

SUPPLEMENTARY MATERIALS

Table S1. Summary of mispairing method validation results.

Validation Parameter	Mispairing				
Speicificity	The formulation buffer shows no interfering peaks in the integration region.				
Precision	Heavy Chain I Impurity		Heavy Chain II Impurity		
	Repeatabili ty %CV (3 injections)	Intermediate precision %CV (6 injections, different analysts,days, instruments, and columns)	Repeatabi lity %CV (3 injections)	Intermediate precision %CV (6 injections, different analysts,days, instruments, and columns)	
	Low (HC II: 0.5%, HC I: 8.0%)	0.8	1.1	4.1	4.1
	Middle (HC II: 1.3%, HC I: 16.0%)	1.4	1.2	3.3	3.0
	High (HC II: 2.0%, HC I: 24.0%)	0.2	1.5	0.7	1.5
Linearity and range (5 levels)					
r²	0.9998		0.9969		
Range	0.5%-24.0%		0.5%-2.1%		
Accuracy (3 levels, 3 replicates each)	% Recovery	% CV	% Recovery	% CV	
Low (HC II: 0.5%, HC I: 8.0%)	100.2~101.6	0.8	89.3~96.9	4.1	
Middle (HC II: 1.3%, HC I: 16.0%)	99.5~102.0	1.4	99.6~106.1	3.3	
High (HC II: 2.0%, HC I: 24.0%)	99.6~100.0	0.2	96.9~98.6	0.7	
LOQ	0.5%		0.5%		
Robustness	Test solution stable at 8°C for 48 h in autosampler.				

Table S3. Summary of charge variants method validation results.

Validation Parameter	Charge variants					
Speificity	The formulation buffer shows no interfering peaks in the integration region, Thermal stress sample shows a trend of change with increasing degradation.					
Precision (3 levels)	Acid species		Main peak		Basic species	
	% CV					
Repeatability (3 replicates each)	2.9		1.1		4.1	
Intermediate precision (different analysts, days, instruments, and columns, 6 injections/level)	2.6		1		3	
Linearity and range (5 levels)						
r ²	1.0000		1.0000		1.0000	
Range	0.4%-38.2%		≥1.5%		0.6%-37.5%	
Accuracy (3 levels, 3 replicates each)	%	% CV	%	% CV	%	% CV
	Recovery		Recovery		Recovery	
Low (0.05 mg/ml)	95.9~97.8	1.0	98.5~100.3	1.0	100.7~105.8	2.80%
Middle (2.0 mg/ml)	100.0	0.0	99.9~100.1	0.1	99.7~100.2	0.3
High (4.0 mg/ml)	100.4~100.5	0.1	99.7~99.8	0.1	100.1~100.5	0.2
LOQ	0.40%		1.50%		0.60%	
Robustness	Test solution stable at 8°C for 48 h in autosampler.					

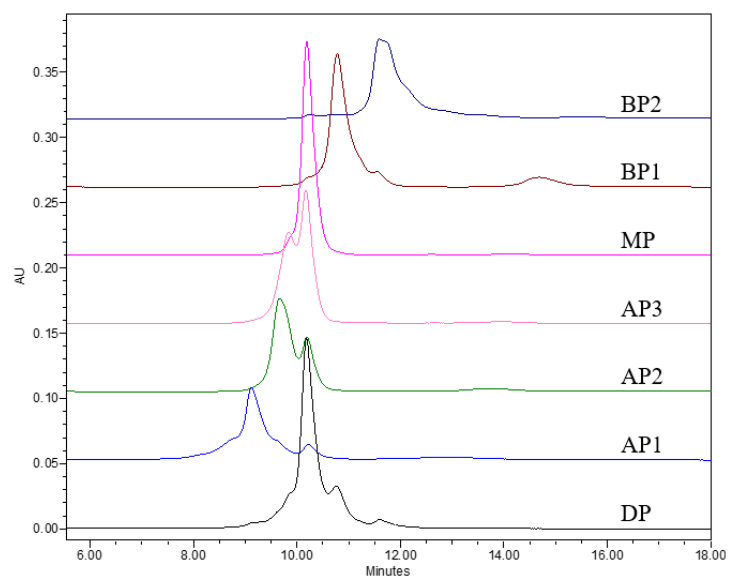


Figure S1 Reinjection WCX-HPLC chromatograms of the collected fractions.

Table S4. Purity of the collected fractions.

