Supplementary information

Branched BBB-shuttle peptides. Chemoselective modification of proteins to enhance blood-brain barrier transport

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MATERIALS AND METHODS

Solvents and reagents

Solvents and reagents were obtained from commercial suppliers such as Iris Biotech (Marktredwitz, Germany), Aldrich (Milwaukee, WI), GL Biochem Shangai Ltd. (Shangai, China) and Fluka Chemika (Buchs, Switzerland), and were of the highest purity commercially available. Protected amino acids were supplied by Iris Biotech. RinkAmide ChemMatrix® resin was purchased from PCAS BioMatrix Inc. (Quebec, Canada). Syringes, eppendorfs and falcons were acquired from Scharlau (Barcelona, Spain) and Deltalab.
Cell culture-treated plates and flasks were purchased from Corning Costar. Culture medium was acquired from Sigma Aldrich. Pierce™ iodination beads were obtained from Pierce. Desalting columns (PD MiniTrap and PD MidiTrap G-25) were obtained from GE-Healtcare.

Peptide synthesis and characterisation

Solid-phase peptide synthesis

Peptides were assembled using Fmoc/*t*Bu solid-phase peptide synthesis (SPPS), manually or with an automated microwave-assisted peptide synthesiser (Liberty Blue synthesiser, CEM). The peptides were synthesised in a 250-µmols scale on RinkAmide ChemMatrix® resin or RinkAmide AM resin. Manual synthesis was performed on polypropylene syringes with a polypropylene porous filter. The stirring was done manually with a Teflon stirring bar and reagents and solvents were eliminated under vacuum filtration.

Temporary protecting group removal

The Fmoc group was removed by treating the resin with a solution of 20% piperidine in DMF (1 x 1 min and 2 x 10 min) or with 10% (w/v) piperazine in 90:10 (NMP/EtOH) + 0.1 M HOBt (1:05 min, 90ºC).[1]

Coupling methods

Manual synthesis

Several coupling methods were used depending on the amino acids involved. Method 1 was used as default, while Method 2 was used for milder couplings and the incorporation of fluorophores.
Method 1 – HBTU/DIEA: 4 eq Fmoc-AA-OH, 4 eq HBTU, 8 eq DIEA, DMF (20-40 min).
Method 2 – DIC/Oxyma: 4 eq Fmoc-AA-OH, 4 eq DIC, 4 eq Oxyma, DMF (30-45 min).

Automated microwave-assisted peptide synthesiser – Liberty Blue

The protected amino acid (0.2 M in DMF), DIC (0.5 M in DMF), Oxyma (1 M in DMF) and HOBt (1M in DMF) were added to the reaction vessel. The mixture was allowed to react for 2 min with a microwave potency of 170 V (90ºC). The solvents were

removed by filtration, and the resin was washed with DMF (3 x 30 s). For His the maximum temperature was reduced to 50°C. The removal of the Fmoc group was performed with piperidine (20% in DMF) or piperazine (10% in NMP/EtOH [9/1]) for 1 min with a microwave potency of 170 V (90°C).

Monitoring of coupling and deprotection

The Kaiser colorimetric[2] test assay was used to detect primary amines bound to solid phase and the chloranil test for the detection of secondary amines.

Alloc deprotection

This group was removed using tetrakis(triphenylphosphine)palladium(0) (0.2 eq) and phenylsilane (10 eq) in DCM under argon atmosphere. Three treatments of 15 min were performed and the deprotection was assessed by the Kaiser test and mini-cleavage.

Mini-cleavage

Few beads of dried but DCM solvated resin were transferred into an eppendorf. The procedure was the same as for the cleavage described in the following section, using 95% of trifluoroacetic acid (TFA), 2.5% H_2O and 2.5% of triisopropylsilane (TIS), for 15-30 minutes. Then the peptide was washed with diethyl ether and dissolved in H_2O/ACN (1:1).

Cleavage from the resin/side-chain deprotection

After completion of the peptide chain, the resin was washed with DCM (5 x 30 s) and dried by suction for 15 min. The peptides were cleaved from the resin with concomitant removal of the side-chain protecting groups using the following cleavage cocktail: TFA, H_2O and TIS (95:2.5:2.5); incorporating EDT (2.5%) when Cys was present. After 2h of cleavage, the solvent was evaporated by applying a stream of N_2. The residue was washed 3 times by suspension in *tert*-butyl methyl ether and subsequent centrifugation. Finally, the cleaved peptides were dissolved in H_2O/MeCN (1:1) and lyophilised.

Peptide purification

Peptides were purified on a Waters system with ChromScope software, a 2707 Autosampler, a Prep Degasser, a 2545 binary gradient module, a 2489 UV/Visible Detector, and a Fraction Collector III. A XBridge C_{18} column (150 x 19 mm x 5 µm, Waters) or an Aeris Peptide XB C_{18} column (250 x 21.1 mm x 5 µm, Phenomenex) was used, with MeCN (0.1% TFA) and H_2O (0.1% TFA) as solvents and a flow rate of 15-20 mL/min.

Purity assessment

UPLC-PDA

UPLC chromatograms were obtained on an Acquity high class (PDA detector, sample manager FNT and Quaternary solvent manager), using an Acquity BEH C_{18} (50 x 2.1 mm x 1.7 µm) column. The flow rate was 0.61 mL/min and MeCN (0.036% TFA) and H_2O (0.045% TFA) were used as solvents. 2-min linear gradients were used in all cases. Detection was performed at 220 nm.

Characterisation of peptides

UPLC-MS

Chromatograms and spectra were recorded on a Waters high class (PDA detector, sample manager FNT and Quaternary solvent manager) coupled to an electrospray ion source ESI-MS Micromass ZQ and using the MassLynx 4.1 software (Waters, Milford, MA), using a BEH C_{18} column (50 x 2.1 mm x 1.7 µm, Waters). The flow rate was 0.6 mL/min, and MeCN (0.07% formic acid) and H_2O (0.1% formic acid) were used as solvents. Samples were analysed with positive ionisation: the ion spay voltage was 30 V and the capillary temperature was 1 kV.

LTQ-FT MS

A high-resolution mass spectrometer was used to determine the exact mass of the peptides. The samples were diluted in H_2O/MeCN (1:1) with 1% formic acid and analysed with an LTQ-FT Ultra (Thermo Scientific). They were introduced by automated nanoelectrospray. A NanoMate (Advion BioSciences, Ithaca, NY) infused the samples through the nanoESI Chip (which consisted of 400 nozzles in a 20 x 20 array). The spray voltage was 1.70 kV, and the delivery pressure was 0.5 psi. MS conditions were as follows: Nano-ESI, positive ionisation, capillary temperature 200°C, tube lens 100 V, ion spray voltage 2 kV, and *m/z* 200–2000 a.m.u.

Amino acid analysis

Amino acid analysis was performed to assess the amino acids present and the amount obtained for each peptide. For this purpose, ion exchange chromatographic analysis after acid hydrolysis was performed. The samples were hydrolysed with 6 M HCl at 110ºC for 16 h. They were then evaporated to dryness at reduced pressure and dissolved in 20 mM of aqueous HCl. Finally, the amino acids were derivatised using the AccQ Tag protocol from Waters, which uses 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as a derivatisation reagent, and analysed by ion exchange HPLC.

