Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Access to site-specific Fc-cRGD peptide conjugates through Streamlined Expressed Protein Ligation

Silvia Frutos,[a] John B. Jordan,[b] Matthew M. Bio, [b], [c] Tom W. Muir,[d] Oliver R. Thiel*[b] and Miquel Vila-Perelló*[a]

* Co-corresponding authors.

[a] ProteoDesign S.L., Baldiri Reixac 10-12, 08028 Barcelona, Spain.

Email: miquel.vila@proteodesign.es.

[b] Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, USA.

Email: othiel@amgen.com

[c] Current address: Snapdragon Chemistry Inc., 85 Bolton Street, Cambridge, MA 02140.

[d] Department of Chemistry, Princeton University, Princeton, NJ 08544, USA.

Table of Contents

General Materials

All buffering salts, isopropyl-β-D-thiogalactopyranoside (IPTG), and *N,N*-diisopropylethylamine (DIPEA) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium 2-mercaptoethanesulfonate (MES), ethanedithiol (EDT), Coomassie brilliant blue, were purchased from Sigma-Aldrich (St. Louis, MO). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Thermo Scientific (Rockford, IL). Complete protease inhibitor tablets were purchased from Roche Diagnostics (Mannheim, Germany). The QuikChange XL II site directed mutagenesis kit was from Agilent (La Jolla, CA). DpnI, restriction enzymes and the Phusion High-Fidelity PCR kit were from New England Biolabs (Ipswich, MA). DNA purification kits (QIAprep spin minikit, QIAquick gel extraction kit, QIAquick PCR purification kit) were from Qiagen (Valencia, CA). Sub-cloning efficiency DH5α cells and One Shot BL21(DE3) chemically competent *E. coli* were purchased from Invitrogen (Carlsbad, CA) and used to generate "in-house" high-competency cell lines. The new intein genes, codon optimized for mammalian expression, were generated synthetically and purchased from GENEWIZ (South Plainfield, NJ).

Criterion XT Bis-Tris gels (12%), Immun-blot PVDF membrane (0.2μm), and Bradford reagent dye concentrate were purchased from Bio-Rad (Hercules, CA).

Cys-cRGD peptide was obtained from the Peptide Synthesis facility of the Department of Experimental and Health Sciences of Pompeu Fabra University (Barcelona, Spain).

General Equipment

Size-exclusion chromatography (SEC) was carried out on ÄKTA FPLC or Purifier systems from GE Healthcare (Piscataway, NJ). Preparative SEC was carried out on a Superdex 200 10/300 column (GE Healthcare). Both semi-preparative and analytical RP-HPLC were performed

on Hewlett-Packard 1100. Analytical RP-HPLC was carried out on a Zorbax 300SB C8 column at a flow rate of 1 mL/min. All runs used 0.25 % Formic acid and 0.02 % TFA (trifluoroacetic acid) in water (solvent A) and 90 % isopropanol in water with 0.25 % Formic acid and 0.02 TFA (solvent B). Electrospray ionization mass spectrometric analyses (ESI-MS) were performed on a Bruker Daltonics MicrOTOF-Q II, and a Waters LCT Premier TOF. Cells were lysed using an S-450D Branson Digital Sonifier. Western blots and coomassie-stained gels were imaged on a LI-COR Odyssey Infrared Imager.

Cloning of plasmids for protein expression

Codon optimized Int^N genes were inserted at the C-terminus of the CH3 domain of the pFUSEN-hG1Fc vector from Invivogen (San Diego, CA) via overlap extension PCR[1]. Once the intein for optimal expression levels was identified (see the Expression Tests Section and Figure S1), site-directed mutagenesis was used to modify the linker between the Interleukin 2 (IL2) leader sequence and the N-terminus of the CH2 domain, to ensure homogeneous cleavage of the IL2 signal peptide. The resulting plasmid encoded for the following sequences:

Fc-Ava^N:

MYRMQLLSCIALSLALVTNSAPLEPKSADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE
YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGKASGGCLSYDTEVLTVEYGFVPIGEIVDKGIECSVFSIDSNGIVYTQPIAQWHHRGKQEVF
EYCLEDGSIIKATKDHKFMTQDGKMLPIDEIFEQELDLLQVKGLPEGHHHHHHG

IL2 Signal Peptide
Linker
Fc domain (CH2-CH3)
AvaN Intein
His tag

Expression and purification of proteins

Expression of Fc-Int^N

CHO-S cells were transiently transfected with Fc-Int^N constructs using FreeStyle MAX reagent (Invitrogen), according to the manufacturer's instructions. Cells were grown in suspension in serum-free media, in vented-cap shaker flasks for 4 days at 37 ºC. Expression tests were performed using 30 mL of culture. Transfection complexes were prepared by mixing 37.5 μl of FreeStyle MAX reagent with 37.5 μg of DNA in 1.2 mL of OptiPro media. After 20 min incubation at r.t., transfection mixtures were added to a suspension FreeStyle CHO-S cells in 30 mL volume.

After 4 days incubation at 37 °C with 5% CO_2, cell supernatants were harvested and spun down at 2000 rcf for 20 min at 4 °C and filtered through a 0.22 mm filter to remove any cell debris. After addition of Complete protease inhbitors cell supernatants were flashed frozen or directly purified over the Protein G using standard protocols. After Protein G purification the eluted Fc-Int^N was dialyzed into PBS and 1 mM EDTA.

Larger scale expressions were performed using up to 150 mL culture volumes and scaling up all other reagents linearly.

