55329, and 55492 Da appear to match M+0G-N, M+1G-N, M+2G-N, and M+3G-N, respectively.

General procedure for PLP transamination

The 2x protein stock solutions were prepared at 10-40 μM using 25 mM phosphate buffer at pH 6.5. The 2x (200 mM) PLP stock solutions were prepared in 25 mM phosphate buffer and the pH of the solution was adjusted to 6.5 using NaOH solution. Protein and PLP stock solutions were mixed in equal volumes. The reaction mixture was briefly agitated to ensure mixing and then incubated without further agitation at 37 ˚C for 1 h. After incubation, the PLP was removed using NAP Sephadex size exclusion columns (GE Healthcare). The resulting keto-Fc solution was then concentrated and the buffer was exchanged with 25 mM phosphate buffer (pH 6.5), using Millipore 0.5 or 4 mL spin concentrators (MWCO 10 kDa), following the protocol from the manufacturer.

General procedure for hydrazone and oxime formation

The reaction was performed with 10-40 μM keto-Fc and $RONH_2$ or $R(CO)NHNH_2$ at varied concentrations. For the analysis of PLP transamination efficiency, $BnONH_2$ and Alexfluor 488-ONH_2 were added to keto-Fc to a final concentration of 100 mM and 80 μM, respectively. For the attachment of an oxidative coupling partner, aniline-ONH_2 was added to a final concentration of 25 mM. To make Fc-aptamer constructs, keto-Fc was mixed with the hydrazide-aptamer at a final concentration of 100 μM in the presence of 100 mM aniline, which is known to enhance the rate of hydrazone formation.[2] The reaction mixture was incubated at RT for 18-50 h. All the reactions were carried out in 25 mM phosphate buffer (pH 6.5), except for oxime formation with aniline-ONH_2, which was done in 25 mM phosphate buffer (pH 5). Following the reaction, the small molecules were removed using NAP Sephadex size exclusion columns (GE Healthcare) and the resulting product mixtures were concentrated using Millipore 0.5 or 4 mL spin concentrators (MWCO 10 kDa), following the protocol from the manufacturer. The percent reaction conversion for the Fc samples with small molecules was analyzed using LCMS and the modification with large molecules was analyzed using SDS-PAGE with Coomassie staining.

The general procedure for oxidative coupling

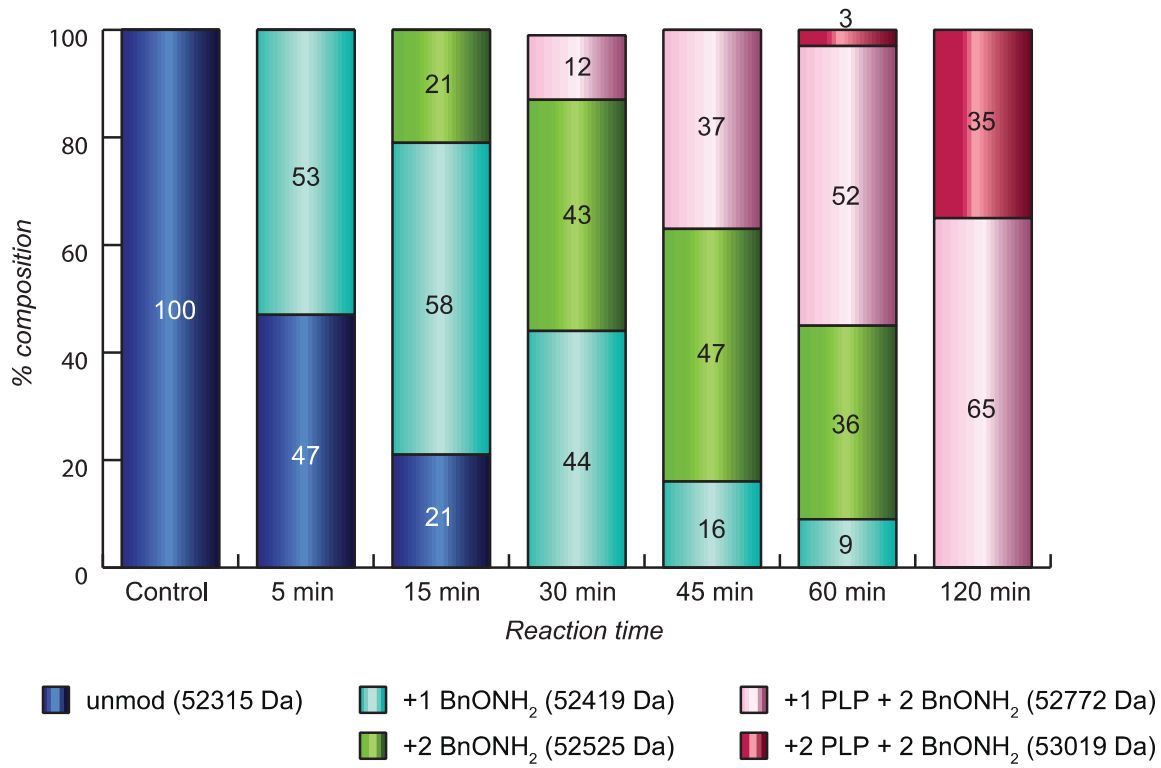
To a solution of 10-40 μM Fc-aniline (**9**) in 25 mM phosphate buffer (pH 6.5) was added a solution of aminophenol-aptamer (**10b**) or aminophenol-2k PEG (**10a**) to a final concentration of 100 μM. Sodium periodate (Sigma-Aldrich) was dissolved to a concentration of 10 mM in 25 mM phosphate buffer (pH 6.5). The sodium periodate was then added to the reaction mixture to reach a final concentration of 1 mM, and the reaction was allowed to proceed for 2 min at RT. In some cases, a solution of mannose was also added to a final concentration of 10 mM or 100 mM before addition of the periodate solution. The resulting protein samples were purified on NAP Sephadex size exclusion columns (GE Healthcare) and concentrated using Millipore 0.5 or 4 mL spin concentrators (MWCO 10 kDa), following the protocol from the manufacturer.