Protein conjugation and characterisation

Expression of GFP

20 μL of *E. coli* B834 cells were transfected by heat shock with pOPINF plasmid carrying the gene for the desired protein and plated onto carbenicilin containing plates (50 μg/mL). After an overnight incubation, one single colony was used to inoculate starter culture of LB media (10 mL), containing carbenicilin (50 μg/mL). The starter culture was grown overnight and then used to inoculate autoinduction media (1L), containing carbenicilin (50 μg/mL). The cells were incubated at 37°C for 6 h, and grown overnight at 25ºC. The cells were then harvested by centrifugation (JLA 9.10 rotor, 10 min, 8000 rpm, 4°C), were collected and stored at -20ºC.

The cell pellet was thawed in binding buffer (50 mM NaPi, 15 mM imidazole, 300 mM NaCl, pH 8 and 10% glycerol, 10 mL). Then, they were incubated on ice with stirring for 1 h with additional protease inhibitor cocktail (EDTA free), DNase and lysozyme. The cell suspension was then sonicated (Recall 9 function, 30 s on, 60 s off, 5 times). The resulting cell debris was removed from the lysate by centrifugation (JA 25.5 rotor, 30 min, 20000 rpm) and the lysate purified by FPLC using a HisTrap HP column (5 mL). The resin was washed with binding buffer (5 column volumes (CV)) and the protein then eluted with elution buffer (50 mM NaPi, 500 mM imidazole, 300 mM NaCl, pH 8 and 10% glycerol, 3 CV) and the fractions collected analysed by SDS-PAGE. Fractions containing protein were combined and the imidazole removed using a PD MidiTrap column (GE healthcare) following manufacturer instructions. The resulting protein solution was analysed by LCT-MS and stored at -20°C until used.

GFP sequence:

AHHHHHHSSG GLCTPSRMVS KGEELFTGVV PILVELDGDV NGHKFSVSGE GEGDATYGKL TLKFICTTGK
LPVPWPTLVT TLTYGVQCFS RYPDHMKQHD FFKSAMPEGY VQERTIFFKD DGNYKTRAEV KFEGDTLVNR
IELKGIDFKE DGNILGHKLE YNYNSHNVYI MADKQKNGIK VNFKIRHNIE DGSVQLADHY QQNTPIGDGP
VLLPDNHYLS TQSALSKDPN EKRDHMVLLE FVTAAGITLG MDELYK

GFP modification

Thioether formation

GFP (2 mg/mL) was reduced by adding TCEP (1.1 eq) in NaPi (50 mM, pH 8, 150 mM NaCl, 1 mM EDTA) for 1 h at 37ºC. The maleimido-peptide (1.1 eq) was added directly and left to react for 1-2 h at RT. The resulting protein was purified by SEC (PD MiniTrap or PD MidiTrap, GE Healthcare, following manufacturer´s instructions) and stored at 4ºC in NaPi buffer with 10% glycerol.

Disulphide formation

GFP (1 mg/mL) was reduced by adding TCEP (1.1 eq) in sodium borate buffer (25 mM, pH 8, 25 mM NaCl, 1 mM EDTA, pH 8) for 1 h at 37ºC. The excess of reagent was removed using a SEC column. Then, Ellman's reagent (1.1 eq) was added for 15-30 min at RT and the Cys-peptides (1.1 eq) were added directly for 1-2 h. Finally, the conjugates were purified by SEC and stored at 4ºC in NaPi buffer with 10% glycerol.

S-alkylation with iodoacetamide

GFP (1 mg/mL) was reduced by adding TCEP (1.1 eq) in sodium borate buffer (25 mM, pH 8, 25 mM NaCl, 1 mM EDTA, pH 8) for 1 h at 37ºC. The excess of reagent was removed using a SEC column. Then, iodoacetamide (30 eq, 0.1 M) was added for 1 h at RT. Finally, the modified protein was purified by SEC and stored at 4ºC in NaPi buffer with 10% glycerol.

Modification of GFP in a random fashion

First, the solvent exposed lysines of GFP were activated as thiols by addition of different amounts of 2-iminothiolane (2IT). Then, 3 different strategies were attempted:

- Activation of thiol groups by reaction with Ellman´s followed by addition of cysteine modified THRre (THRre_1c) (strategy A)
- Activation of thiol groups followed by addition of activated cysteine modified THRre (THRre_1c) (strategy B)
- Addition of maleimide modified peptide (THRre_1m) (strategy C)

In all the cases, the protein precipitated upon addition of the peptide. Only when little modification occurred (average of 1.1 peptide/protein) the protein was stable in solution.

SDS-PAGE

SDS-PAGE electrophoresis was carried out using BioRad system (Miniprotean cell) in a 15% Tris gel using the following running buffer: 1.92 M Gly, 0.25 M Tris, 1% SDS, pH 8.3-8.8. The gel run at 140 V until the marker reached the bottom of the gel. Protein molecular weights were approximated by comparison to a protein marker (Perfect Protein Markers 15-150 kDa from Novagen). Proteins were visualised by coomassie staining (staining solution: 10% AcOH, 0.25 g brilliant blue; discoloration solution: 20% MeOH, 3% AcOH in water). Image Studio Software was used for densitometric analysis.

LC-MS

The characterisation of the protein conjugates was performed using LCT-Premier (Waters). Samples were injected automatically to a BioSuite pPhenyl 1000 (Waters, 10 μm RPC 2.0x75mm) column at a flow rate of 100 μL/min using an Acquity UPLC System (Waters). Intact protein was eluted using a linear gradient from 5% to 80% B in 60 min (A = 0.1% formic acid in H_2O, B = 0.1% formic acid in MeCN). The column outlet was directly introduced into the electrospray ionisation (ESI) source of an LCT-Premier XE mass spectrometer (TOF) (Waters). Capillary and cone voltage were set to 3000 V and 100 V, respectively. Desolvation and source temperature were set to 350°C and 120°C. Cone gas and desolvation gas flow were set to 50 and 600 L/h. The mass spectrometer acquired full MS scans (400-4000 m/z) working in positive polarity mode. Data was acquired with MassLynx software.

Stability of protein constructs in the presence of GSH

To a solution of GFP-THRre_1c or GFP-THRre_1m (20 μL, 10 μM, PBS), spiked with 1 μL of GFP(IAM) (50 μM, PBS), a 23-fold solution of GSH (230, 23 or 0.046 mM, 1 μL) was added. The incubation was performed at 37°C. At different time points, the protein was analysed by UPLC-MS. Quantification was done using GFP(IAM)/GFP-THRre_1c or GFP-THRre_1m ratio.

^{125}I protein labelling and quantification

Pierce™ Iodination Beads (Life Technologies) were used to radiolabel the protein conjugates. Briefly, two beads per protein were washed with 500 μL of reaction buffer (50 mM NaPi, pH 6.5) and dried on filter paper. In a glass vial, the beads were added with the sufficient amount of carrier-free Na^{125}I (1 mCi/mg protein) in 200 μL of reaction buffer. The reaction was incubated for 5 min. The proteins were then added and the reaction was carried out for 15 min with occasional mixing. The reaction was stopped by removing the solution from the reaction vessel and adding it to a PD MiniTrap G-25 column (GE Healtcare) previously equilibrated with PBS. The iodinated protein was dialysed (Slide-A-Lyzer® minidialysis devices, 20 K, 0.5 mL) overnight against PBS to further remove the unincorporated ^{125}I. The radioactivity of 10-μL fractions was measured for 2 min using a Packard Cobra II Gamma Counter, and the protein concentration was determined using BCA analysis (Thermo Scientific).