Characterization of Fc-Int^N constructs

Fc-Ava^N was analyzed by RP-HPLC and MS. 50 μg of Fc-Ava^N were used for RP-HPLC/MS analysis. Prior to RP-HPLC analysis, half the amount of sample (25 μg) was completely reduced and the other half was degycosylated and fully reduced [2,3]. Deglycosylation was performed using 2 U of PNGase F (New England Biolabs) per μg of Fc and incubated at 37 °C overnight. Deglycosylated and glycosylated Fc samples were denatured by exchanging sample buffer to 6 M Gn·HCl, 100 mM phosphate, 150 mM NaCl, 1 mM EDTA at pH 7.2 and fully reduced by treatment with 10 mM DTT at 37 °C for 1 hr. The fully denatured and reduced Fc samples were analyzed by RP-HPLC on a Zorbax 300SB C8 column using a 15-70 % linear gradient of solvent B over 30 min at 1 mL/min flow rate and 55 °C, preceded by a 3 minute isocratic phase at 15% B. Solvent A is 0.02 % TFA and 0.25% FA (formic acid) in water, solvent B is 90 % isopropanol in water with 0.02% TFA and 0.25% FA. HPLC peaks were collected and analyzed by mass spectrometry

(Figure S3).

General Western blot analysis procedure

Gel samples were loaded onto 12% acrylamide Bis-Tris gels and run in MES-SDS running buffer (Boston Bioproducts, Ashland, MA). The resolved proteins were transferred from the gel onto a PVDF membrane (BioRad) in Tris-Gly transfer buffer (25 mM Tris, 192 mM glycine, 15% (v/v) methanol, pH 8.5) at 100 V for 90 minutes. Membranes were blocked with 4% milk in PBS buffer, then blotted with an IRDye labeled anti-human IgG antibody (Li-Cor 800, 1:15,000). The blots were imaged using the Li-Cor Odyssey IR scanner.

Generation of Npu^{C}-Cys-OMe (IntC) and H-Cys-Gly-Lys(Fluorescein)-NH_2 (CGK(Fl)) peptides.

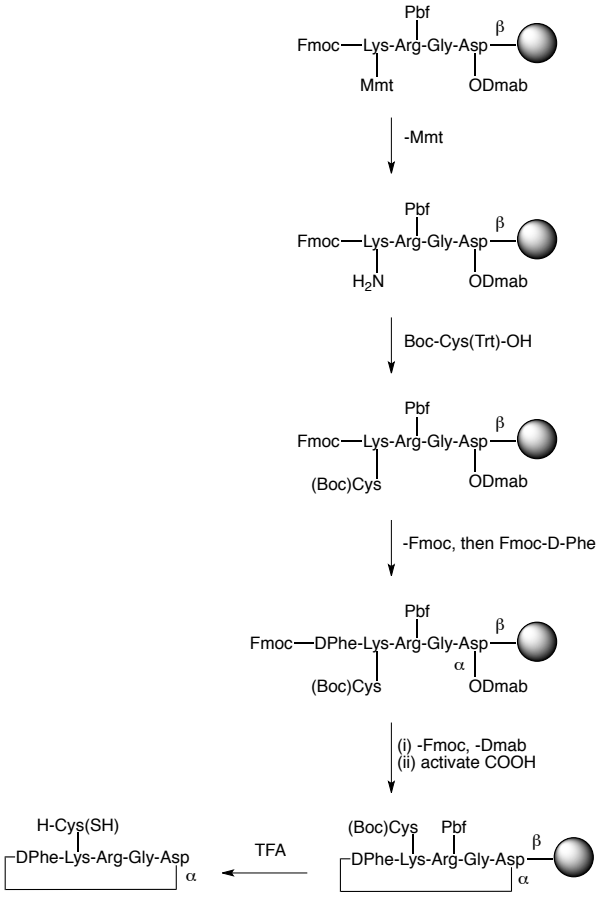
Npu^{C}-Cys-OMe was obtained as previously reported via thiolysis from a Mxe GyrA intein fusion [4]. CGK(Fl) was also obtained as previously described[4] via solid phase peptide synthesis (SPPS).

Synthesis of Cys-cRGD Peptide.

cyclo[DPhe-Lys(Cys)-Arg-Gly-Asp] was custom-made at the Pompeu Fabra University Peptide Synthesis Facility (Barcelona, Spain) by solid phase methods (Scheme S1). First, Fmoc-Asp-ODmab was esterified through its ß-carboxyl group (DIC/DMAP activation) to a pMBHA (p-methylbenzhydrylamine) resin functionalized with the HMPB linker (4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid). The derivatives of Gly, Arg(Pbf) and Lys(Mmt) were then sequentially incorporated using standard Fmoc protocols, with HBTU/DIEA activation and 20% piperidine/DMF deprotection. Orthogonal deprotection of the Mmt group (1% TFA/DCM)

allowed the coupling of Boc-Cys(Trt) to the side chain of Lys. Deprotection of the N-terminus followed by Fmoc-DPhe coupling yielded the target linear sequence in protected form. Sequential removal of the Fmoc and Dmab (2% hydrazine/DMF) groups made possible on-resin cyclization mediated by PyAOP (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate). Finally, the resin was treated with 95% TFA for full deprotection and cleavage. The crude material was purified to >95% homogeneity by RP-HPLC and characterized by RP-HPLC and MS-ESI ($[M+H]^+$observed = 707.33, $[M+H]^+$calculated = 707.32).

Scheme S1: Synthesis of Cys-cRGD (*cyclo*[DPhe-Lys(Cys)-Arg-Gly-Asp])



Thiolysis/ligation tests using CGK(Fl) peptide

Thiolysis/ligation reaction tests were performed mixing protein G purified Fc-AvaN (250

μg/mL) with IntC (14 μM) in the presence of MESNa (100 mM) and the model CGK(Fl) at different concentrations from 12.5 to 500 μM in 100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2. Reactions were allowed to proceed protected from light at room temperature for 24h. Reactions were monitored by SDSPAGE (Figures 2A and S4) and RP-HPLC (Figures 3B and S5) analysis.

Optimized Thiolysis/ligation with Cys-cRGD peptide and purification of Fc-cRGD conjugate.

Protein G purified Fc-AvaN at 500 μg/mL was mixed with 25 μM of IntC peptide and 0.5 mM Cys-cRGD in 100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 100 mM MESNa 1 mM TCEP, pH 7.2. Reaction was allowed to proceed protected from light at room temperature for 24h.