Fraction ID	AP1	AP2	AP3	MP	BP1	BP2
Reinjection purity / %	72.3	64	44	89.8	77.8	92.2

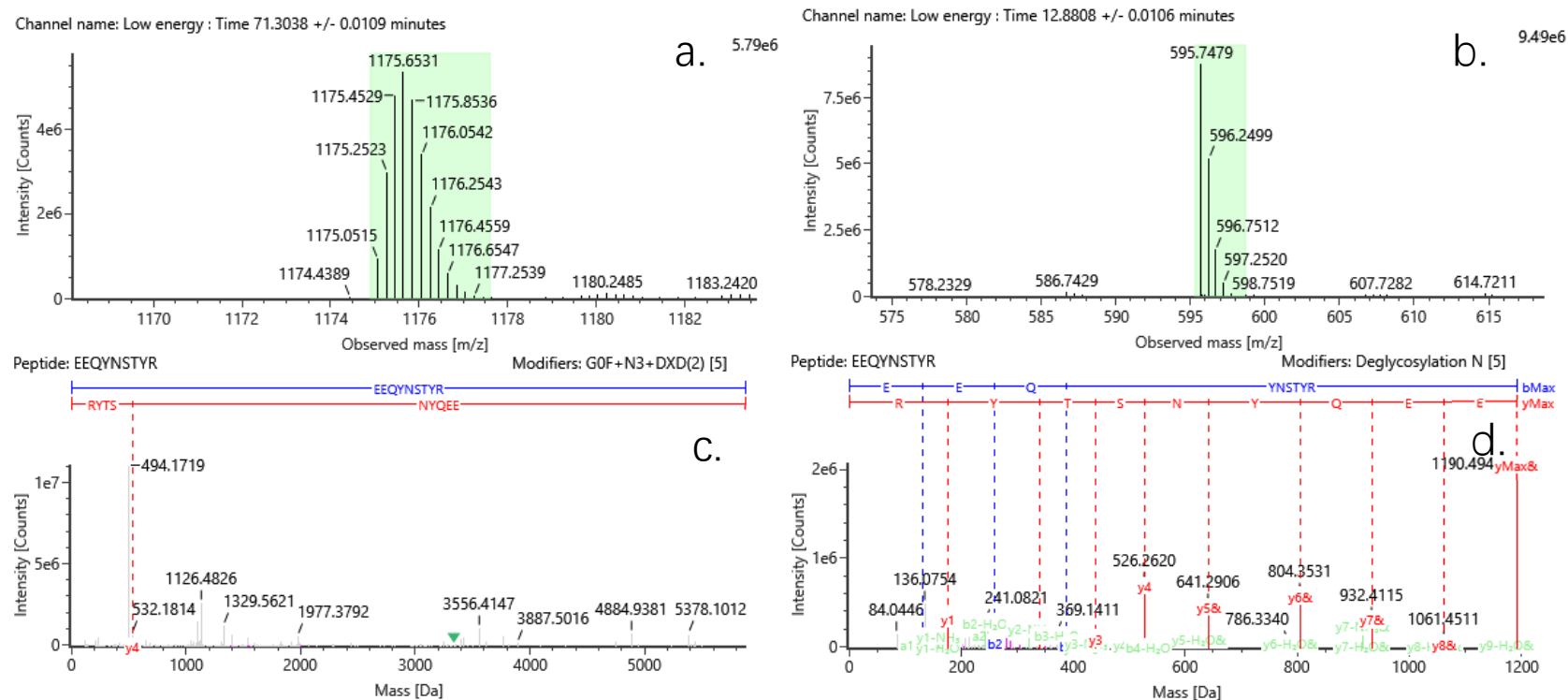


Figure S2. Mass spectra of the linker-payload conjugation site-containing peptide (EEQYNSTYR) before and after deglycosylation. (a) MS spectrum of the peptide before deglycosylation. (b) MS spectrum of the peptide after deglycosylation. (c) MS/MS spectrum of the peptide before deglycosylation. (d) MS/MS spectrum of the peptide after deglycosylation.

Table S5. Mass errors of the conjugation site-containing peptide (EEQYNSTYR) before and after deglycosylation.

Peptide	Modifiers	Expected m/z	Observed m/z	Charge	Expected mass (Da)	Observed mass (Da)	Mass error (ppm)
EEQYNSTYR	Deglycosylation N [5]	595.7517	595.7480	2	1190.4960	1190.4888	-6.1
EEQYNSTYR	G0F+2*LP [5]	1175.0717	1175.0516	5	5871.32916	5871.2290	-17.1

Table S6. Quantitative analysis of conjugation occupancy based on MS spectral data.