Cell-based assays

Internalisation experiments

bEnd.3 cells were cultured in DMEM complete medium (glucose 4.5 g/L, 2 mM glutamine, 10% FBS and 0.5% Pen/Strep) (Sigma). Medium was changed 3 times per week and cells were detached using 0.05% trypsin/EDTA.

Analysis using flow cytometry

Two days before the experiment, 100000 bEnd.3 cells/well were seeded in 24-well plates. On the day of the assay, the cells were washed with Ringer HEPES (RH) for 15 min and CF-labelled peptides were added to reach a final concentration of 25 μM. After 30 min of incubation at 37°C, the cells were washed 5 times with Gly buffer (pH 3) at 4°C, trypsinised and kept on ice.

Cells were immediately analysed using a Gallios Beckman Coulter flow cytometer with a 488nm laser. The samples were analysed in triplicates. These results were obtained by dissolving THRre_2f with the buffer at pH 4.5 instead of at 7.4 as this fluorophore-labelled peptide is insoluble at higher pHs.

Analysis using radioactivity

The same methodology as before was used adding ^{125}I-labelled GFP conjugate at a concentration of 100 nM. The amount of protein internalised was measured using a Packard Cobra II Gamma Counter and the total amount of proteins was determined using the BCA kit (Thermo Scientific). These experiments were repeated 3 independent times with 2-3 replicates each time. The protein uptake was calculated using the following formula:

$$Protein\ uptake = \frac{amount\ of\ labeled\ protein\ (fmol)}{total\ amount\ of\ protein\ in\ cells\ (mg)}$$

Analysis using confocal microscopy

For confocal microscopy experiments, the cells (5000 cells/dish) were seeded on collagen precoated MatTek glass-bottom culture dishes (MatTek Corporation, Ashland, MA) and incubated for 48 h at 37°C. They were given a complete media change before being treated with the labelled peptide in reduced 1% serum media. After 30 min, cells were washed 3 times with 1 mL of media and immediately imaged.

Fluorescence was detected on a Zeiss LSM 780 laser scanning confocal microscope equipped with temperature-controlled environmental chamber and CO_2 for live-cell imaging. Images were taken with a Zeiss Plan-Apochromat 63x/1.40 oil DIC M27 objective. Carboxyfluorescein and Hoechst were excited with an Ar laser at 488 and 405 nm, and emission was recorded at 546 and 458 nm, respectively. Pinholes were set to equate optical slice thicknesses. The images were processed using the ImageJ 1.49b software.

Permeability assays in the *in vitro* human BBB cellular model

These experiments were performed using the model developed in Prof. R. Cecchelli's laboratory.[3] In brief, endothelial cells derived from pluripotent stem cells and bovine pericytes were defrosted in gelatin-coated Petri dishes (Corning). Pericytes were cultured in DMEM pH 6.8 while endothelial cells were cultured in supplemented endothelial cell growth medium (sECM) (Sciencells). After 48 h, endothelial cells were seeded in 12-well Transwell inserts (8000 cell/well) and pericytes were plated in 12-well plates (50000 cells/well) previously coated with Matrigel and gelatin, respectively. sECM medium was used for both cell lines and changed every 2-3 days. The assays were performed 7-8 days after seeding by placing the endothelial cells into new wells without pericytes.

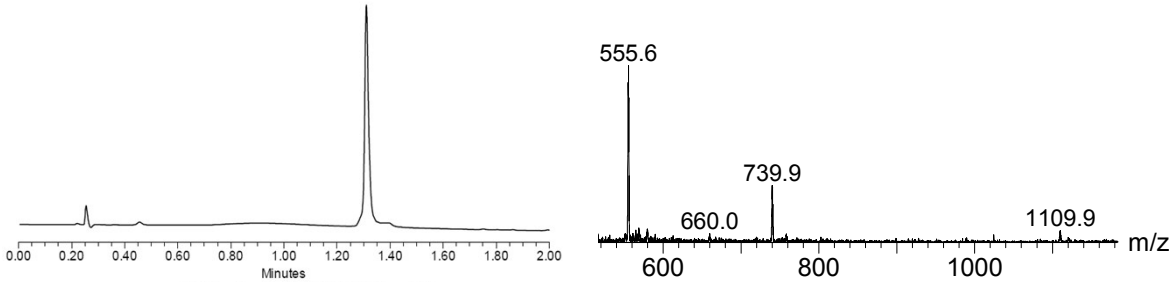
To perform the assay, 500 μL of the ^{125}I labelled protein (100 nM) in Ringer HEPES was added to the donor compartment and 1500 μL of Ringer HEPES was introduced into the acceptor compartment. Lucifer Yellow (50 μM) was added as a control of barrier integrity (Papp < $15 \cdot 10^{-6}$ cm/s). The plates were incubated for 2 h at 37°C, and the solutions from both compartments were recovered and analysed. The samples were evaluated in triplicates. The amount of protein was quantified using a gamma counter and the apparent permeability using the following formula:

$$Papp = \frac{Q_A(t) \cdot V_D}{t \cdot A \cdot Q_D(t_0)}$$

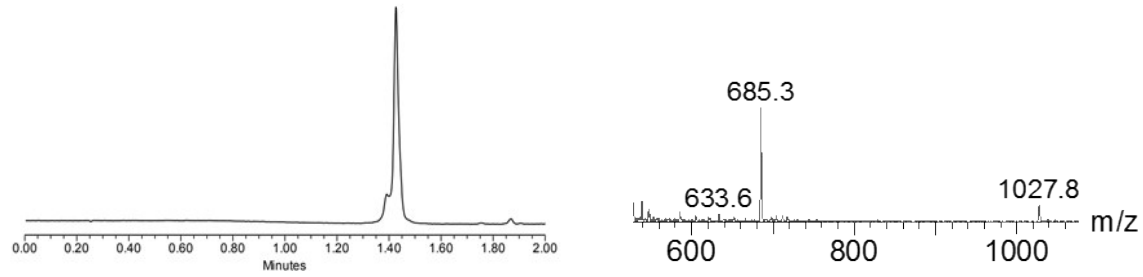
where P_{app} is obtained in cm/s, $Q_A(t)$ is the amount of compound at the time t in the acceptor well, V_D is the volume in the donor well, t is time in seconds, A is the area of the membrane in cm and $Q_D(t_0)$ is the amount of compound in the donor compartment at the beginning of the experiment.