After 24h the reaction mixture was extensively dialyzed into PBS to remove excess peptide and MESNa and the Fc-cRGD conjugate purified over a Superdex S200 column on an AKTA Purifier system.

Characterization of Fc-peptide conjugates

RP-HPLC/MS of Fc-peptide conjugates

30 to 50 μg of ligated Fc-peptide were typically used for RP-HPLC/MS analysis. Before RP-HPLC analysis, Fc conjugate was fully deglycosylated and fully reduced as described above for Fc-AvaN constructs.

For the analysis of Fc-peptide dimers, 30 to 50 μg of the conjugate were analyzed without prior treatment with DTT.

Size-exclusion analysis of Fc-peptide conjugates

The integrity of Fc-peptide was confirmed by injecting 25-50 μg of ligated and dialyzed conjugate in running buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2)

into an S200 size-exclusion column using the AKTA Purifier system.

FcRn and integrin binding measured by Surface Plasmon Resonance (SPR).

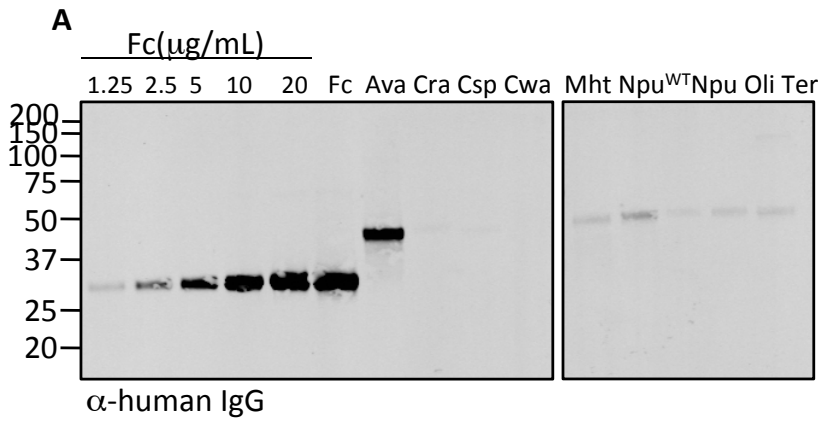
Fc-cRGD and Fc-OH binding to the FcRn

FcRn was immobilized to a COOH5 sensor chip (SensiQ Technologies, Oklahoma City, OK) using standard amine coupling at a concentration of 25 μg/mL and 10 mM Sodium Acetate, pH 4.0. The final surface density of the target was approximately 2300 RU. Analytes were injected over the surface using 2-fold serial dilutions at top concentrations of 1 μM in 25 mM Sodium Phosphate, pH 6.0, 150 mM NaCl and in 25 mM HEPES, pH 7.4, 150 mM NaCl. Compounds were injected for 1 min at 30 μL/min flow rates and utilized a 240s dissociation time. KD values were determined using a single-site steady-state affinity model.

Fc-cRGD and Fc-OH binding to integrin αVβ3

Binding of the Fc-cRGD and Fc-OH consturcts to integrin αVβ3 was performed using previosuly published assay conditions.[4] Integrin αVβ3 (R&D Systems, Minneapolis, MN) was immobilized to a COOH5 sensor chip (SensiQ Technologies, Oklahoma City, OK) using standard amine coupling at a concentration of 25 μg/mL and 10 mM Sodium Acetate, pH 4.0. The final surface density of the target was approximately 1300 RU. Analytes (Fc-OH and Fc-Peptide) were injected over the surface using 2-fold serial dilutions at top concentrations of 1 μM in 25 mM Sodium Phosphate, pH 6.0, 150 mM NaCl, 1mM $MnCl_2$, 1mM $MgCl_2$, 0.005% P20, and 5mM Octylglucoside. Analytes were injected for 1 min at 30 μL/min flow rates and utilized a 240s dissociation time. K_D values were determined using a single-site kinetic fit model.

Supplementary Figures



B

Construct	yield (μg/mL)
Fc	22.5
Fc-Ava	8.1
Fc-Cra	< 0.5
Fc-Csp	< 0.5
Fc-Cwa	< 0.5
Fc-Mcht	1.0
Fc-NpuWT	1.3
Fc-NpuMut	0.8
Fc-Oli	0.9
Fc-Ter	0.9

Figure S1: Quantification of Fc-IntN recombinant expression in CHO cells. A) Western blot analysis of samples from the production media of transiently transfected CHO cells with different Fc-IntN constructs. A calibration curve using purified an Fc standard was used for quantification. an Fc standard curve from 1.25 to 20 μg/mL, and samples from the production media of transiently transfected CHO cells. B) Production yields of the different Fc constructs calculated by western blot quantification.

A

B

MYRMQLLSCIALSLALVTNS-APLEPKSA-DKTHTCPPCPAPELLGGPSVF
LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK-ASGG-CLSYDTEVLTVEYGFVPIGEIVDKGIECSVFSIDSNGIVYTQP
IAQWHHRGKQEVFEYCLEDGSIIKATKDHKFMTQDGKMLPIDEIFEQELDL
LQVKGLPE-GHHHHHHG

Figure S2: Selected Fc-IntN construct. A) Schematic representation of the Fc constructs used in this study. The C_H2-C_H3 domains were fused at their N-terminus to a Signal Peptide (SP) via a short linker and at their C-terminus to an ultrafast DnaE N-intein. B) Amino acid sequence of the selected construct containing the Interleukin 2 signal peptide, and the AvaN intein. The linker sequence, shown in green, was selected to ensure homogeneous cleavage from the signal peptide.

