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Supporting Information

Reconstructing the Discontinuous and Conformational β1/β3-Loop Binding Site on hFSH/hCG by Using Highly Constrained Multicyclic Peptides

Linde E. J. Smeenk,[a] Drohpatie Timmers-Parohi,[b] Joris J. Benschop,[b] Wouter C. Puijk,[b] Henk Hiemstra,[a] Jan H. van Maarseveen,[a] and Peter Timmerman*[a, b]

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TABLE OF CONTENTS

EXPERIMENTAL

All chemical reagents were used as received. UPLC analysis was performed on a Waters Acquity Ultra Performance LC System, equipped with a Waters Acquity UPLC BEH130 C18 1.7 μm column. A linear gradient of 5-55% MeCN (0.05% TFA) in H_2O (0.05% TFA) was used. Preparative HPLC purification was performed on a Waters Prep LC System equipped with a Waters Delta-Pak C18 column (15 μm, 25x10 mm). Depending on the UPLC retention time, a gradient over 25 min of H_2O (0.05% TFA) and MeCN (0.05% TFA) was used, starting at 100:0 and going to a maximum of 70:30 at 40 mL/min with simultaneous UV detection at 215 nm. Photo induced reactions were carried out in a glass vial (diameter: 2 cm; wall thickness: 0.55 mm), located 10 cm away from the UV lamp (irradiation on sample: 365 nm). Mass spectra and accurate mass determinations were performed on a JEOL JMX SX/SX102A, coupled to a JEOL MSMP7000 data system.

Procedures for Peptide Synthesis

Automated peptide synthesis was performed following the standard Fmoc strategy on a 0.05 mmol scale. The first amino acid was coupled to a Rink Amide Resin (0.55 mmol/gr) or a Tentagel R RAM Resin (0.18 mmol/gr) by a double coupling procedure, using amino acid (4 equiv, 2 mL of a 100 mM solution), HBTU/HOBt (4 equiv, 1 mL of a 200 mM solution) and DIEA (4 equiv, 0.5 mL of a 800 mM solution) in 3.5 mL NMP by reacting 2 x 30 min. Subsequent amino acid couplings were performed using single amino acid couplings. Fmoc deprotection of the resin and after each amino acid coupling step was achieved by addition of 5 x 1 mL of 20% piperidine in NMP and reacting 5 x 2.5 min. Final peptide capping was achieved by reacting the peptide with 1 mL acetic anhydride for 20 min. Peptides were manually cleaved from the resin for 2 h using the reagent cocktail TFA/water/EDT/TIS 94/2.5/2.5/1 (10-25 mL/g resin). Post-cleavage work-up was done by ether precipitation using centrifugation. Peptides were washing 3x with cold ether, followed by freeze-drying and HPLC purification. See Table S1 for peptide sequences.

Synthesis of Mimic I-a, I-b, I-c, V-a and Control 1, 2

Sequence of chemistries (procedures described in the article):

i) Two separate CLIPS reactions (Table S2), ii) oxime ligation (Table S3), iii) Acm removal + full SH reduction iv) intramolecular SS formation (Table S4)

Synthesis of Control 3 and Control 4

Sequence of chemistries (as described in the article):

i) Two separate CLIPS reactions (only for Control **3**, Table S2), ii) Acm removal + full SH reduction ii) intermolecular SS formation* (Table S4)

** Procedure for intermolecular SS formation*

A solution of fully reduced β3 peptide (1 equiv) in H_2O (4/5 volume units) was added dropwise to a solution of sodium tetrathionate (100 equiv) in H_2O (1/5 volume units) to obtain a final solution of 1 mM. The reaction was stirred for 30 min at room temperature. Excess sodium tetrathionate was washed away using Sep-Pac purification of the complete reaction mixture. Finally, a solution of fully reduced β1 peptide (1.1 equiv) was added dropwise to the collected fractions from Sep-Pac purification containing the activated β3 peptide (1 equiv). The reaction was followed on HPLC-MS and freeze-dried after reversed phase HPLC purification.

TABLES

Table S1: Linear hFSH and hCG derived peptide lengths, sequences, and scaffolds used for cyclization in the CLIPS reaction.