SUPPLEMENTARY FIGURES AND TABLES

THRre_1f MW = 2218.5



THRre_1m MW = 2053.4



THRre_1c MW = 1849.2

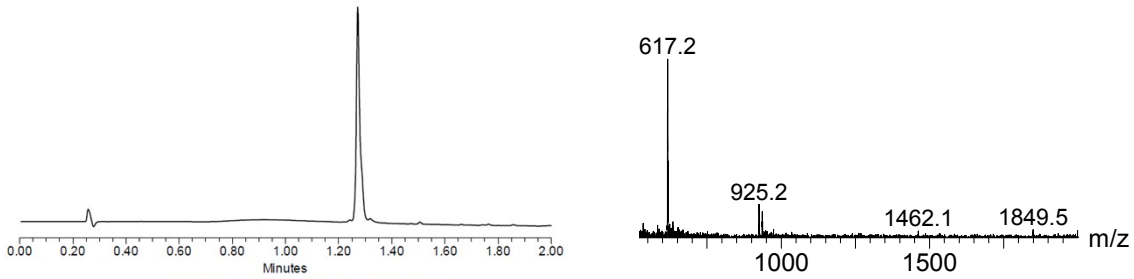
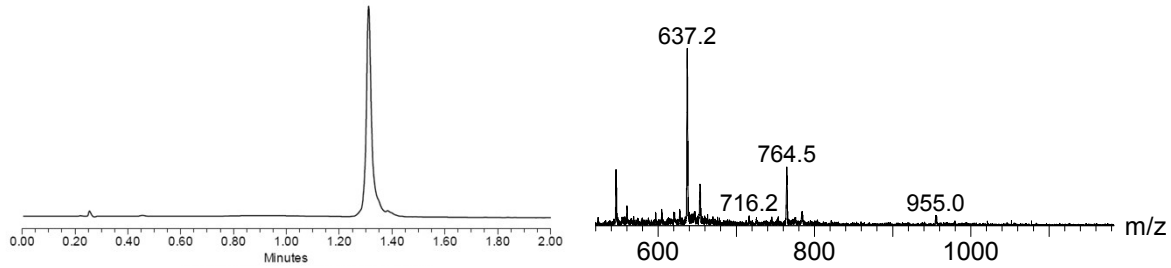


Fig. S1 (continued in the following page) UPLC traces and MS spectra of all peptides described in this paper. UPLC chromatograms are recorded at 220 nm in a 2-min linear gradient from 0 to 100% of MeCN (0.036% TFA) in H_2O (0.045%TFA). The MW of each peptides is shown above the mass spectra.

THRre_2f

MW = 3819.4



THRre_2m

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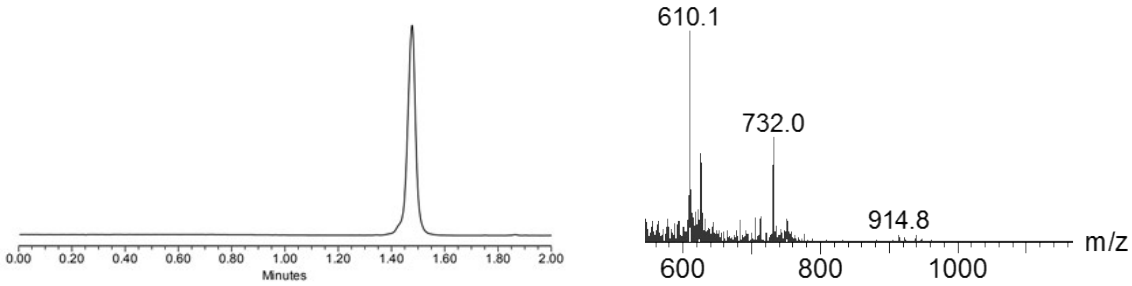
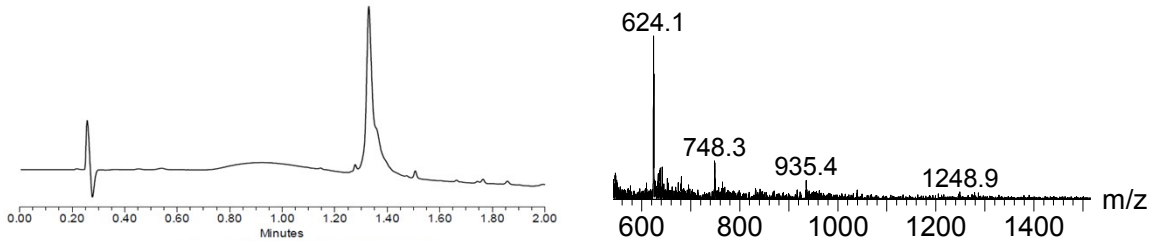


THRre_2c

MW = 3740.4



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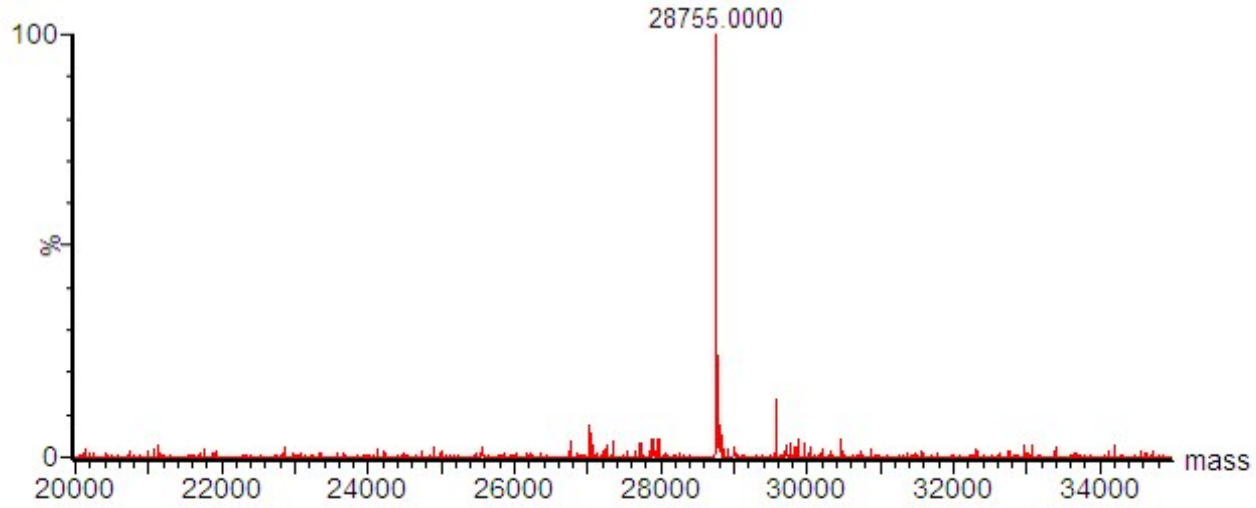
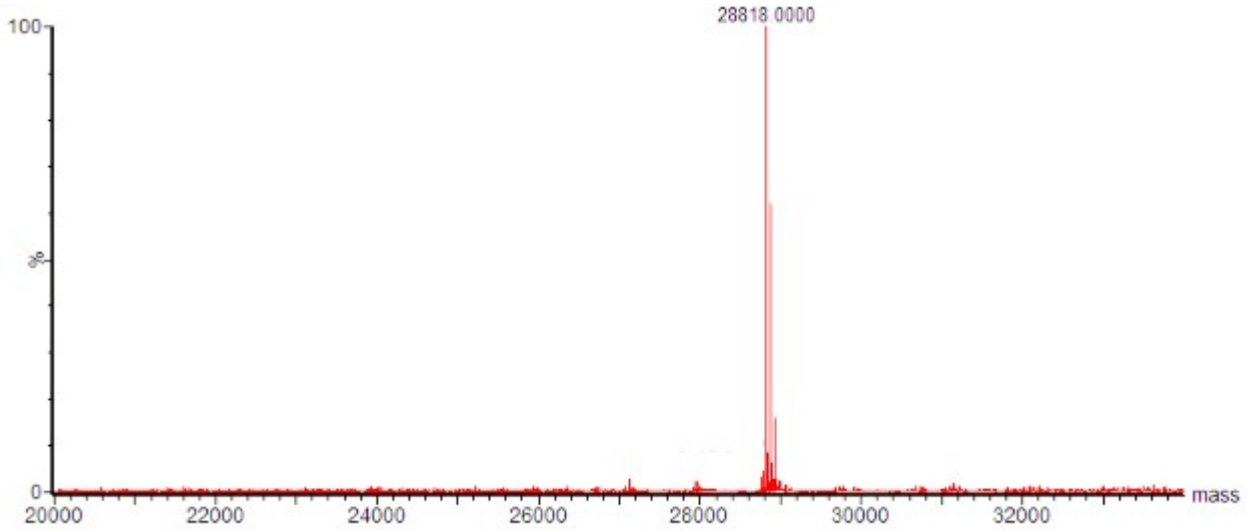
GFP