Purification of Fc-aptamer constructs

The resulting Fc-DNA conjugates from both hydrazone formation and oxidative coupling were purified from unreacted Fc and DNA using anion exchange HPLC with a 20-min gradient of 100 % buffer A to 5% buffer A 95% buffer B, where buffer A is 25 mM sodium phosphate buffer pH 6.5 and buffer B is 25 mM sodium phosphate buffer pH 6.5 with 1 M NaCl. The fractions collected were analyzed using SDS-PAGE and those containing Fc-DNA constructs were combined and concentrated using Millipore 0.5 mL spin concentrators (MWCO 10 kDa).

Table 2. Sequence of DNA oligonucleotides used in the protein modification.

DNA oligonucleotides	Sequence
Sgc8c (41-mer)	5'-ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA-3'
TD05.1 (37-mer)	5'-AGGAGGATAGTTCGGTGGCTGTTCAGGGTCTCCTCCT-3'
M2M2 (41-mer)	5'-CCCTAGAGTGAGTCGTATGACCCTAGAGTGAGTCGTATGAA-3'



Supplementary Figure S4. Product distribution of PLP-mediated transamination of the Fc domains, followed by oxime formation with benzyl hydroxylamine (**4a**).

Flow cytometry experiments

Flow cytometry was used to determine binding ability of all the Fc-aptamer constructs. All experiments were carried out in triplicate. For all samples, 100 µL of $3x10^6$ cells/mL of Jurkat, Ramos, and U266 was used, suspended in binding buffer (4.5 g/L glucose, 5 mM $MgCl_2$, 0.1 mg/mL yeast tRNA (Sigma) and 1 mg/mL BSA (Fisher) in Dulbecco's PBS with calcium chloride and magnesium chloride (Invitrogen)). To these cells were added 10 µL of a series of 400 nM Fc-aptamer construct solutions. The samples were then incubated on ice for 1 h. The resulting cells were washed with 500 µL of binding buffer and resuspended in an additional 100 µL of binding buffer. Anti-human IgG1 antibody (specific for the Fc domain) with FITC conjugated (Sigma) was then added to a final concentration of ~ 0.30 µM. Cells were incubated for 1 h on ice in the dark, then washed with 500 µL of binding buffer, and resuspended in 200 µL binding buffer. The cells were analyzed by flow cytometry to determine the amount of FITC fluorescence. For each sample, 10,000 cells were counted.

ELISA for C1q binding

The binding of human C1q to AKT-Fc, chemically-modified Fc, and Fc-DNA conjugates were assessed by an ELISA binding assay, adjusted from a published procedure.[3] High binding Costar 96-well plates (Corning, NY) were coated with varying concentrations of Fc samples in coating buffer (0.05 M sodium carbonate buffer, pH 9) overnight at 4 °C. All samples were run in duplicate. The plates were washed three times after each incubation step with 300 µL of PBS+0.05% Tween 20, pH 7.4. After coating, the plates were blocked with 200 µL of ELISA diluent (0.1 M $NaPO_4$/0.1 M NaCl/0.1% gelatin/0.05% Tween 20/0.05% ProClin300) for 1 h at RT, followed by incubation with 100 µL of 2 µg/mL human C1q (Quidel, San Diego, CA) in ELISA diluent for 2 h. Then, 100 µL of a 1:400 dilution of sheep anti-human C1q peroxidase-conjugated antibody (Abcam) in ELISA diluent was added and incubated at RT for 1 h. The plates were developed with 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate buffer (Sigma) at RT for 15 min. The reaction was stopped upon the addition of 100 µL of stop reagent for TMB substrate (Sigma), and the OD was measured at 450 nm using a Spectramax M3 microplate reader (Molecular Devices, Sunnyvale, CA). The obtained Hill plots displayed different amplitudes for the binding curves, which were taken to arise from different amounts of Fc samples binding to the wells. The plots were therefore normalized based on their maximum overall absorbance.