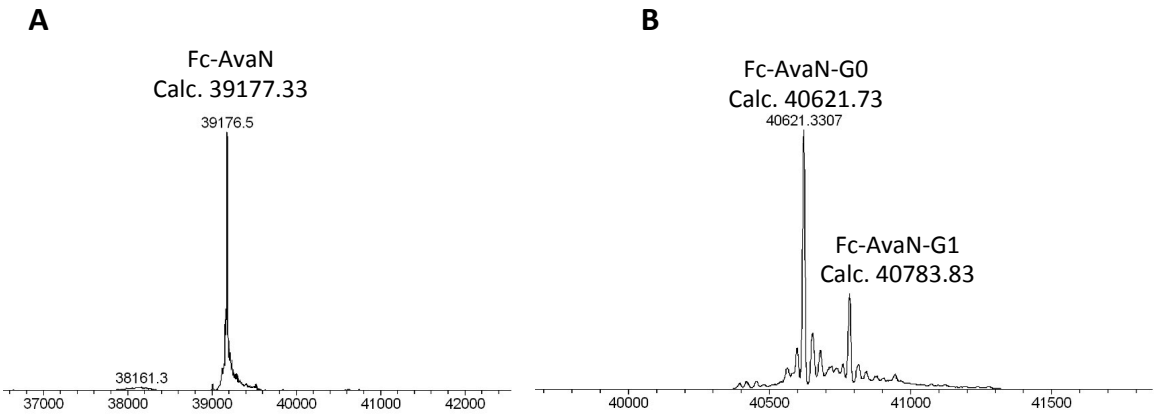


Figure S3: ESI-MS analysis of Fc-AvaN constructs. Protein G purified Fc-AvaN was analyzed by ESI-MS. A) MS analysis of fully reduced and deglycosylated Fc-AvaN. Prior to analysis the Fc-AvaN was deglycosylated by treatment with PNGase F, denatured and fully reduced. B) MS analysis of fully reduced glycosylated Fc-AvaN. Two major peaks were detected corresponding to G0 and G1 glycans (see Figure S10A).

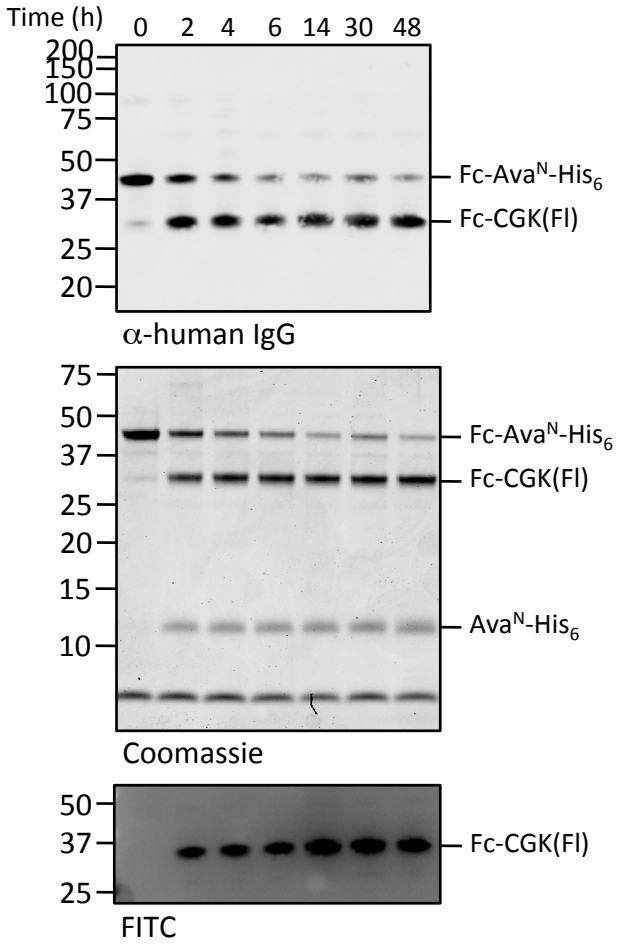


Figure S4: In solution thiolysis/ligation reactions of Fc-AvaN and CGK(Fl). SDSPAGE analysis of one pot thiolysis/ligation reactions. Samples of a reaction mixture (250 μg/mL FcAvaN, 14 μM IntC, 1 mM CGK(Fl) in 100 mM phosphate, 150 mM NaCl, 1 mM TCEP and 100 mM MESNa) were taken at the indicated timepoints, and analyzed by SDSPAGE and imaged using western blot with an anti-human IgG antibody (top panel), Coomassie staining (middle panel) and FITC fluorescence (bottom panel).