Label	Peptide	Modifiers	Observed RT (min)	Response	Percentage
P:T24	EEQYNSTYR	/	11.46	135868	0.17%
P:T24&	EEQYNSTYR	Glycosylation Man5 N [5]	11.74	558275	0.68%
P:T24&	EEQYNSTYR	Glycosylation G2F N [5]	12.58	71807	0.09%
P:T24&	EEQYNSTYR	G1F+N3+LP(1) [5]	49.52	774783	3.08%
P:T24&	EEQYNSTYR	G1F+N3+ LP (1) [5]	49.77	1754306	
P:T24&	EEQYNSTYR	G0F+N3+ LP (2) [5]	70.98	18761180	95.99%
P:T24&	EEQYNSTYR	G0F+N3+ LP (2) [5]	71.30	40639208	
P:T24&	EEQYNSTYR	G0F+N3+ LP (2) [5]	71.53	19517716	

SUM (EEQYNSTYR G0F/G1F+N3+LP) =99.1%

Table S7. Summary of free drugs method validation results.

Validation Parameter	Free drug			
Speicificity	Formulation Buffer and 80% DMSO shows no interfering peaks in the integration region, Distinct degradation peaks observed in thermal stress sample vs. control, demonstrating stability-indicating(see Figure 3).			
	Payload		Linker-Payload	
Intermediate precision (different analysts, days, instruments, and columns, 6 injections/level)	% CV			
Low (0.5 ug/ml)	1.1		1.7	
Middle (5.0 mg/ml)	1.3		1.2	
High (25.0 mg/ml)	1.7		1.1	
Linearity and range (5 levels)				
r ²	1.0000		0.9999	
Range	0.5µg/ml~25.0µg/ml		0.5µg/ml~25.0µg/ml	
Accuracy (3 levels, 3 replicates each)	% Recovery	% CV	% Recovery	% CV
Low (0.5 ug/ml)	98~105	3.6	91~94	1.9
Middle (5.0 mg/ml)	103	0	103~104	0.6
High (25.0 mg/ml)	103	0	102	0
LOQ	0.5µg/ml		0.5µg/ml	
Robustness	Test solution stable at 8°C for 29 h in autosampler.			

Table S8. Summary of residual GalT enzyme method validation results.

Validation Parameter	Residual GalT enzyme	
Speicificity	Formulation Buffer shows no interfering peaks in the integration region	
Precision	Repeatability %CV (3 replicates)	Intermediate precision %CV (2 analysts on 2 days, 6 replicates)
Low (0.5 ng/ml)	1	2
Middle (1 ng/ml)	1	2
High (3 ng/ml)	3	4
Linearity and range (5 levels)		
r²	0.9998	
Range	0.25 ng/ml~5 ng/ml	
Accuracy (3 levels, 3 replicates each)	% Recovery	% CV
Low (0.5 ng/ml)	87	1
Middle (1 ng/ml)	89	1
High (3 ng/ml)	94	3
LOQ	0.25 ng/ml	

Table S9. Summary of relative binding activity method validation results.

Validation Parameter	Relative Binding Activity	
Speicificity	Formulation buffer shows no typical S-shaped curve, Relative binding activity of thermal stress sample changed with increasing degradation.	
Precision (3 levels)	Repeatability %CV (3 replicates)	Intermediate precision %CV (2 analysts on 2 days, 6 replicates)
Potency level (50%)	4	6
Potency level (100%)	8	6
Potency level (201%)	4	5
Linearity and range (5 levels)		
r²	0.999	
Range	50%~201%	
Accuracy (3 levels, 3 replicates each)	RB/%	GCV/%
Potency level (50%)	-4~2	4
Potency level (100%)	-9~4	8
Potency level (201%)	-4~1	4

Table S10. Summary of biological activity method validation results.

Validation Parameter	Biological Activity	
Speicificity	Formulation buffer shows no typical S-shaped curve, Biological activity of thermal stress sample changed with increasing degradation.	
Precision (3 levels)	Repeatability %CV (3 replicates)	Intermediate precision %CV (2 analysts on 2 days, 6 replicates)
Potency level (50%)	4	6
Potency level (100%)	4	5
Potency level (201%)	4	6
Linearity and range (5 levels)		
r²	0.9965	
Range	50%~201%	
Accuracy (3 levels, 3 replicates each)	% RB	% GCV
Potency level (50%)	-2~0	4
Potency level (100%)	5~10	4
Potency level (201%)	-2~1	4