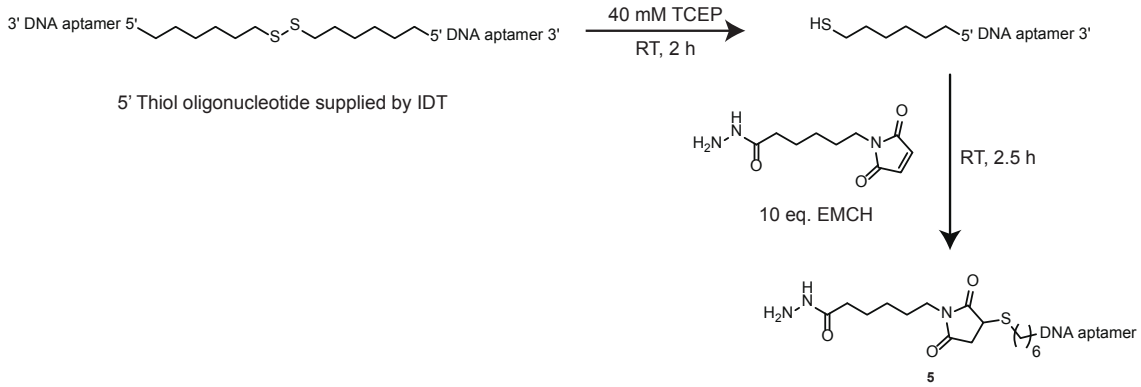
Synthesis of hydrazide-DNA (6)

The 5' thiol DNA oligonucleotide supplied by IDT was reduced in 40 mM TCEP in PBS, pH 7.4 for 2 h at RT. The TCEP was removed using NAP Sephadex size exclusion columns (GE Healthcare). Ten equivalents of 3, 3´-*N*-[ε-maleimidocaproic acid] hydrazide, trifluoroacetic acid salt (EMCH, Pierce), were added to a sample of reduced 5' thiol oligonucleotide in PBS, pH 7.4. The reaction mixture was incubated for 2.5 h at RT. The resulting hydrazide-DNA (**6**) was purified using NAP Sephadex size exclusion columns (GE Healthcare), followed by spin concentration with Millipore 0.5 mL spin concentrators (MWCO 10 kDa).

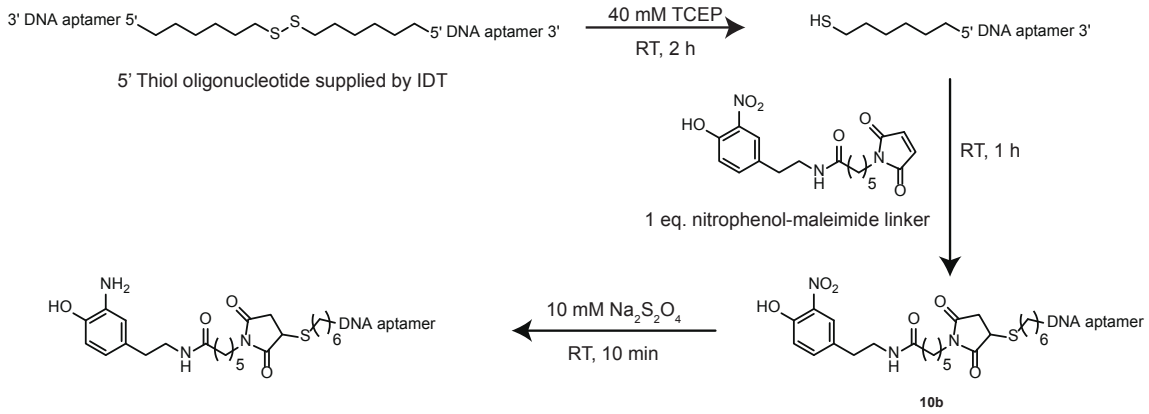
Synthesis of aminophenol-DNA (11b)

The 5' thiol DNA oligonucleotide supplied by IDT was reduced in 40 mM TCEP in PBS, pH 7.4 for 2 h at RT. The TCEP was removed using NAP Sephadex size exclusion columns (GE Healthcare). The resulting product was buffered exchanged into 25 mM phosphate buffer at pH 8 by spin concentration with Millipore 0.5 mL spin concentrators (MWCO 10 kDa).

