Supporting Information
For
Genetic PEGylation of different lengths on one polypeptide backbone

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1. Materials and Methods

1.1 Prepared DNA templates

The DNA templates for the cell-free translation encoding peptides were prepared. The plasmid DNA was prepared by pROX-FL92.1amber as a template, and the primers are shown below. The PCR products were purified using the QIA-quick PCR Purification Kit (Qiagen) and used as the templates for cell-free translation reactions.

1.2 Primer sequences

Primer fp-(FL92-T7prom): 5′- CGCGAAATTAATACGAGTCAC-3′

Primer rp-(Prox)FLAG-TGA: 5′-
TCACTTGTCATCGTCATCCTTGTAGTCCTCATTAGACTAGTTTACTTCGATTT
G-3′

Primer rp(ProX)4bFLAG-TGA: 5′-
TCACTTGTCATCGTCATCCTTGTAGTCCCCGCTCATTAGACTAGTTTACTTCG
ATTTG-3′

Primer rp-(Prox)FLAG-CGGG:
5′-
TCACTTGTCATCGTCATCCTTGTAATCCTCATTAGACCCGGTTTACTTCGATT
TG-3′

Primer rp(Prox-4b)-amb-FLAG-TGA:

5′-

TCACTTGTCATCGTCATCCTTGTAGTCCTACTCATTAGACCCGGTTTACTTCG

ATTTG-3′

1.3 *In vitro* translation

The *in vitro* translation assays were performed using a RYTS kit (Proteins Express, Chiba, Japan) according to the manufacture's protocol. The translation reaction was performed in the presence of 200 pmol of each PEG-AF-tRNA, unless otherwise stated. The reaction mixture was incubated at 30 °C for 2 h.

1.4 Mass spectra measurements

The samples were prepared for mass spectrometry as previously reported.[1] The translated peptides were purified from 25-μL reactions using prewashed Anti-DDDDK-tag mAb-Magnetci Agarose (MBL). After two washes with buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6), the peptides were eluted from the matrix with 0.2% TFA. Before freeze drying, 3xFlag Peptide (Sigma) (1 nmol or 10 fmol) was added to each sample as the internal standard. For the mass analysis, the peptides were desalted using ZipTip μ-C18 (Millipore) and mixed with 2,5-dihydroxybenzoic acid or 3-hydroxy-2-pyridinecarboxylic acid as the matrix. The samples were subjected to MALDI–TOF–MS analysis on an Ultraflex spectrometer (Bruker Daltonics) in linear or reflector mode. Each sample was analyzed three times and the amount of each peptide was estimated by the area intensity comparing to 3xFlag peptide.

78 **2. Table S1**: MS data of PEGylation product. X and B indicate AF-PEG (4, 8, 12, 24)