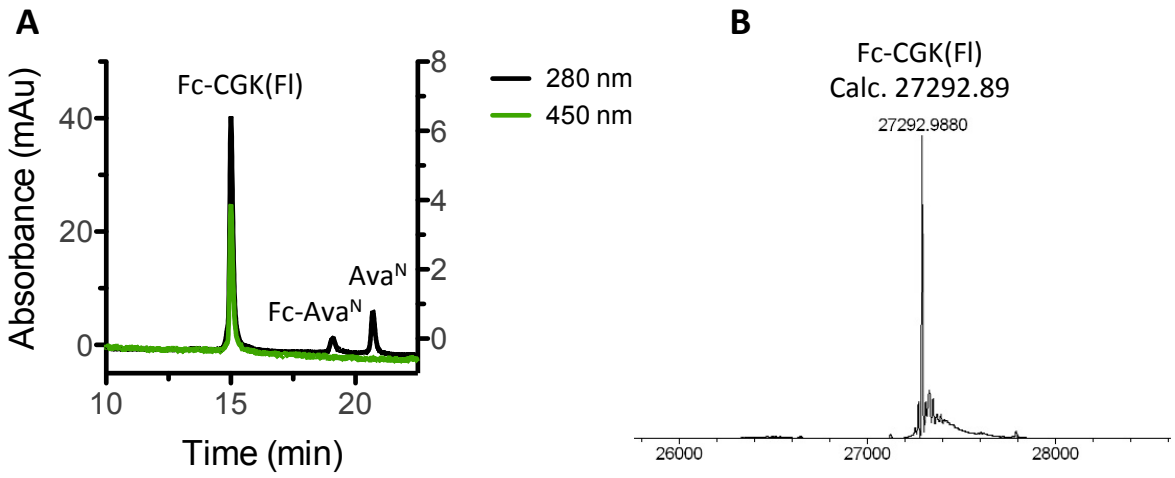


Figure S5: RP-HPLC and ESI-MS analysis of Fc-CGK(Fl) reaction mixture. A) RP-HPLC analysis of the thiolysis/ligation reaction. Reaction mixture was deglycosylated using PNGase F and fully reduced with DTT under denaturing conditions (6M GnHCl, 10 mM DTT, pH 7.5, 1h at 37 ºC). RP-HPLC analysis of the crude reaction mixture was performed over a 15- 40%B gradient on a Zorbax 300SB C8 column. B) ESI-MS of the HPLC peak labeled Fc-CGK(Fl). The entire peak was analyzed and the presence of the desired Fc-CGK(Fl) (MW_{Obs}: 27292.99Da, MW_{Calc}: 27292.89Da) was confirmed, as well as the absence of hydrolyzed Fc-OH byproduct (MW_{Calc}: 26647.19Da).

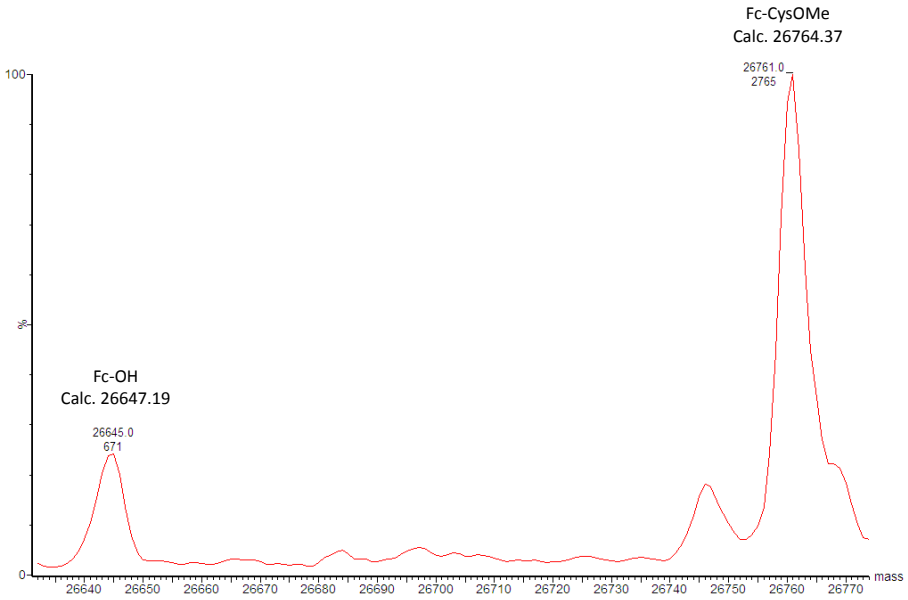


Figure S6: Detection of unreacted Fc α-thioester in ligation reactions using low concentrations of N-terminal Cys-containing peptide.
Thiolysis/ligation reactions were carried out with low concentrations of N-terminal Cys-containing peptide (below 25 μM), as described in the Experimental Section. After 24h, 10 mM of CysOMe were added to the reaction mixture and incubated at r.t for 1 h to quench any unreacted Fc α-thioester. This treatment results in the ligation of any residual Fc α-thioester to CysOMe to generate Fc-CysOMe. Pre-hydrolyzed Fc (Fc-OH) cannot react. Next, samples were deglycosylated using PNGase F (o/n at 37 ºC) and fully reduced with DTT under denaturing conditions (6M GnHCl, 10 mM DTT, pH 7.5, 1h at 37 ºC). Finally, samples were analyzed by RP-HPLC (15-40%B gradient on a Zorbax 300SB C8 column, monitored at 280 nm) followed by ESI-MS to confirm identity of the peaks. A zoomed in area of the MS spectrum is shown, which shows that although after 24h reaction there is already small amount of hydrolyzed Fc-OH, most of the unligated Fc is in the form of Fc-CysOMe, which corresponds to the former Fc α-thioester. These results indicate, as expected, that under the thiolysis/ligation conditions used there is Fc α-thioester thioester available for reaction after 24h incubation with low concentration of N-terminal Cys-containing peptide, and so that the low reaction yields at those concentrations are due to reduced reaction kinetics rather than to premature thioester hydrolysis.

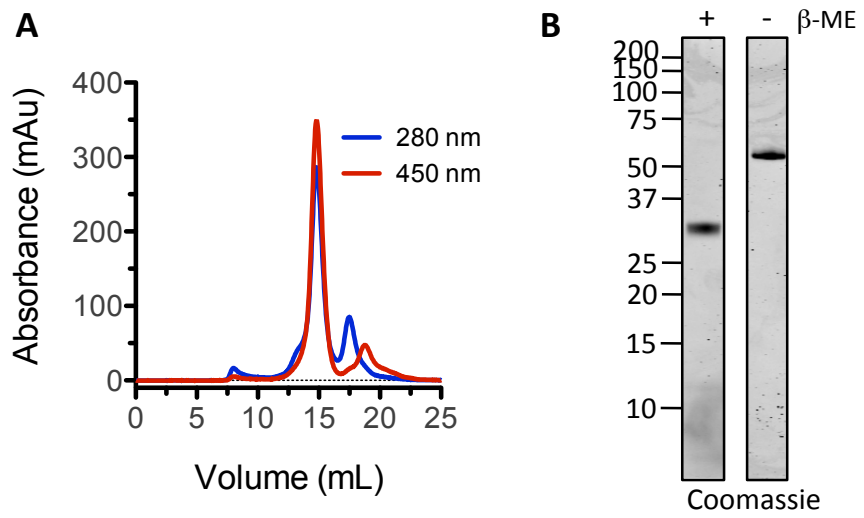


Figure S7: Size-exclusion chromatography (SEC) purification of Fc-CGK(Fl) conjugate. A) SEC purification of thiolysis/ligation reaction mixture over an S200 column in phosphate buffer containing 1 mM EDTA monitored at 280 and 450 nm. B) Coomassie stained SDSPAGE analysis of purified Fc-CGK(Fl) under reducing (left) and non-reducing (right) conditions.