Approximately one equivalent of a nitrophenol-maleimide linker was added to the reduced 5' thiol DNA oligonucleotide and the reaction was carried out in 25 mM phosphate buffer pH 8 at RT for 1 h. The resulting nitrophenol-DNA was then purified using NAP Sephadex size exclusion columns (GE Healthcare), followed by spin concentration with Millipore 0.5 mL spin concentrators (MWCO 10 kDa) into PBS. The nitro group was reduced to amine in the presence of 10 mM sodium dithionite at RT for 10 min. The final product aminophenol-DNA was purified using NAP Sephadex size exclusion columns (GE Healthcare), followed by spin concentration with Millipore 0.5 mL spin concentrators (MWCO 10 kDa) into 25 mM phosphate buffer pH 6.5 for subsequent conjugation with Fc proteins.



Supplementary Figure S5. Scheme for the synthesis of hydrazide-DNA oligonucleotide **5**.



Supplementary Figure S6. Scheme for the synthesis of aminophenol-DNA oligonucleotide **10b**.

Synthesis of aniline-ONH$_2$ (8)

To (boc-aminooxy)acetic acid (280 mg, 1.5 mmol) dissolved in methylene chloride was added dicyclohexylcarbodiimide (362 mg, 1.7 mmol) and *N*-hydroxysuccinimide (168 mg, 1.5 mmol). After 15 min with stirring, the precipitate was filtered through Celite, followed by a 0.22 µm PVDF syringe filter. To the remaining solution was added 2-(4-aminophenyl)ethylamine (200 mg, 1.5 mmol) and triethylamine (400 mg, 4 mmol). After 1 h of stirring the solution was concentrated under reduced pressure and applied to a silica gel column. Purification using ethyl

acetate as the mobile phase afforded approximately 200 mg of the product (45% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.4 (s, 9H), 2.7 (t, 2H), 3.4 (q, 2H), 3.5 (br s, 2H), 4.2 (s, 2H), 6.6 (d, 2H), 7.0 (d, 2H), 8.1 (br s, 1H), 8.5 (s, 1H). The Boc group was removed via exposure to 1:1 trifluoroacetic acid:methylene chloride for 10 min, after which the solvent was removed under a stream of nitrogen. The resulting oil was placed under vacuum overnight. It was then dissolved to 100 mM in water and stored frozen until used. To avoid precipitation, it was necessary to neutralize the residual trifluoroacetic acid by adding phosphate buffer before addition to protein-containing solutions.

Synthesis of 2k PEG-aminophenol (10a)

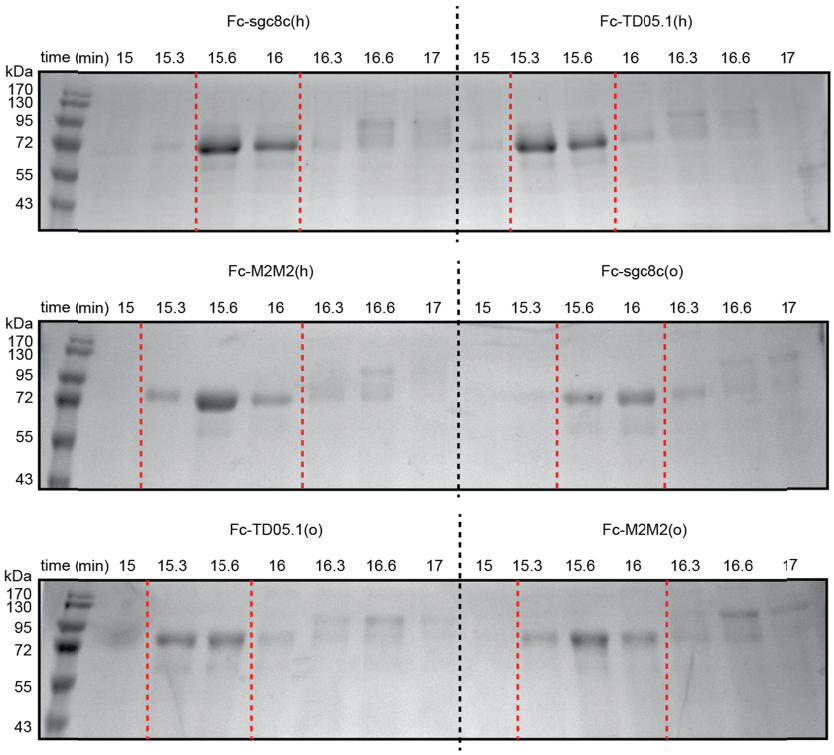
2k PEG-aminophenol (**10a**) was synthesized according to published protocol.[4]