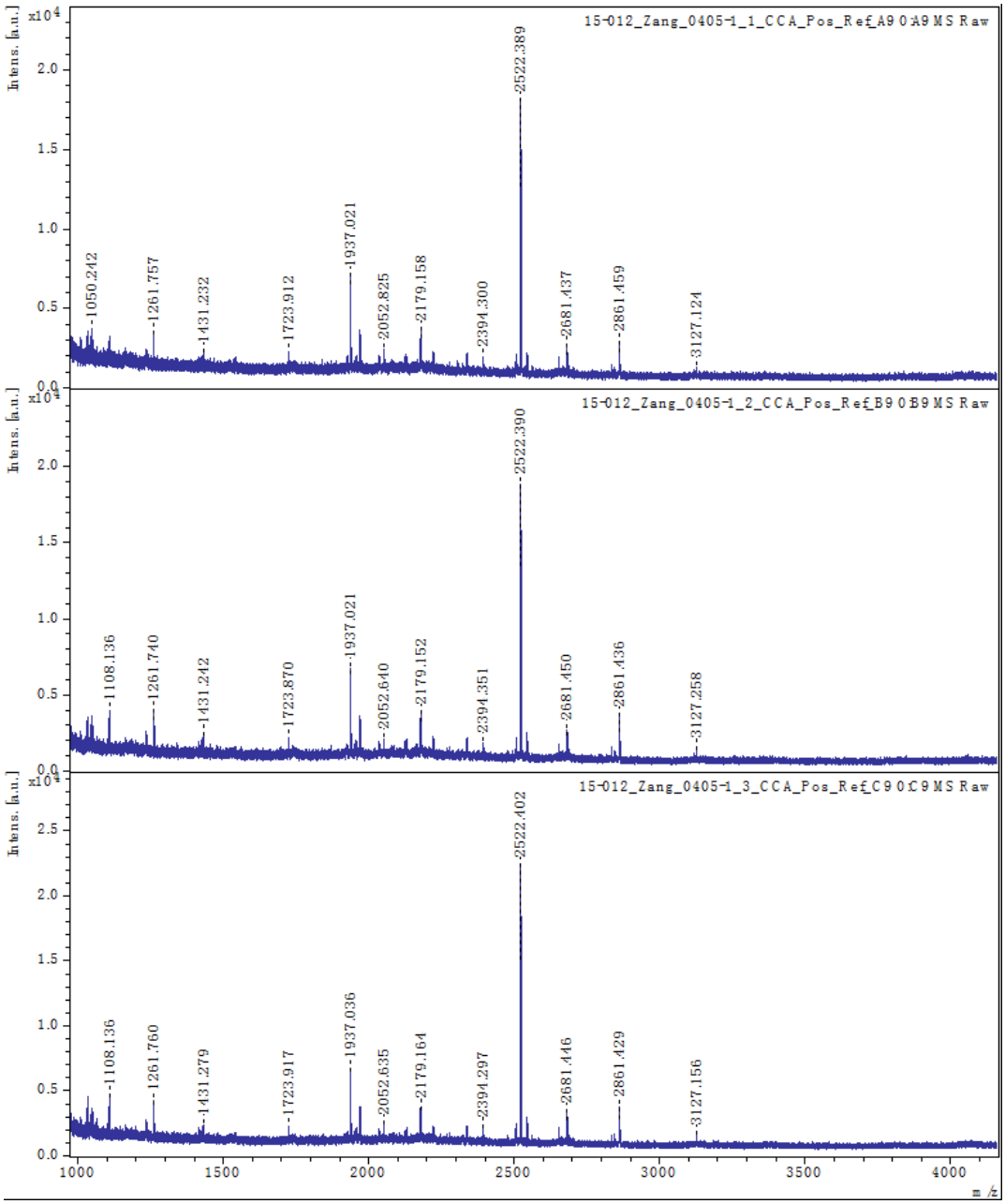
position	sequence	M.W.Calculated [M+H]+	MW. found
PEG4-amber	fMQKIEVNXSNEDYKDDDDK	2681.178	2681.437
	QKIEVNXSNEDYKDDDDK	2522.142	2522.389
	XSNEDYKDDDDK	1723.719	1723.021
PEG8-amber	fMQKIEVNBSNEDYKDDDDK	2857.282	2857.416
	QKIEVNBSNEDYKDDDDK	2698.247	2698.390
	BSNEDYKDDDDK	1899.824	1899.948
PEG4-CGGG	fMQKIEVNXSNEDYKDDDDK	2681.178	2861.451
	QKIEVNXSNEDYKDDDDK	2522.142	2522.428
	XSNEDYKDDDDK	1723.719	1723.977
PEG8-CGGG	fMQKIEVNBSNEDYKDDDDK	2857.282	2857.623
	QKIEVNBSNEDYKDDDDK	2698.247	2698.562
	BSNEDYKDDDDK	1899.824	1900.075
PEG12-amber	fMQKIEVNXSNEDYKDDDDK	3033.387	3033.970
	QKIEVNXSNEDYKDDDDK	2874.352	2874.890
	XSNEDYKDDDDK	2075.928	2076.311
PEG24-amber	fMQKIEVNXSNEDYKDDDDK	3561.702	3562.222
	QKIEVNXSNEDYKDDDDK	3402.667	3403.168
	XSNEDYKDDDDK	2604.243	2604.622
PEG4(amber)--PEG4(CGGG)	fMQKIEVNXSNEXDYKDDDDK	3061.372	n.d.
	QKIEVNXSNEXDYKDDDDK	2902.337	2903.113
PEG4(CGGG)--PEG4(amber)	fMQKIEVNXSNEXDYKDDDDK	3061.372	3062.304
	QKIEVNXSNEXDYKDDDDK	2902.337	2903.271
PEG4(amber)--PEG8(CGGG)	fMQKIEVNXSNEBDYKDDDDK	3237.477	n.d.
	QKIEVNXSNEBDYKDDDDK	3078.442	n.d.
PEG8(amber)--PEG4(CGGG)	fMQKIEVNBSNEXDYKDDDDK	3237.477	3239.239
	QKIEVNBSNEXDYKDDDDK	3078.442	3079.390
PEG4(CGGG)--	fMQKIEVNXSNEBDYKDDDDK	3237.477	n.d.