GFP(IAM)



GFP-THRre_1m

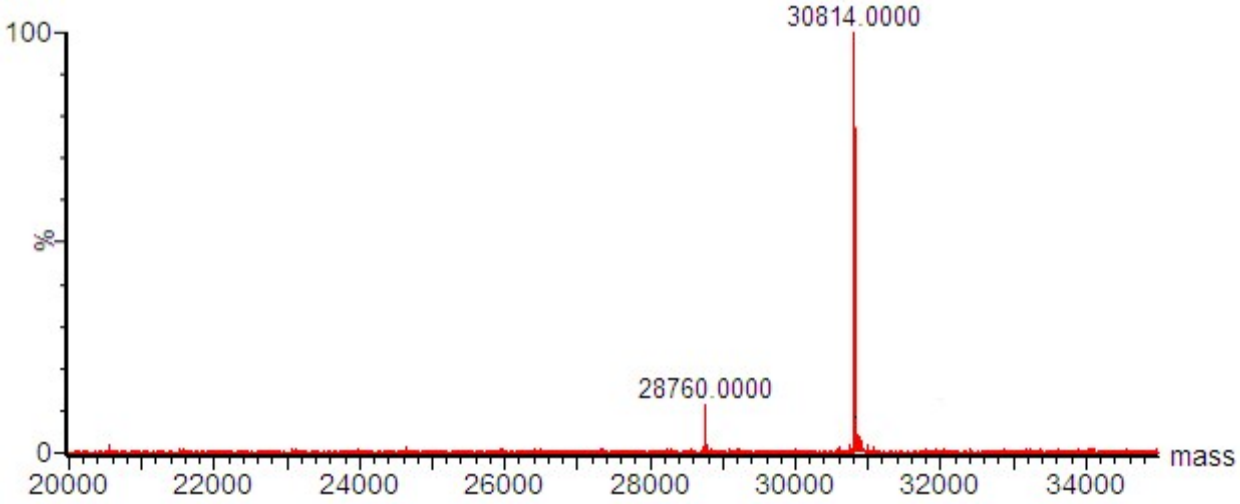
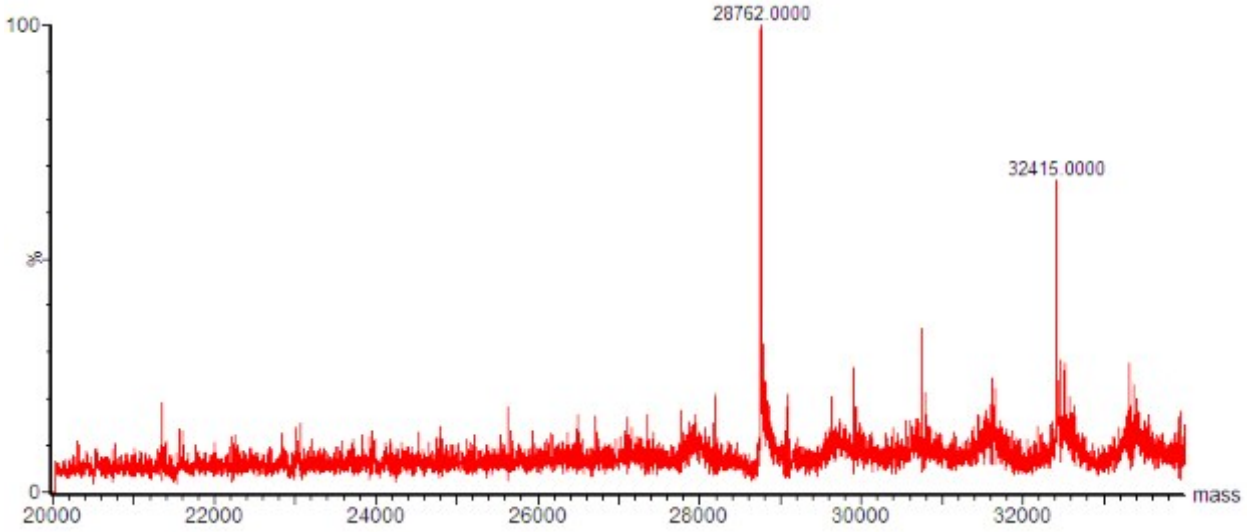
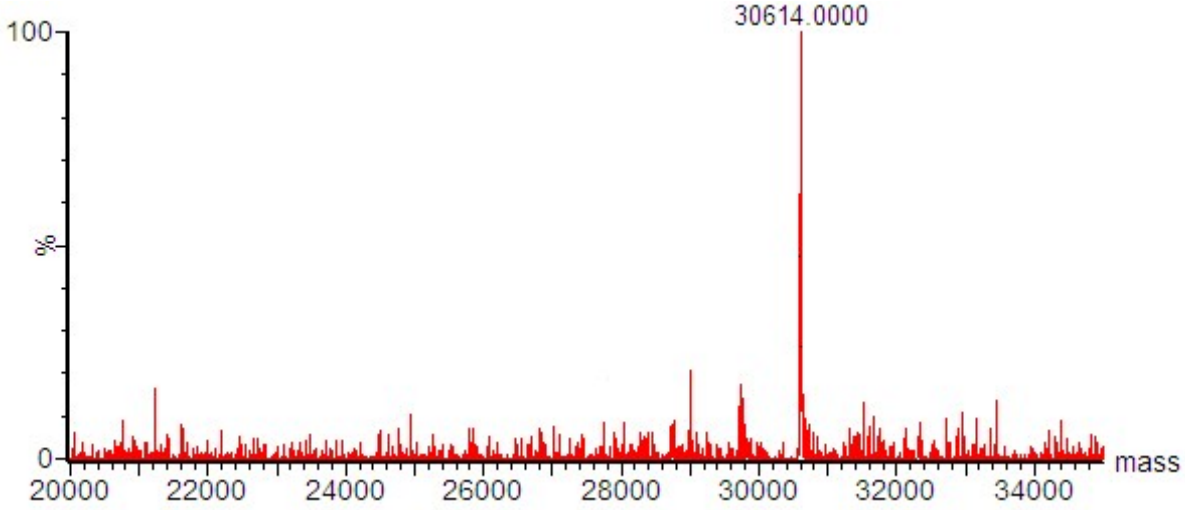


Fig. S2 (continued in the following page) Mass characterisation of the proteins by LCT-Premier or UPLC-MS. The deconvoluted spectra are shown. GPF(IAM) is GFP reacted with iodoacetamide to alkylate the sulfhydryl group of Cys.