Synthesis of nitrophenol-maleimide linker

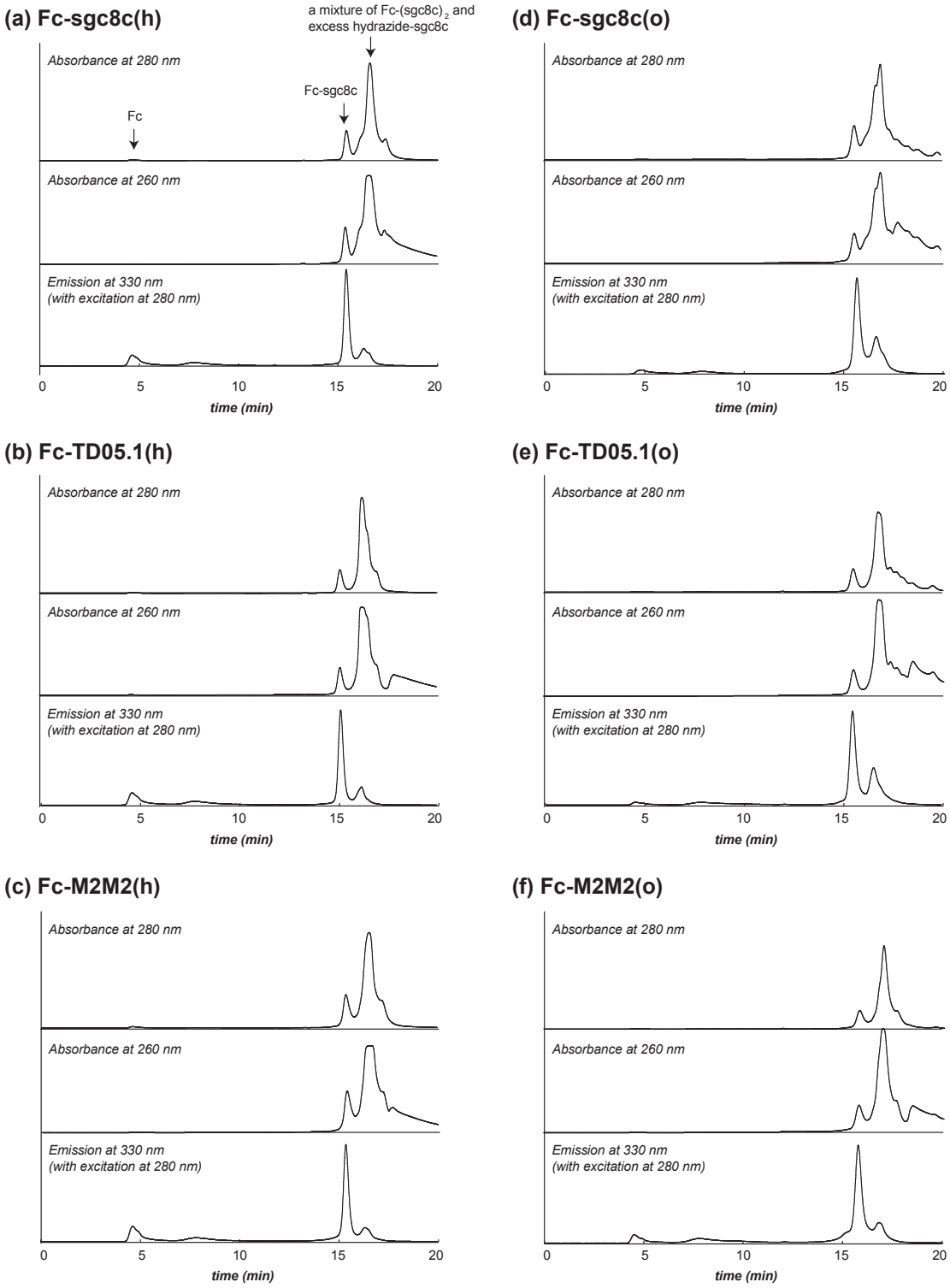
To tyramine was added dropwise one equivalent of fuming nitric acid at 4 °C using trifluoroacetic acid as the solvent and this resulted in quantitative conversion to *o*-nitrotyramine. *O*-nitrotyramine (50 mg, 0.27 mmol) was dissolved in 10 mL DMF and treated with one equivalent of succinimidyl-6-*N*-maleimidohexanoate (as described in reference [5]) along with sufficient triethylamine to reach pH 8. Multiple equivalents of triethylamine were required due to residual trifluoroacetic acid from the nitration step. After 45 min, 20 mL of 0.1 M $NaHSO_4$ was added to the reaction. The product was extracted with methylene chloride, dried over Na_2SO_4, and purified on a silica column using ethyl acetate as the mobile phase. Isolated yield was 33%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.2 (m, 2H), 1.6 (m, 4H), 2.1 (t, 2H), 2.8 (t, 2H), 3.5 (t, 4H), 5.6 (br s, 1H), 6.6 (s, 2H), 7.0 (d, 1H), 7.5 (d, 1H), 8.0 (s, 1H), 10.5 (br s, 1H).