Name/Length	Sequence	Scaffold
FSH-β1 8-mer	Ac-**C**EKEE**J**R**C**-NH_2	**oS2-ONH$_2$**
FSH-β1 8-mer	Ac-**C**EKEE**J**R**C**-NH_2	**oS2-CHO**
FSH-β1 8-mer	Ac-**C**EKEE**J**R**C**-NH_2	**oS2-OH**
FSH-β1 8-mer	Ac-**C**EKEE**A**R**C**-NH_2	**oS2-ONH$_2$**
FSH-β1 8-mer	Ac-**A**EKEE**C**R**A**-NH_2	-
FSH-β1 12-mer	Ac-**C**IEKEE**J**RFAI**C**-NH_2	**oS2-ONH$_2$**
FSH-β1 21-mer	Ac-**C**NITIAIEKEE**J**RFSISINT**C**$\mathbf{K_6}$-NH_2	**oS2-ONH$_2$**
FSH-β3 18-mer	Ac-**C**TVRVPG**J**AHHADSLYT**C**-NH_2	**oS2-ONH$_2$**
FSH-β3 18-mer	Ac-**C**TVRVPG**J**AHHADSLYT**C**-NH_2	**oS2-CHO**
FSH-β3 18-mer	Ac-**C**TVRVPG**J**AHHADSLYT**C**-NH_2	**oS2-OH**
FSH-β3 18-mer	Ac-**C**TVRVPG**A**AHHADSLYT**C**-NH_2	**oS2-CHO**
FSH-β3 24-mer	Ac-**C**VYETVRVPG**J**AHHADSLYTYPV**C**-NH_2	**oS2-CHO**
FSH-β3 24-mer	Ac-**J**VYET**C**RVPG**J**AHHADSL**C**TYPV**J**-NH_2	**oS2-CHO**
CG-β1 8-mer	Ac-**C**EKEG**J**P**C**-NH_2	**oS2-ONH$_2$**
CG-β1 8-mer	Ac-**C**EKEG**J**P**C**$\mathbf{K_6}$-NH_2	**oS2-ONH$_2$**
CG-β1 12-mer	Ac-**C**VEKEG**J**PVSI**C**-NH_2	**oS2-ONH$_2$**
CG-β1 20-mer	Ac-**C**ATLAVEKEG**J**PVSITVNT**C**$\mathbf{K_6}$-NH_2	**oS2-ONH$_2$**
CG-β3 18-mer	Ac-**C**SIRLPG**J**PRGVNPVVS**C**-NH_2	**oS2-CHO**
CG-β3 22-mer	Ac-**C**FESIRLPG**J**PRGVNPVVSYA**C**-NH_2	**oS2-CHO**

Amino Acid "J" stands for Cys(Acm)

Table S2: Products after CLIPS reaction. R_f-values (UPLC 5-55% MeCN in 2 min) and Molecular Weights of hFSH and hCG derived cyclized peptides.

Name	Mw calc	Mw found	R_f-value
FSH-β1 8-mer C--J--C ONH_2	1397.5	1397.68	0.35
FSH-β1 8-mer C--J--C CHO	1408.5	1408.38	0.40
FSH-β1 8-mer C--J--C OH	1343.5	1343.44	0.38
FSH-β1 8-mer C--A--C ONH_2	1294.2	1294.43	0.35
FSH-β1 12-mer C--J--C ONH_2	1842.1	1842.43	0.90
FSH-β1 21-mer C--J--C-K_6 ONH_2	3555.1	3555.34	1.02
FSH-β3 18-mer C--J--C ONH_2	2331.6	2331.58	0.76
FSH-β3 18-mer C--J--C CHO	2342.6	2342.72	0.81
FSH-β3 18-mer C--J--C OH	2277.6	2277.58	0.78
FSH-β3 18-mer C--A--C CHO	2239.3	2239.60	0.79
FSH-β3 24-mer C--J--C CHO	3093.4	3093.79	1.00
FSH-β3 24-mer J--C--J--C--J CHO	3179.7	3179.83	0.89
CG-β1 8-mer C--J--C ONH_2	1266.4	1266.10	0.44
CG-β1 8-mer C--J--C-K_6 ONH_2	2035.4	2035.12	0.32
CG-β1 12-mer C--J--C ONH_2	1664.9	1664.68	0.81
CG-β1 20-mer C--J--C-K_6 ONH_2	3205.8	3205.35	0.88
CG-β3 18-mer C--J--C CHO	2266.6	2266.53	1.02
CG-β3 22-mer C--J--C CHO	2777.1	2776.41	1.18