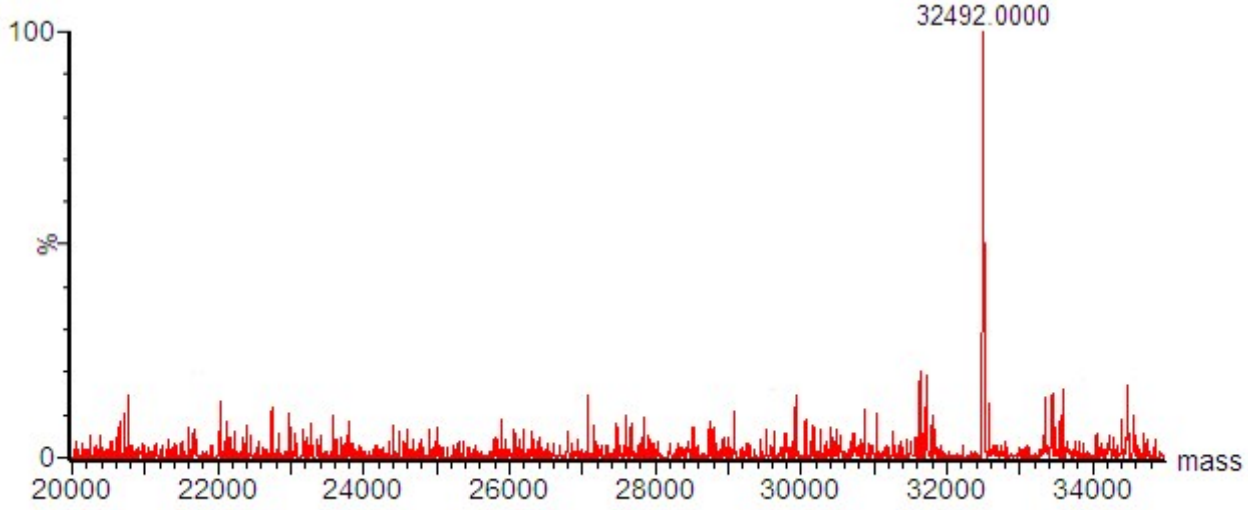
GFP-THRre_2m



GFP-THRre_1c



GFP-THRre_2c



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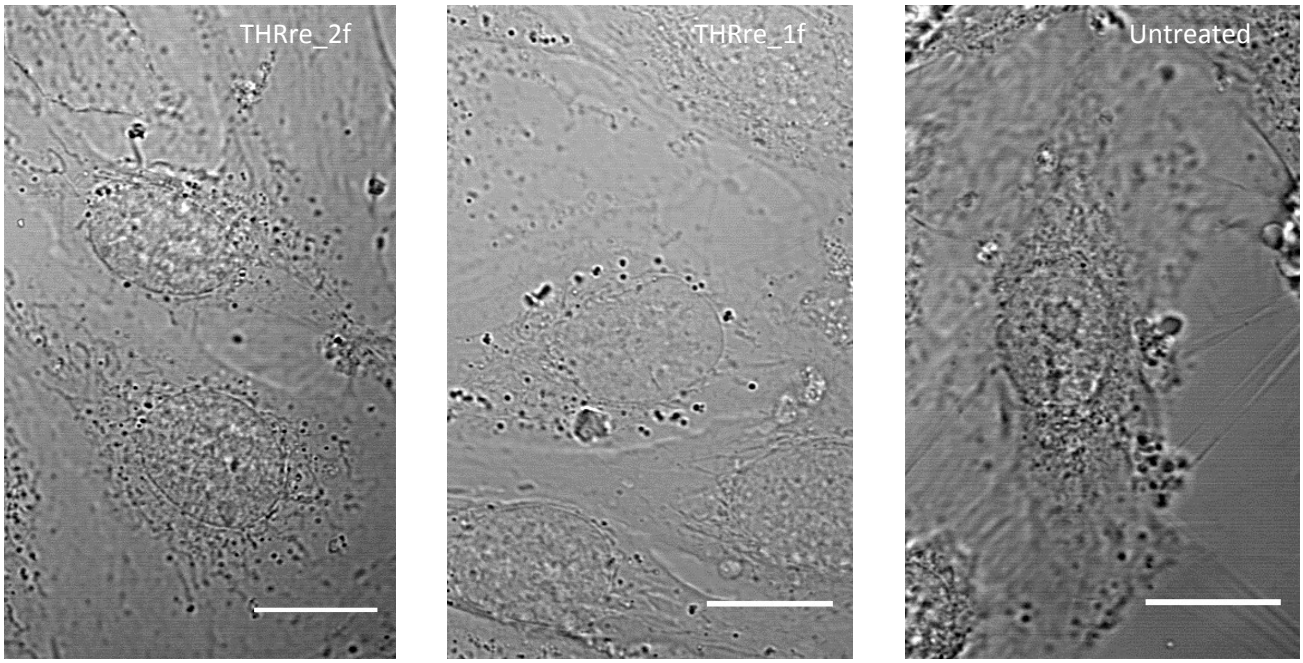

Fig. S3 Bright field images of bEnd.3 cells after 30 min of incubation with THRre_2f, THRre_1f or without peptide (at 37ºC in RH pH 4.5). The scale bar represents 15 µm.

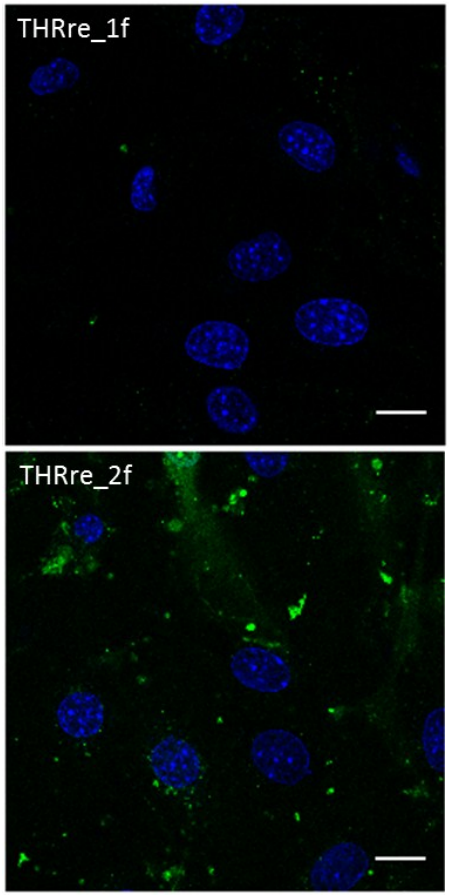

Fig. S4 Confocal fluorescent microscopy images of bEnd.3 cells after 30 min of incubation with THRre_1f or THRre_2f. CF-labeled peptides are shown in green and cell nuclei (Hoechst staining) in blue. The scale bar represents 15 µm.