Supporting Information References

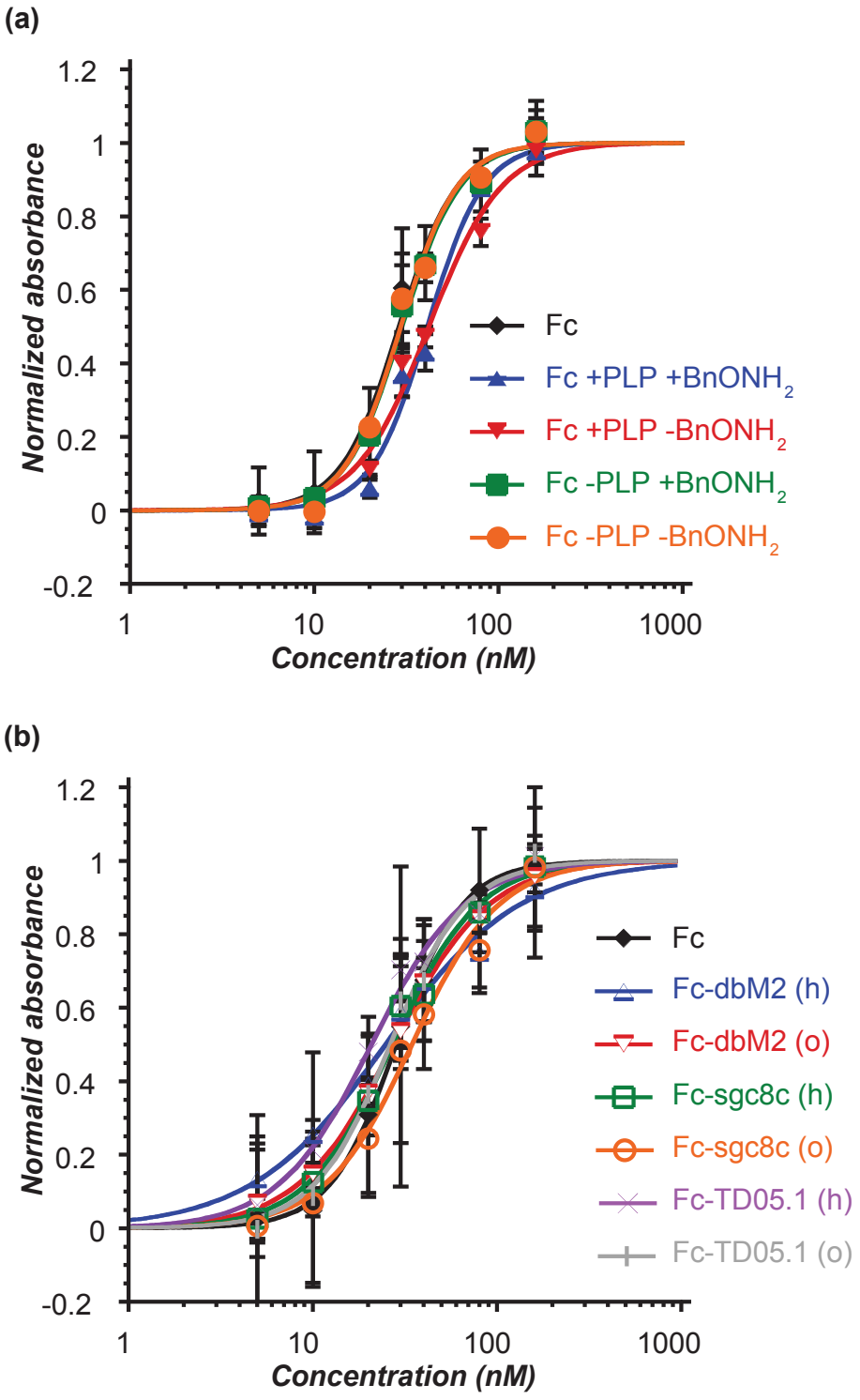
[1] Laemmli, U. K. *Nature*. **1970**, *227*, 680-685.
[2] Dirksen, A.; Hackeng, T. M.; Dawson, P. E. *Angew. Chem. Int. Ed.* **2006**, *45*, 7581-7584.
[3] Idusogie, E. E.; Presta, L. G.; Totpal, K.; Wong, P. Y.; Meng, Y. G.; Mulkerrin, M. G.; Alerts, E. *J. Immunol* **2000**, *164*, 4178-4184.
[4] Behrens, C. R.; Hooker, J. M.; Obermeyer, A. C.; Romanini, D. W.; Katz, E. M.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133*, 16398-16401.
[5] Nielsen, O.; Buchardt, O. *Synthesis* **1991,** *10*, 819-821.



