One-pot synthesis of dual functional peptides by Sortase A-mediated on-resin cleavage and ligation

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I. General Experimental Methods

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification.

**RP-HPLC.** Analytical scale HPLC were performed with Waters 2695 system and Diamonsil C18 column (250 mm×4. 6 mm, 3.5 μm,100 Å). Semi-preparative purifications were achieved using Waters 1525 and Diamonsil C18 column (250 mm×10 mm, 3.5 μm,100 Å).

**Mass Spectrometry.** Electrospray ionization-mass spectrometry (ESI-MS) analysis was carried out with a Bruker Amazon SL.

**MALDI TOF-MS.** Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry was performed on Bruker Ultraflex mass spectrometer.

**NMR:** Proton chemical shifts are reported in ppm (δ) downfield from tetramethylsilane (TMS). Carbon-13 chemical shifts are reported in ppm (δ) in reference to CDCl₃ (δ 77.16) or in reference to the proton signal of the solvent CD₃OD (δ 49.00).

II. Synthesis and characterization of peptides

i. Synthesis of AbzLPETGGK(DNP)S-HMBA, NH₂-BK-Acp-LPETGGS-HMBA and DNS-LPETGGS-HMBA on resin

The resin (PEGA or MBHA resin) was swollen in dry DMF (5ml), then treated with a DMF solution of hydroxymethyl benzoic acid (HMBA) (3 eq) in presence of TBTU (2.85 eq) and DIEA (6 eq). After shaking 4 h at room temperature, the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried in vacuo. The attachment of first amino acids (Fmoc-Ser(otBu)-OH) was performed using 1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT)/methylimidazole (MeIm) esterification. The resin was swollen in dry DCM, Fmoc-Ser(otBu)-OH) (3 eq), MSNT (3.75 eq) and MeIm (5 eq) was dissolved in DCM and added to the resin. After 1 h, the resin was filtered and washed with DMF (10×), MeOH (10×), DCM (10×). A second coupling was performed with same amount of reagents. The Fmoc group was removed by 20% piperidine/DMF for 20 min. The assembly of the rest Fmoc amino acids or Fmoc amino acid derivatives (Abz,K-Dnp,Acp) was performed using the CEM Liberty automated microwave peptide synthesizer. The coupling reagents was the same as above described. As to DNS (dansyl chloride) coupling, the peptide resin was treated with 5 eq of DNS and 2 eq of DIPEA in 3 ml dry DMF for 4 h at 35°C. After the incorporation of all amino acids, the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried. The resin was treated with a solution of TFA (95%), water (2.5%) and Tis (2.5%) for 2 h at room temperature for removing all the side-chain protection groups, then resin was washed with DMF (10×), MeOH (10×), DCM (10×). A few beads were collected and was cleaved with 0.1 M NaOH for 2 h at room temperature. The resin was filtered and the filtrate was neutralized with 0.1 M HCl. The product was analysed by HPLC and MALDI-TOF or ESI.
Figure S1. HPLC diagrams of peptide AbzLPETGGK(DNP)S-CO₂H, NH₂-BK-Acp-LPETGGS-CO₂H and DNS-LPETGGS- CO₂H, and monitored with UV at 220 nm. HPLC conditions: 10% ACN in H₂O (both containing 0.1% TFA) to 90% ACN in 20 min; flow rate: 1 mL/min; R.T.: AbzLPETGGK(DNP)S-CO₂H (11.84 min); NH₂-BK-Acp-LPETGGS-CO₂H (10.13 min); DNS-LPETGGS- CO₂H (9.39 min).
ii. Synthesis of ACE inhibitory peptides

ACE inhibitory peptides were prepared using the CEM Liberty automated microwave peptide synthesizer. Deprotection was performed with 20% piperazine in DMF. Coupling reactions were performed with 5 fold excess of Fmoc-AA-OH with 1:0.9:2 AA/HBTU/DIEA. After the incorporation of all amino acids, the resin was washed with DMF (10x), MeOH (10x), DCM (10x) and dried. The resin was treated with a solution of TFA (95%), water (2.5%) and Tis (2.5%) for 2h at room temperature for removing all the side-chain protection groups. Following cleavage the peptide was precipitated and washed in cold diethyl ether. The peptide was analyzed by HPLC and MALDI-TOF-MS. Wang resin preloaded with corresponding amino acid was used to synthesize peptide 6-10 and 2-Chlorotrity chloride resin preloaded with Proline residue was used to synthesize peptide 11.