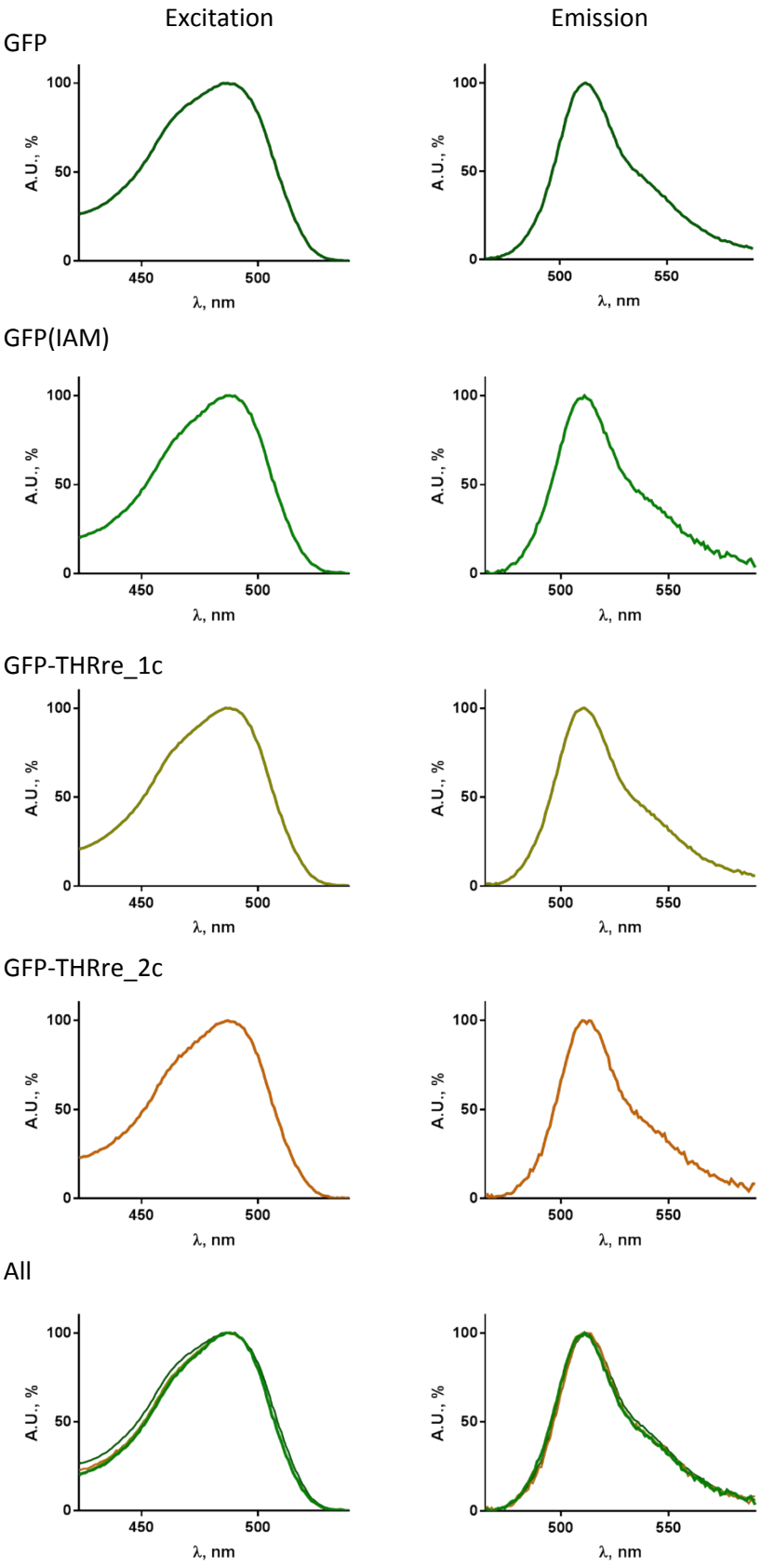

Fig. S5 Excitation and emission spectra of four GFP constructs, relative to the maximum value of each spectra. All four proteins present the same profile, indicating that the protein is well folded and maintains its activity. The excitation maximum is at 586-588 nm and the emission maximum is at 510-512 nm. A.U. = arbitrary units.

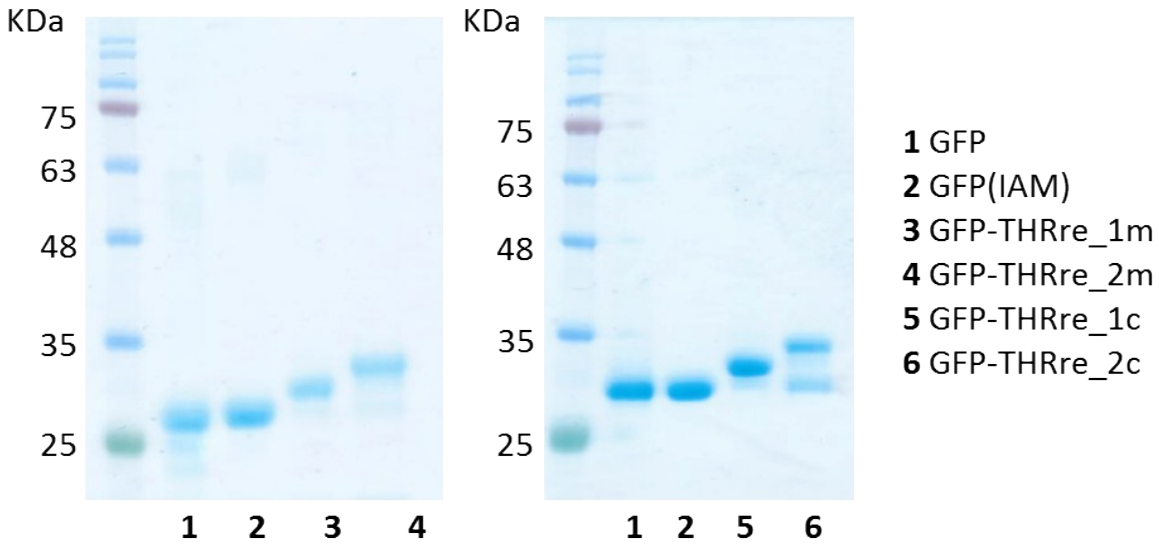

Fig. S6 Coomassie-stained SDS-PAGE for the GFP conjugates with the BBB-shuttle peptides. GPF(IAM) is GFP reacted with iodoacetamide to alkylate the sulfhydryl group of Cys.