Amino Acid "J" stands for Cys(Acm)

Table S3: Detailed structural info for Peptide Mimics of hFSH and hCG

Name	β1-loop length	Simplified Sequence	Scaffold	β3-loop length	Simplified Sequence	Scaffold
hFSH I-a	8-mer	C—J—C	ONH_2	18-mer	C—J—C	CHO
hFSH I-b	8-mer	C—J—C	ONH_2	24-mer	C—J—C	CHO
hFSH I-c	12-mer	C—J—C	ONH_2	24-mer	C—J—C	CHO
hFSH Control 1	8-mer	C—J—C	CHO	18-mer	C—J—C	ONH_2
hFSH Control 2	8-mer	C—A—C	ONH_2	18-mer	C—A—C	CHO
hFSH Control 3	8-mer	C—J—C	OH	18-mer	C—J—C	OH
hFSH Control 4	8-mer	A—C—A		18-mer	A—C—A	
hFSH Control 5	-	-	-	18-mer	C—J—C	CHO
hFSH Control 6	-	-	-	24-mer	C—J—C	CHO
hFSH Control 7	8-mer	C—J—C	ONH_2	-	-	-
hFSH III-a	21-mer	C—J—C—K_6	ONH_2	24-mer	C—J—C	CHO
hFSH V-a	28-mer	C—J—C	ONH_2	24-mer	J—C—J—C—J	CHO
hCG II-a	8-mer	C—J—C	ONH_2	18-mer	C—J—C	CHO
hCG II-b	12-mer	C—J—C	ONH_2	18-mer	C—J—C	CHO
hCG II-c	8-mer	C—J—C—K_6	ONH_2	18-mer	C—J—C	CHO
hCG II-d	20-mer	C—J—C—K_6	ONH_2	22-mer	C—J—C	CHO
hCG Control 2	8-mer	C—A—C	ONH_2	18-mer	C—A—C	CHO
hCG Control 5	-	-	-	18-mer	C—J—C	CHO
hCG Control 7	8-mer	C—J—C	ONH_2	-	-	-

C = Cysteine introduced via Cys(trt); J = Cysteine introduced via Cys(Acm), A = Alanine, K_6 = $(Lysine)_6$

Table S4: Products after oxime formation. Yields, R_f-values (UPLC 5-55% MeCN in 2 min) and Molecular Weights of double-CLIPS compounds derived from the β1- and β3-loop of hFSH and hCG.

Name	**Mw calc**	**Mw found**	**R_f-value**	**Yield**
hFSH **I-a** (Acm)	3722.1	3721.72	0.77	69%
hFSH **I-b** (Acm)	4472.9	4473.92	0.90	68%
hFSH **I-c** (Acm)	4917.5	4918.23	1.05	59%
hFSH **III-a** (Acm)	6630.2	6630.86	1.13	62%
hFSH Control **1** (Acm)	3722.1	3722.54	0.77	87%
hFSH Control **2**	3515.5	3516.40	0.73	60%
hFSH **V-a** (Acm)	5003.8	5003.56	1.06	63%
hCG **II-a** (Acm)	3515.0	3515.91	0.96	54%
hCG **II-b** (Acm)	3913.5	3913.32	1.05	66%
hCG **IV-a** (Acm)	4284.0	4284.92	0.84	59%
hCG **IV-b** (Acm)	5964.9	5965.65	1.07	62%

Table S5: Products after disulfide bond formation. Yields, R_f-values (UPLC 5-55% MeCN in 2 min) and Molecular Weights of double-CLIPS compounds derived from the β1- and β3-loop of hFSH and hCG.