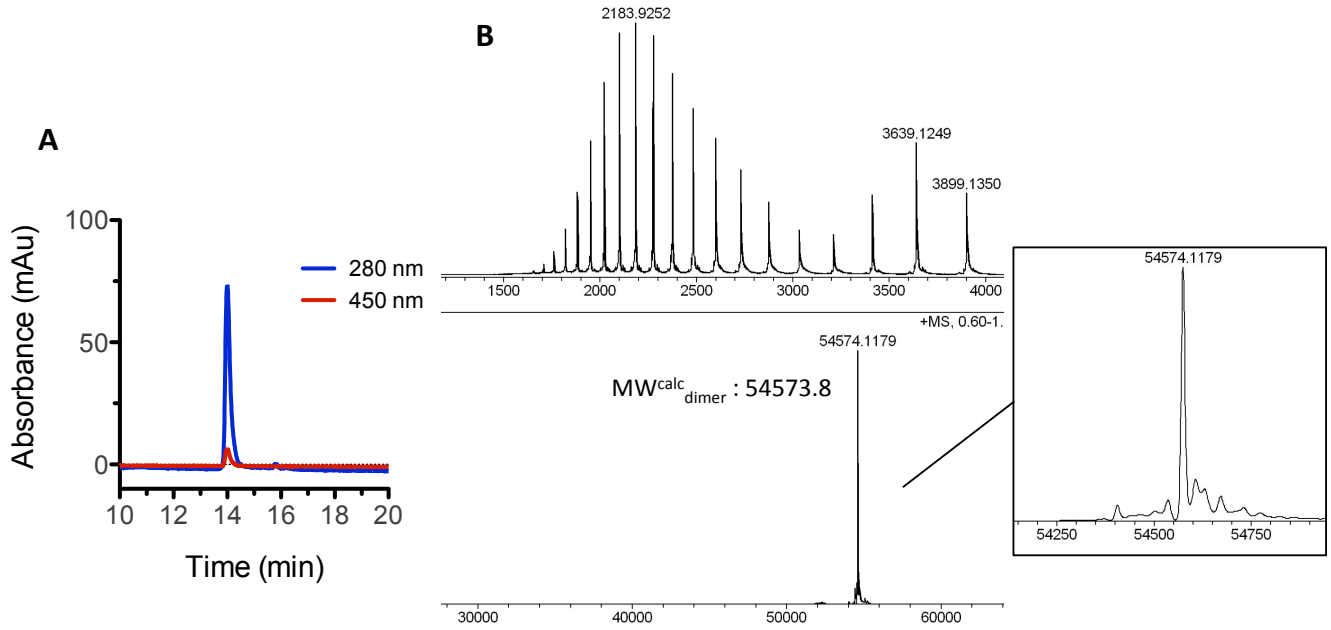


Figure S8: RP-HPLC and MS analysis of de-glycosylated non-reduced Fc-CGK(Fl) conjugate. A) RP-HPLC of purified deglycosylated, non-reduced, Fc-CGK(Fl) over a 10- 75%B gradient on a Zorbax 300SB C8 column (280 and 450 nm detection). B) ESI-MS of the entire HPLC peak. The presence of a species with a MW in good agreement with an Fc-CGK(Fl) dimer with 6 (or 7, see bellow) disulfide bonds was confirmed. No peaks corresponding to heterodimers containing one hydrolyzed chain (MW_{Calc}: 53928.09 Da) nor the completely hydrolyzed dimer were detected (MW_{Calc}: 53282.38 Da).