PEG8(amber)		QKIEVNXSNEBDYKDDDDK	3078.442	3079.491
PEG8(CGGG)	--	fMQKIEVNBSNEXDYKDDDDK	3237.477	3239.195
PEG4(amber)		QKIEVNBSNEXDYKDDDDK	3078.442	3080.503

79

80

81 **3. Figure S1**



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83 **4.** Fig. S1. Mass spectra of the peptide containing PEG4 via the amber codon.

5. Figure S2

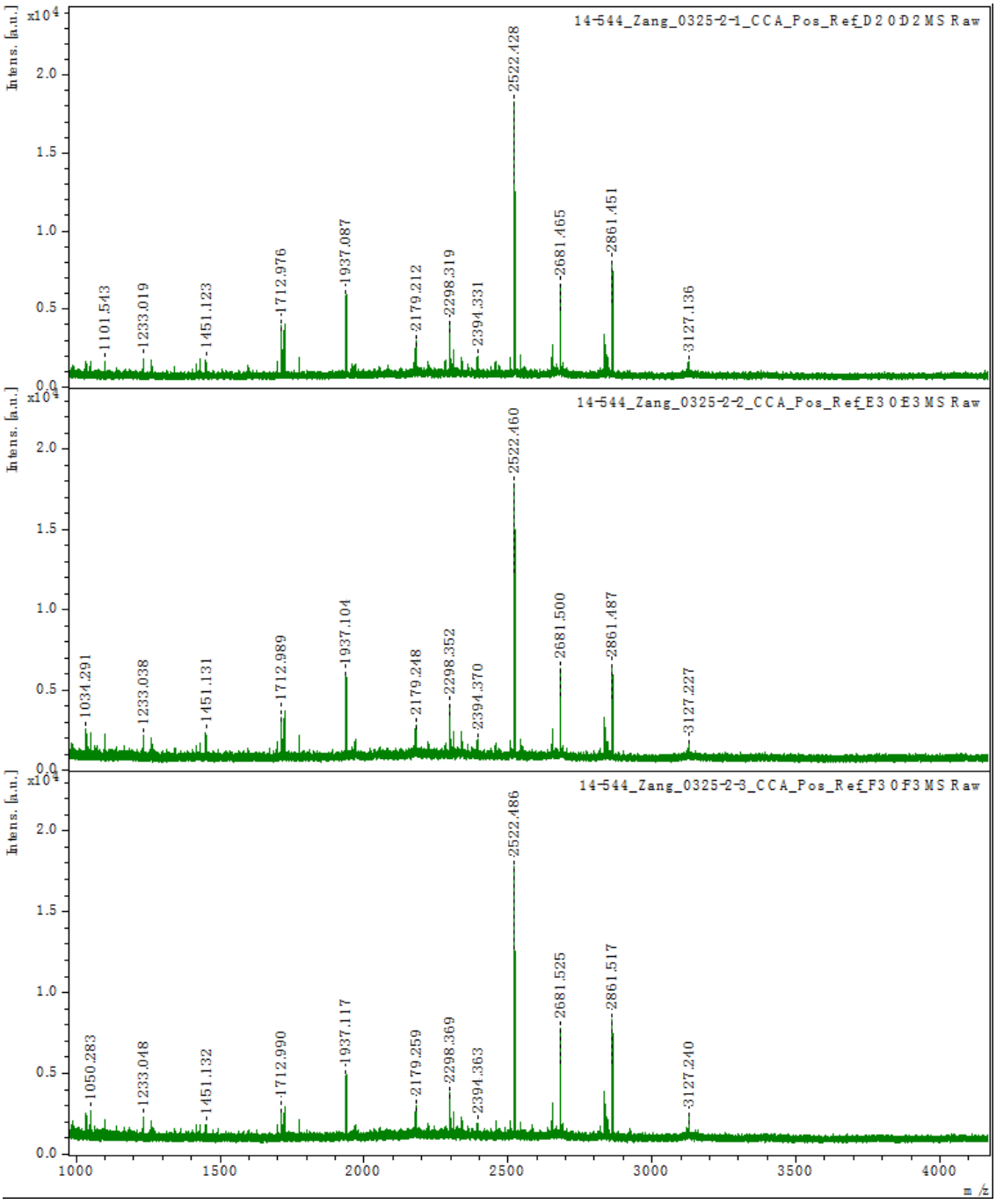

Fig. S2. Mass spectra of the peptide containing PEG4 via the frameshift codon.