Name	Mw calc	Mw found	R_f-value	Yield
hFSH **I-a**	3578.1	3578.75	0.79	93%
hFSH **I-b**	4328.9	4329.22	0.89	99%
hFSH **I-c**	4773.5	4773.55	1.05	68%
hFSH **III-a**	6486.2	6485.67	1.18	76%
hFSH Control **1**	3578.1	3578.53	0.74	85%
hFSH Control **3**	3477.1	3477.38	0.72	52%
hFSH Control **4**	2884.8	2884.60	0.71	39%
hFSH **V-a**	4715.6	4716.55	1.01	72%
hCG **II-a**	3371.0	3370.91	0.90	86%
hCG **II-b**	3769.5	3770.44	1.00	85%
hCG **IV-a**	4140.0	4140.83	0.81	72%
hCG **IV-b**	5820.9	5821.33	1.12	58%

FIGURES

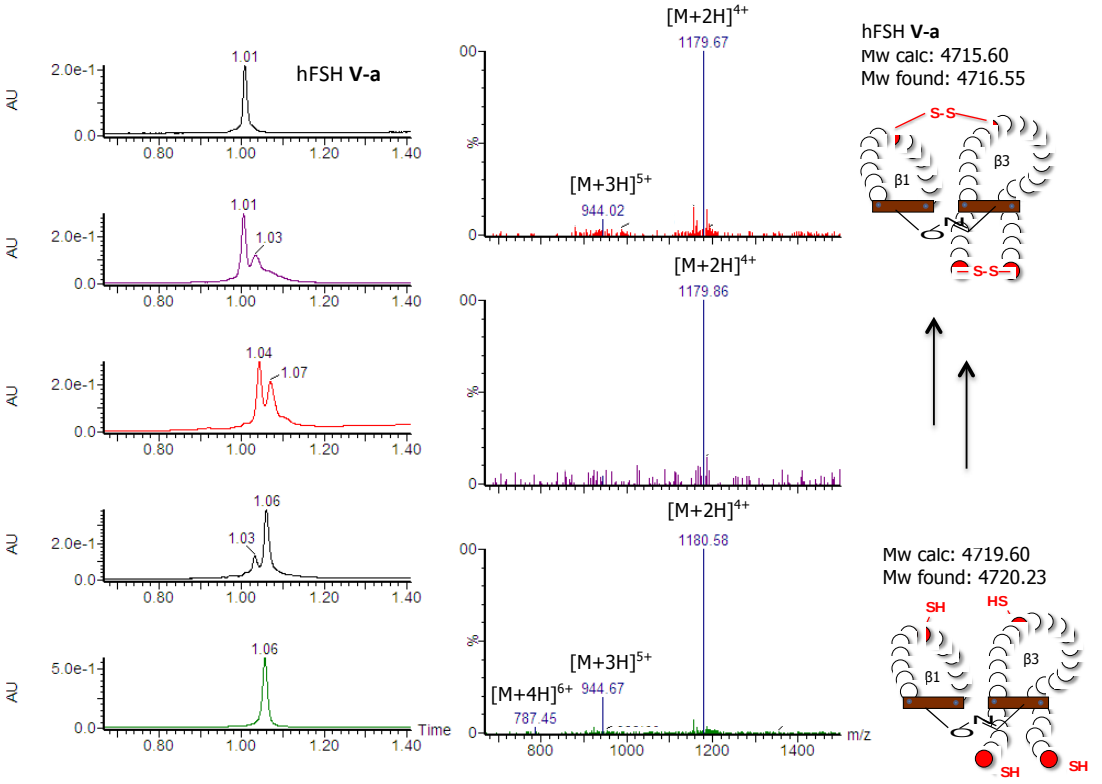
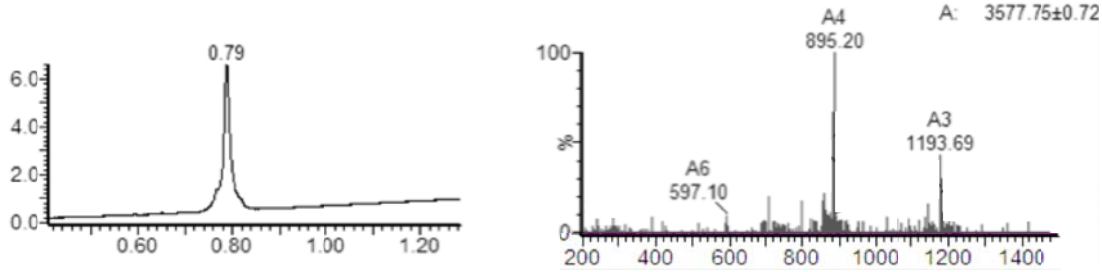


Figure S1: UPLC-MS chromatograms (5-55% MeCN in 2 min) showing the stepwise formation of two disulfide bonds in 3 days.