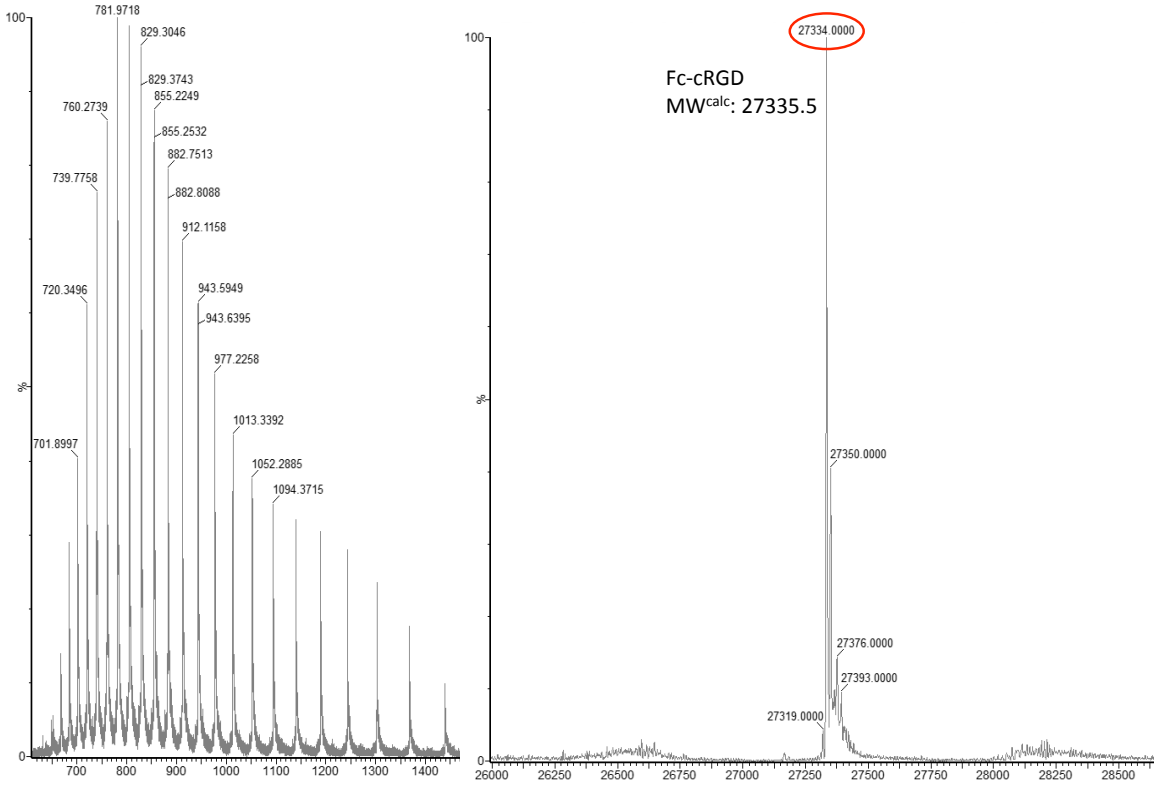


Figure S9: LC-MS analysis of Fc-cRGD thiolysis/ligation reaction. Reaction mixture was deglycosylated using PNGase F and fully reduced with DTT under denaturing conditions (6M GnHCl, 10 mM DTT, pH 7.5, 1h at 37 ºC) prior to ESI-MS analysis. The MS of the entire LC peak is shown. The presence of a species with a MW in good agreement with the desired, fully reduced, Fc-cRGD was confirmed. No peak corresponding to the hydrolyzed Fc could be detected (MW_{Calc}: 26647.19Da).

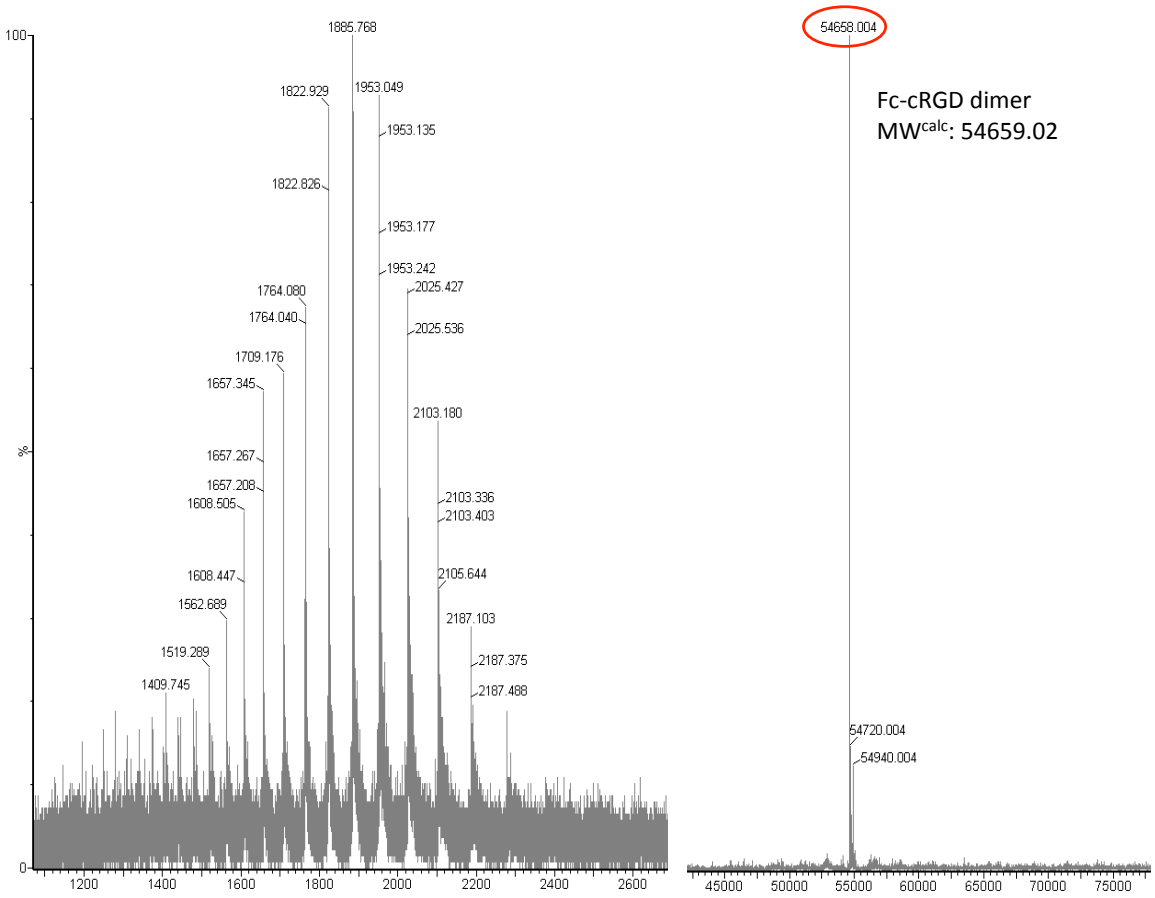


Figure S10: LC-MS analysis of purified deglycosylated Fc-cRGD dimer. The SEC purified Fc dimer was deglycosylated using PNGase F prior to ESI-MS analysis. The MS of the entire LC peak is shown. The presence of a species with a MW in good agreement with the desired Fc-cRGD dimer was observed. No peaks corresponding to heterodimers containing one hydrolyzed C_H2-C_H3 chain (MW_{Calc}: 53970.69 Da) nor the completely hydrolyzed dimer were detected (MW_{Calc}: 53282.38 Da).