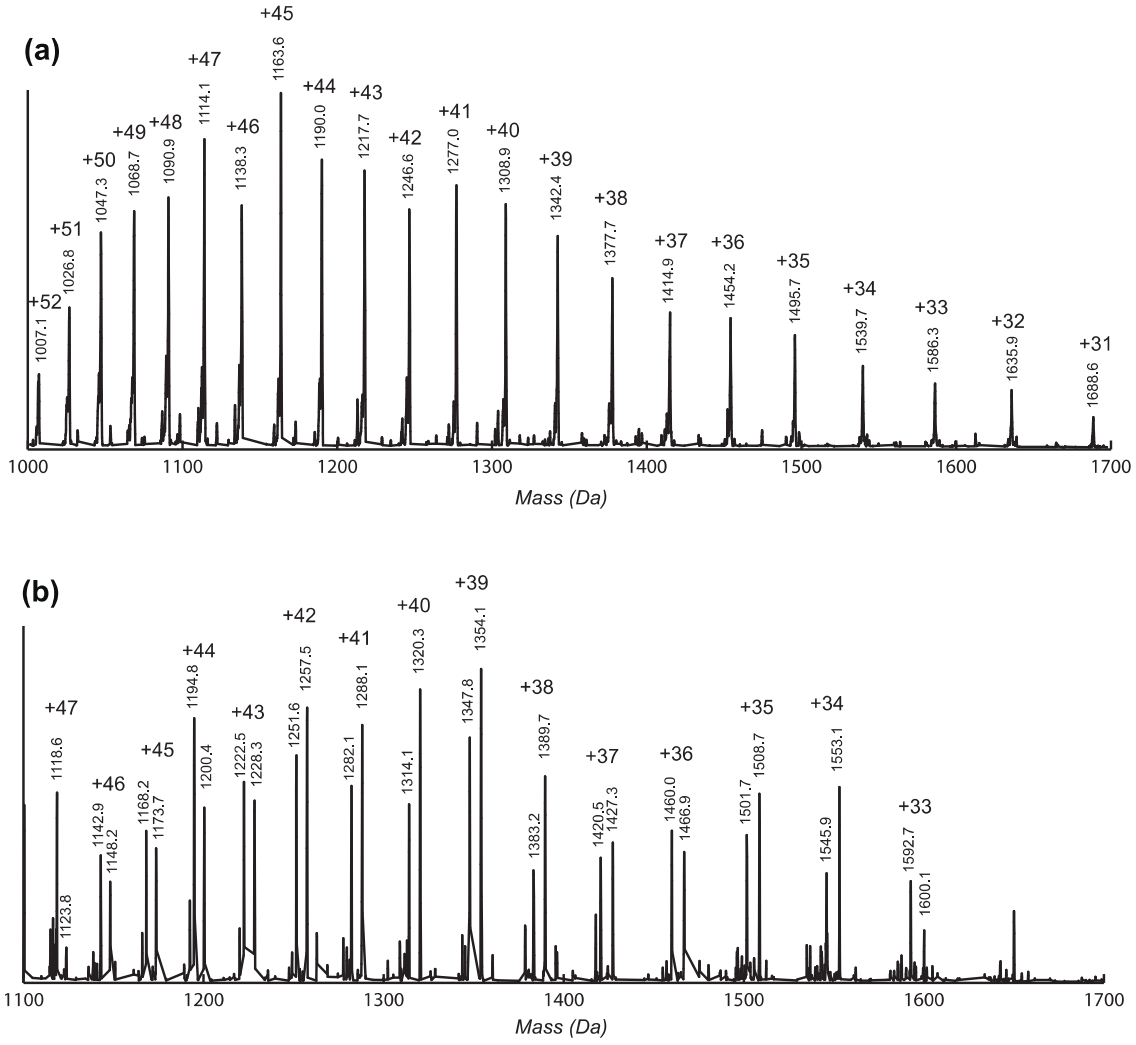
Supplementary Figure S7. SDS-PAGE analysis of Fc-DNA contructs after anion exchange HPLC purification. Fractions at various time points of the HPLC runs were collected every 1/3 min, and small amounts were used for SDS-PAGE analysis to visualize the collected Fc-DNA speciees. The combined fractions are outlined by the red dotted lines.