hFSH **I-a**



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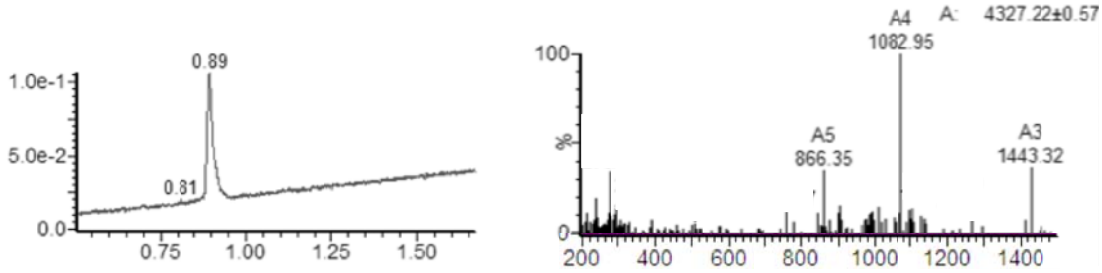
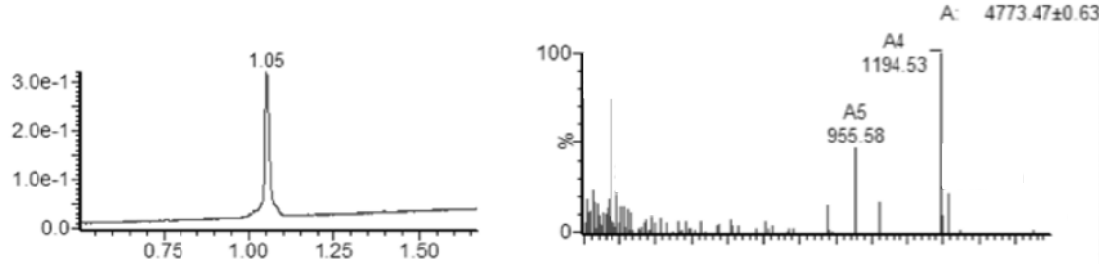
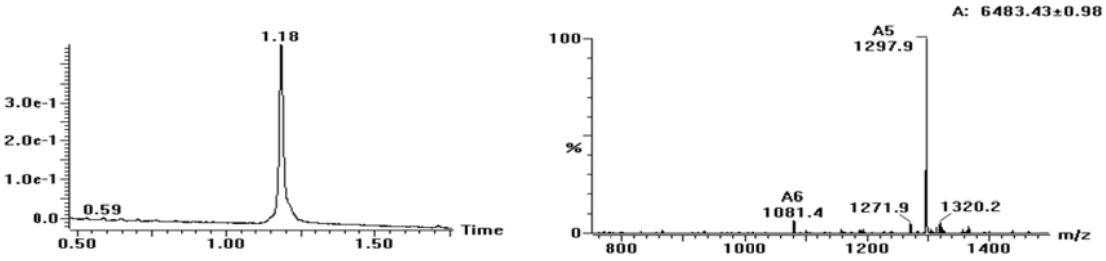