A

Code	Glycan structure	Average mass (Da)
G0	GN – M GN – M M – GN – GN(F) –	1445.4
G1	G – GN – M GN – M M – GN – GN(F) –	1607.5
F	Fucose	146.1
G	Glucose	162.1
GN	N-acetylglucosamine	203.2
M	Mannose	162.1

B

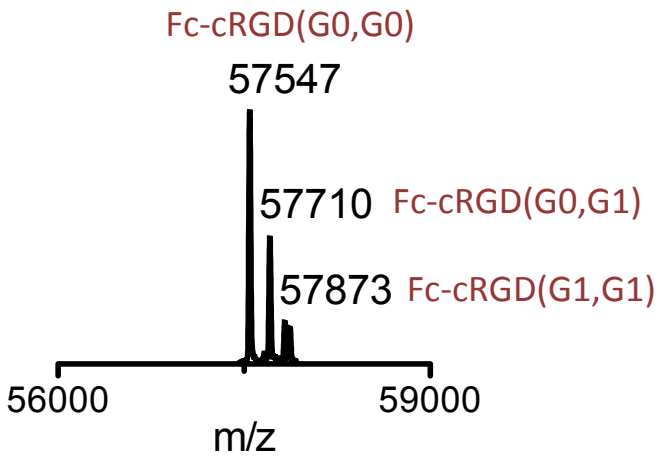
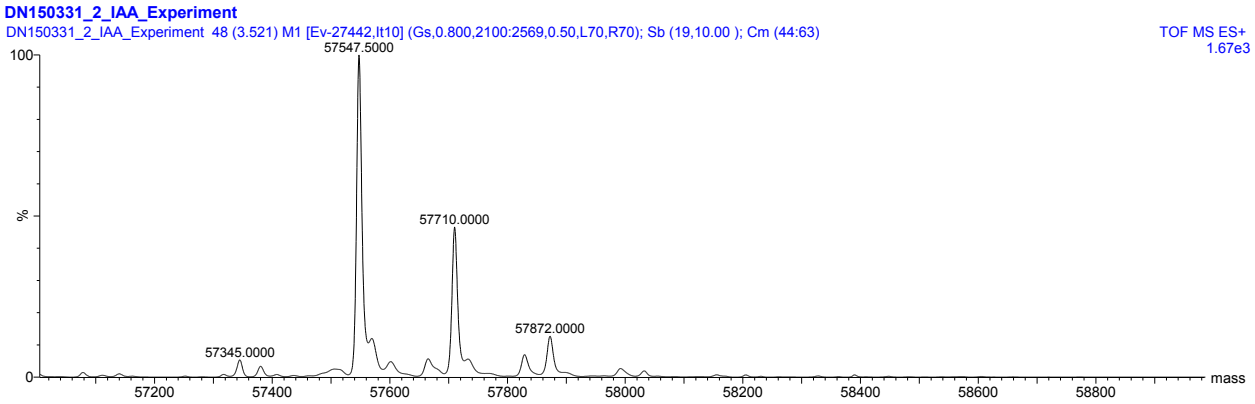


Figure S11: LC-MS analysis of purified Fc-cRGD dimer. Zoomed in spectrum from Figure 3D. The SEC purified Fc dimer was analyzed by LC-MS. A) Table summarizing structure and mass of most common N-glycans found in IgGs[5,6]. B) MS spectrum of the intact Fc-cRGD dimer. The three major peaks were assigned to different glycoforms based on their MW.

A



B

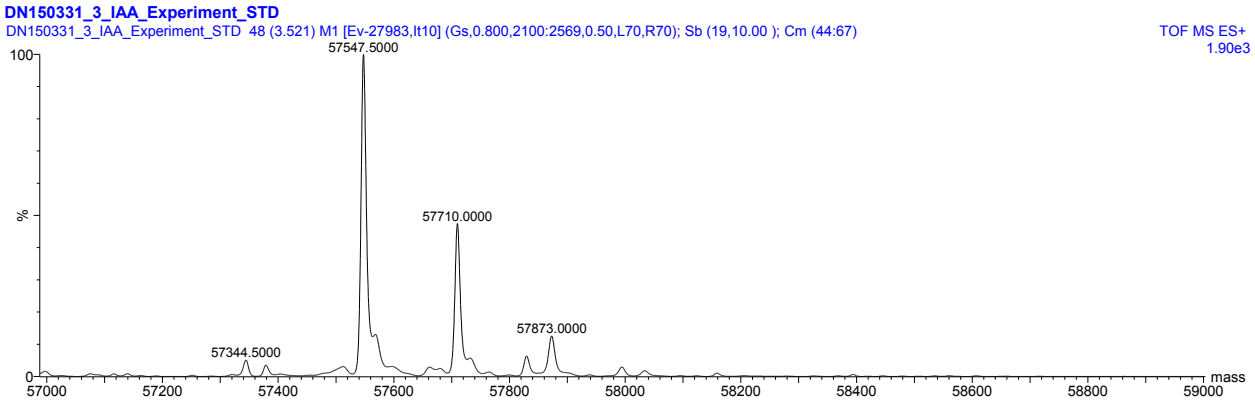


Figure S12: Detection of free Cys on the Fc-cRGD product by MS. The SEC purified Fc dimer was treated with 1 mM iodoacetic acid in 4 M guanidine for 20 minutes at 37 °C. The MS-data (panel A) is identical to the MS-data of the untreated material (panel B), demonstrating absence of free thiols in the conjugate.

References

1 A. V. Bryksin and I. Matsumura, *BioTechniques*, 2010, **48**, 463–465.
2 E. Wagner-Rousset, A. Bednarczyk, M.-C. Bussat, O. Colas, N. Corvaïa, C. Schaeffer, A. Van Dorsselaer and A. Beck, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2008, **872**, 23–37.
3 T. M. Dillon, P. V. Bondarenko, D. S. Rehder, G. D. Pipes, G. R. Kleemann and M. S. Ricci, *J Chromatogr A*, 2006, **1120**, 112–120.
4 M. Vila-Perelló, Z. Liu, N. H. Shah, J. A. Willis, J. Idoyaga and T. W. Muir, *J Am Chem Soc*, 2013, **135**, 286–292.
5 D. S. Rehder, T. M. Dillon, G. D. Pipes and P. V. Bondarenko, *J Chromatogr A*, 2006, **1102**, 164–175.
6 Z. Zhang, H. Pan and X. Chen, *Mass Spectrom Rev*, 2009, **28**, 147–176.