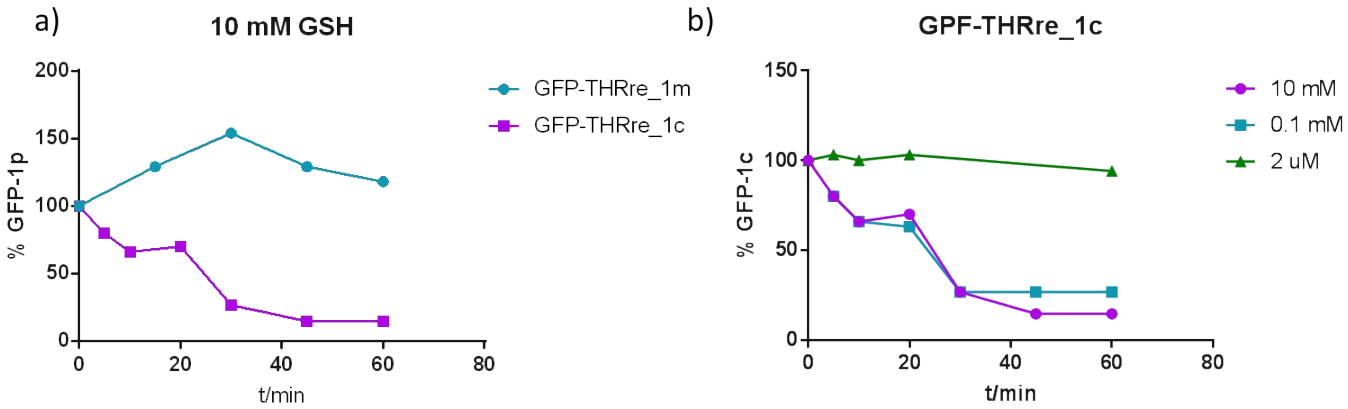

Fig S7. Stability of GFP conjugates in GSH. a) Incubation of GFP-THRre_1c and GFP-THRre_1m with 10 mM of GSH; b) Incubation of GFP-THRre_1c with 10, 0.1 and 0.0002 mM of GSH.

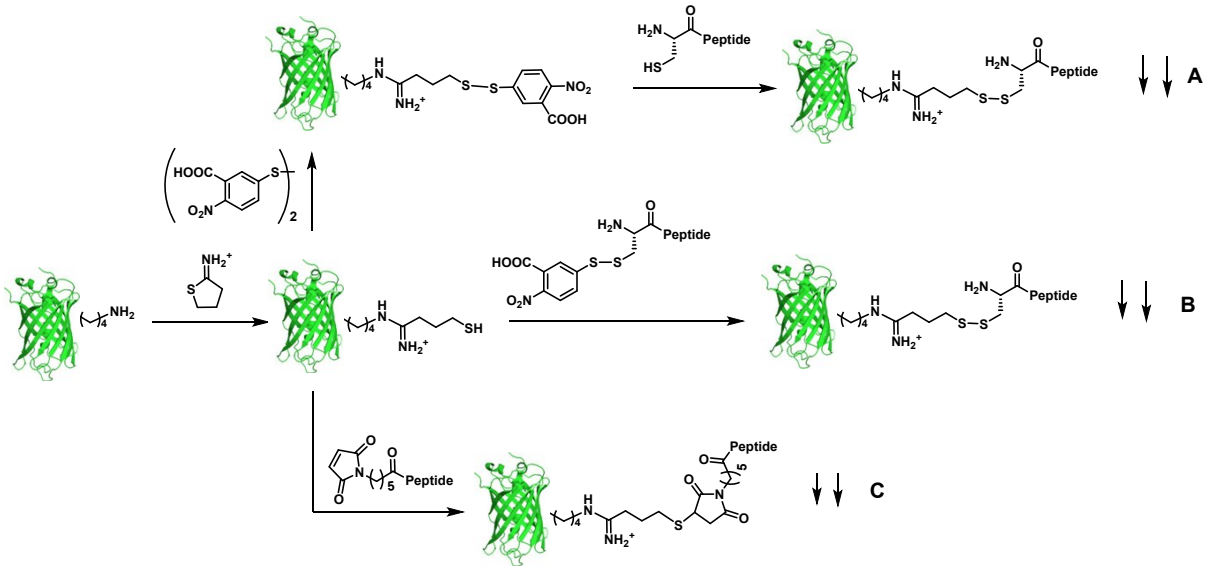

Fig. S8 Strategies to modify GFP with two copies of the monovalent THRre (THRre_1p). The protein was funtionalised with 2- iminothiolane followed by A) activation with Ellman´s and addition of THRre_1c; B) addition of Ellman´s activated THRre_1c; C) addition of THRre_1m.

Peptide	Sequence	Molecular formula	Calc. Mw, Da	Found Mw, Da	t_R UPLC, min	Purity, %	Yield, %
THRre_1f	pwvpswmpprhtKGK(CF)G	$C_{108}H_{140}N_{26}O_{24}S$	2217.01994	2217.02545	1.31	>97	25
THRre_1m	pwvpswmpprhtKGK(Mal)G	$C_{97}H_{141}N_{27}O_{21}S$	2052.04646	2052.05160	1.43	>95	18
THRre_1c	pwvpswmpprhtkkc	$C_{86}H_{129}N_{25}O_{17}S_2$	1847.94397	1847.94371	1.27	>98	32
THRre_2f	$(pwvpswmpprht)_2$KKGK(CF)G	$C_{185}H_{249}N_{47}O_{39}S_2$	3816.83387	3816.83872	1.31	>95	10
THRre_2m	$(pwvpswmpprht)_2$KKGK(Mal)G	$C_{174}H_{251}N_{48}O_{36}S_2$	3652.86407	3652.87270	1.48	>95	7
THRre_2c	(pwvpswmpprht-O_2Oc)$_2$kkkc	$C_{175}H_{260}N_{48}O_{38}S_3$	3737.90503	3737.90479	1.33	>95	2

Table S1 Sequence, molecular formula, molecular weight (MW), UPLC characterisation and purity and yield after synthesis and purification of branched THRre peptides. Mal = maleimide, CF = carboxyfluorescein and O_2Oc = 3,6-dioxaoctanoic acid.

Construct	Calc. MW, Da	Found MW, Da	Conjugate/Protein, %
GFP	28761	28755	-
GFP(IAM)	28817	28817	-
GFP-THRre_1m	30813	30814	100
GFP-THRre_2m	32413	32414	70
GFP-THRre_1c	30606	30614	100
GFP-THRre_2c	32496	32492	80

Table S2 Characterisation of THRre conjugates with GFP. GPF(IAM) is GFP reacted with iodoacetamide to alkylate the sulfhydryl group of Cys.

Entry [a]	Strategy	GFP,	2IT,	Ellmans,	THRre_1p,	THRre_1c-Ellmans,

		eq	eq	eq	eq	eq
1	A	1	5	-	-	3
2	A	1	5	-	-	5
3	A	1	10	-	-	20
4	B	1	5	15	THRre_1c (10)	-
5	C	1	2	-	THRre_1m (2)	-
6	C	1	2	-	THRre_1m (10)	-
7	C	1	2	-	THRre_1m (5)	-
8	C	1	2	-	THRre_1m (2)	-
9	C	1	5	-	THRre_1m (5)	-
10	C	1	5	-	THRre_1m (66)	-
11	C	1	5	-	THRre_1m (16)	-
12	C	1	5	-	THRre_1m (3)	-

[a] Reactions were carried out in NaPi 50 mM, NaCl 150 mM, EDTA 1 mM. After every step the protein was purified using PDminitrap, PDmiditrap or PD10 according to the manufacturer's instructions. The reactions were carried out at r.t.

Table S3. Summary of the conditions evaluated to modify GFP with 2 copies of 1p.

References

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