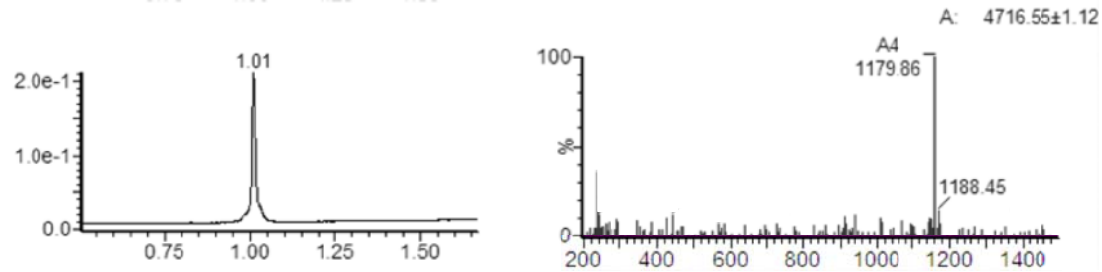
hFSH **I-c**



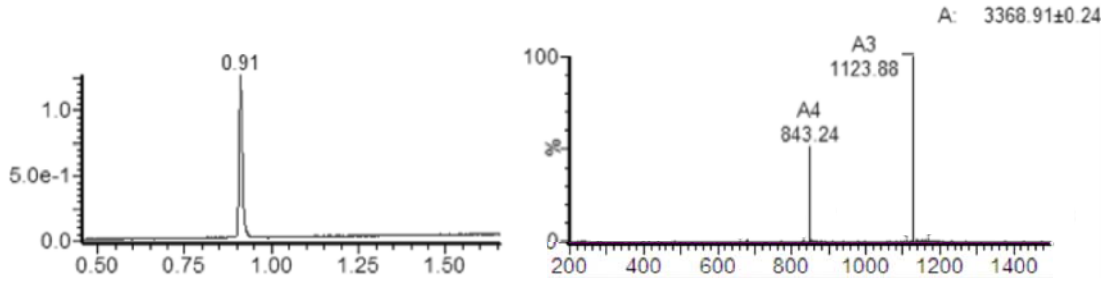
hFSH **III-a**



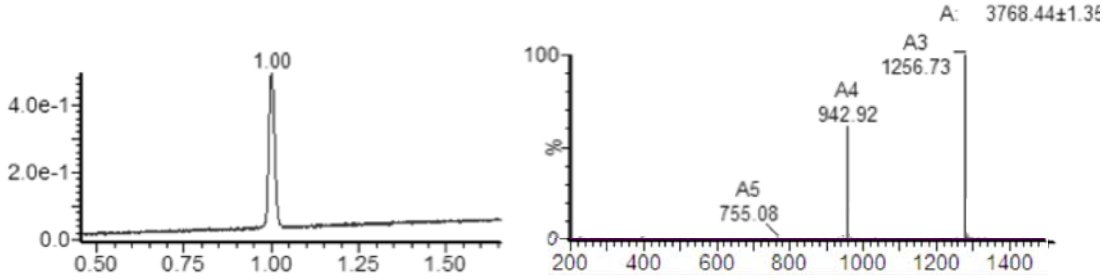
hFSH **V-a**



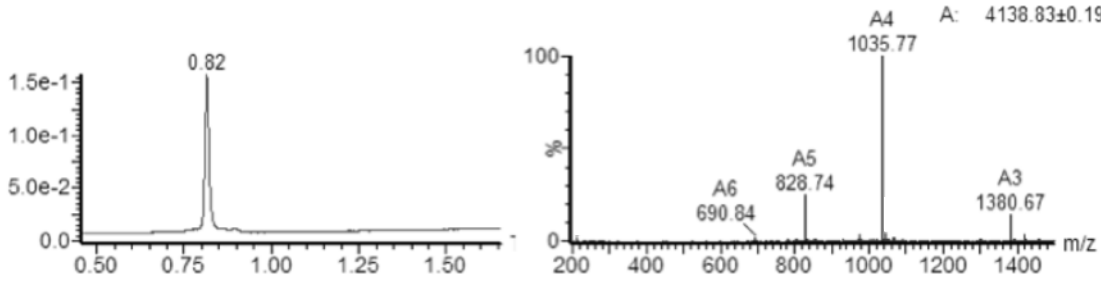
hCG **II-a**



hCG **II-b**



hCG **IV-a**



hCG **IV-b**

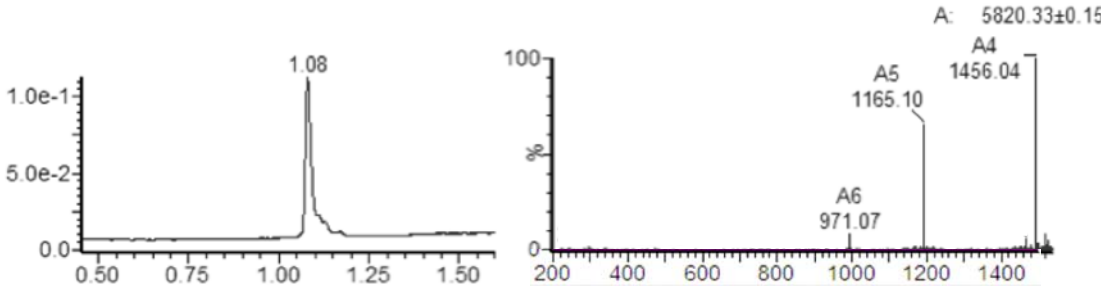


Figure S2: UPLC-MS chromatograms (5-55% MeCN in 2 min) of the final peptide mimics hFSH **I-a, I-b, I-c, III-a**, **V-a**, and hCG **II-a, II-b, IV-a, IV-b**