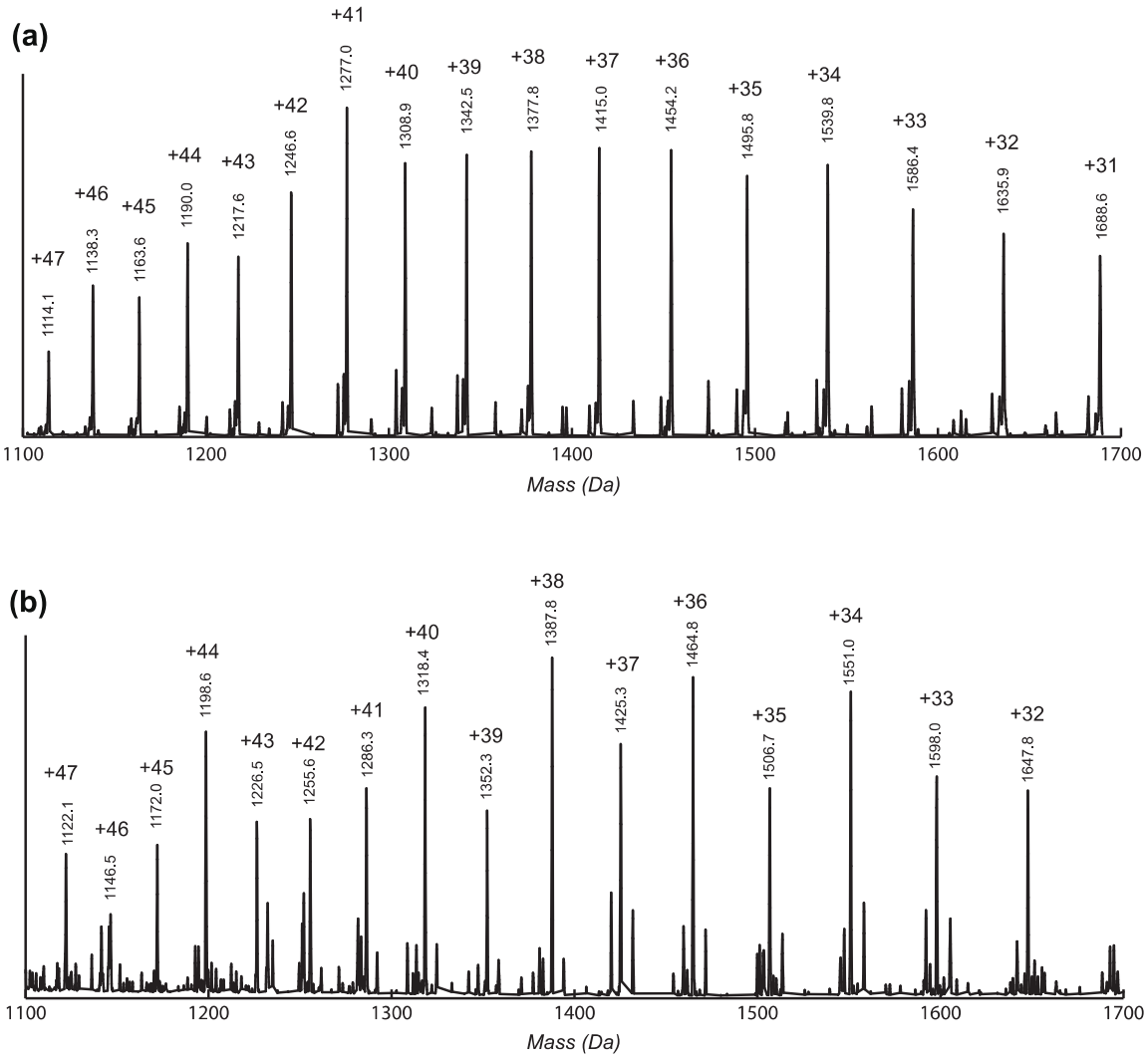
Supplementary Figure S8. Traces from anion exchange HPLC purification of all six Fc-DNA hybrid constructs screened for cell binding in Figure 5 of the main text. Fc proteins were eluted from DEAE column at t = ~ 5 min. Fc-DNA constructs were eluted at t = ~ 15 min, followed by co-elusion of Fc-$(DNA)_2$ and excess hydrazide- or aminophenol-DNA immediately after.



Supplementary Figure S9. Enlarged versions of C1q binding data a ppearing in Figure 5c,d of the main text.



Supplementary Figure S10. Non-deconvoluted mass spectra corresponding to (a) Figure 2a and (b) Figure 2b.



Supplementary Figure S11. Non-deconvoluted mass spectra corresponding to (a) Figure 3a and (b) Figure 3b.