6. Figure S3

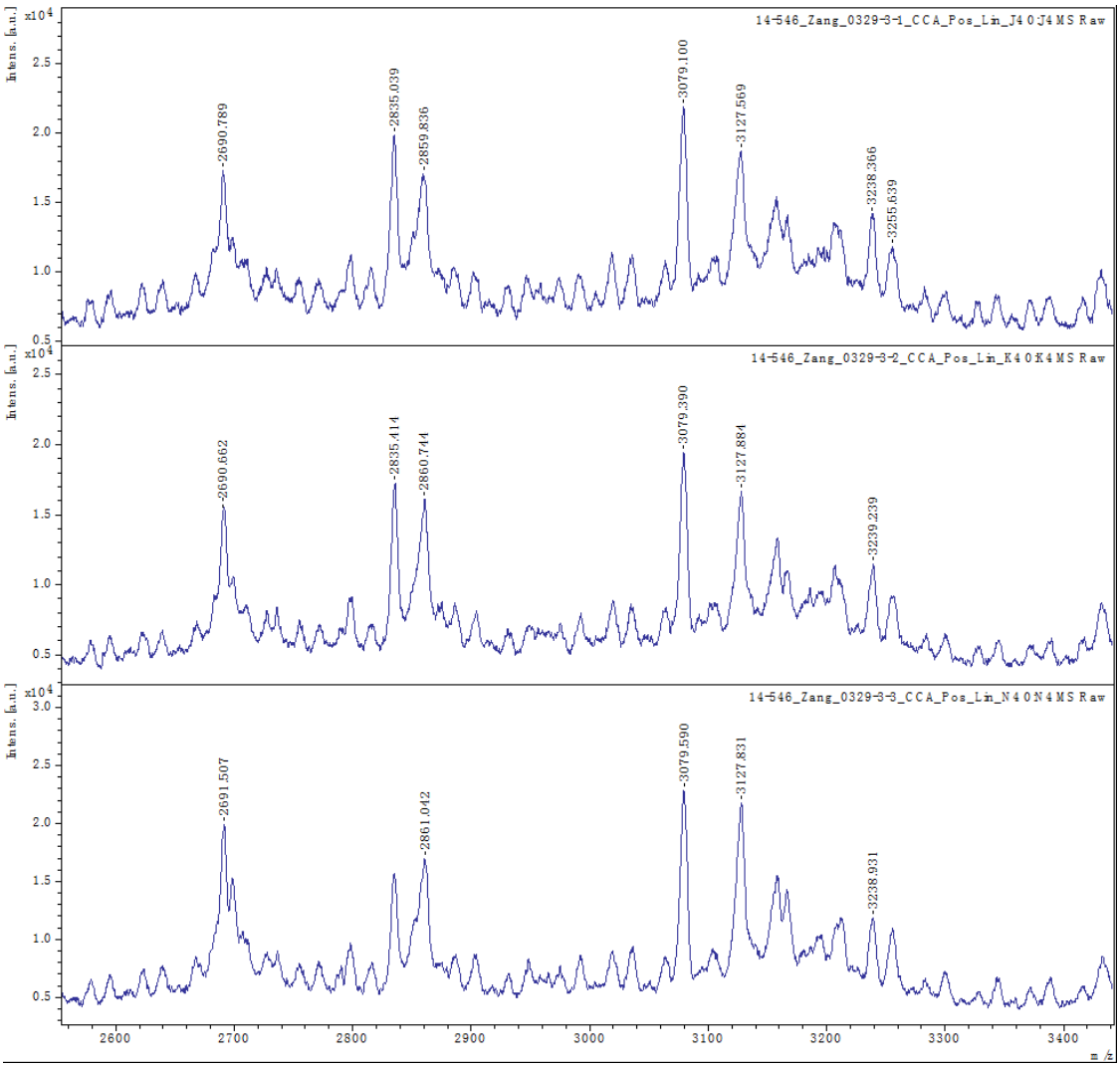

Fig. S3. The mass spectra of PEG8 (amber)—PEG4 (CGGG).

7. References

[1] K. Josephson, M. C. Hartman, J. W. Szostak, *J. Am. Chem. Soc.*, 2005, **127**, 11727-11735.