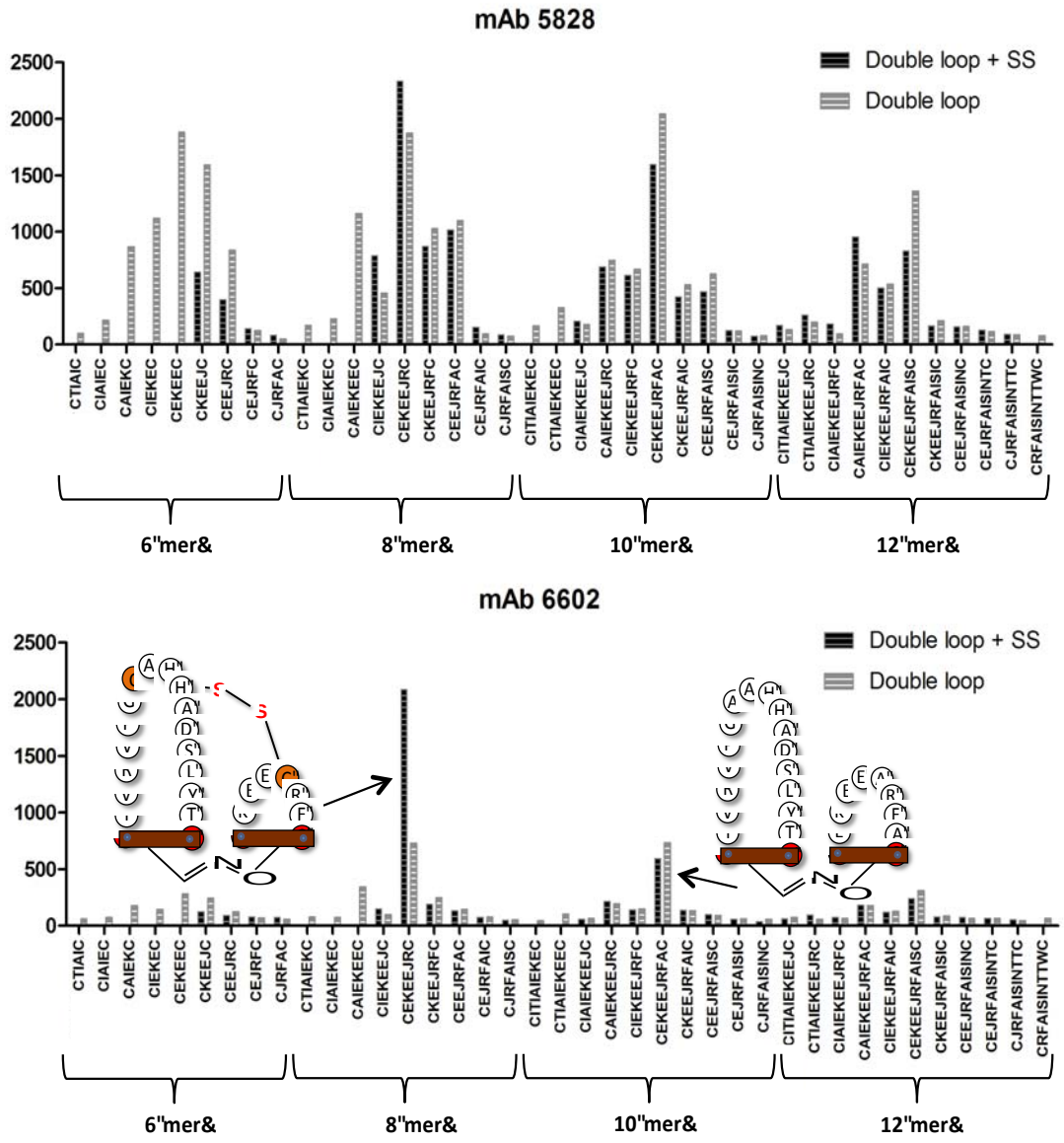


Figure S3: Optical density (OD) at λ=405 nm wavelength for binding to mAb 5828 and 6602 (100 ng/mL) for double-CLIPS constructs (with and without native disulfide bond) covalently attached to the surface via 6-mer, 8-mer, 10-mer and 12-mer β1-loop peptides.