![HPLC diagrams of ACE inhibitory peptides monitored with UV at 220 nm. HPLC conditions: 10% ACN in H₂O (both containing 0.1% TFA) to 90% ACN in H₂O in 20 min; flow rate: 1 mL/min; R.T.: NH₂-GGIKW-CO₂H (9.82 min); NH₂-GGFQKVVA-CO₂H (8.24 min); NH₂-GGGALPHA-CO₂H (7.31 min); NH₂-GGAVPYPQR-CO₂H (7.25 min); NH₂-GGGALKAWSVAR-CO₂H (9.10 min); NH₂-GGHIHPFAQTQLVYP-CO₂H (9.09 min).](image-url)
A

NH$_2$-GGIKW-CO$_2$H

[M+H]$^+$

[M+Na]$^+$

B

NH$_2$-GGFQKVVA-CO$_2$H

[M+H]$^+$

[M+Na]$^+$
C

$\text{NH}_2\text{-GGGALPHA-CO}_2\text{H}$

$[\text{M+H}]^+$

$[\text{M+Na}]^+$

$[\text{M+K}]^+$

D

$\text{NH}_2\text{-GGGAVPYPQR-CO}_2\text{H}$

$[\text{M+H}]^+$
Figure S4. MALDI-TOF MS of ACE inhibitory peptides. A. NH$_2$-GGIKW-CO$_2$H, calcld : 559.6, observed 560.2 [M+H]$^+$, 582.2 [M+Na]$^+$; B. NH$_2$-GGFQKVVA-CO$_2$H, calcld : 804.9, observed 805.6 [M+H]$^+$, 827.4 [M+Na]$^+$; C. NH$_2$-GGALPHA-CO$_2$H, calcld : 678.7, observed 679.3 [M+H]$^+$, 701.2 [M+Na]$^+$, 717.2 [M+K]$^+$; D. NH$_2$-GGAVPYPQR-CO$_2$H, calcld : 1001.1, observed 1001.5 [M+H]$^+$; E. NH$_2$-GGGALKAWSVAR-CO$_2$H, calcld : 1172.3, observed 1172.8 [M+H]$^+$; F. NH$_2$-GGGIHPFAQTQLYV-CO$_2$H, calcld : 1671.8, observed 1672.0 [M+H]$^+$.
III. Synthesis and characterization of GG-HMDA-Biotin (3), GG-Lipid (12) and GG-PEG2000 (13)

i. Synthesis of GG-HMDA-Biotin 3

19: Biotin (0.2 g, 0.81 mmol) was dissolved in dry dimethylformamide (DMF 4 mL), then N-hydroxysuccinimide (0.102 g, 1.1 eq) and dicyclohexycarbodiimide (0.18 g, 1.1 eq) were added. The reaction was carried out at rt overnight, then dicyclohexyl urea was removed by filtration and N-(tert-Butoxycarbonyl)-1,6-diaminohexane (0.19 g, 1.1 eq) and TEA (0.112 mL, 1 eq) were added. After 2 h at rt, the product was precipitated by adding cold water, it was isolated by filtration and rinsed anhydrous diethyl ether and dried in vacuo. Yield 82%.

20: Compound 19 was dissolved in 4 M HCl/MeOH. After 30 min, the solvent was evaporated and the product was redissolved in the minimum amount of DCM, and TEA was added to obtain biotinamidohexylamine as the free base. The insoluble product was isolated by filtration, it was rinsed with DCM and cold diethyl ether and dried in vacuo. Yield 65%.

21: Boc-Gly-Gly-OH (0.135 g, 0.583 mmol) was dissolved in dry dimethylformamide (DMF 2 mL), then N-hydroxysuccinimide (0.073 g, 1.1 eq) and dicyclohexycarbodiimide (0.132 g, 1.1 eq) were added. The reaction was carried out at rt overnight, then dicyclohexyl urea was removed by filtration and compound 20 (0.20 g, 1 eq) and TEA (0.08 mL, 1 eq) were added. After 5 h at rt, the solvent was removed under reduced pressure, then purified by flash column chromatography (DCM:MeOH=15:1 to 12:1). Yield 40%. $^1$H NMR (400 MHz, DMSO) $\delta$ = 8.06 (t, J = 5.6 Hz, 1H), 7.73 (m, 2H), 7.08 (t, J = 5.8 Hz, 1H), 6.45 (s, 1H), 6.38 (s, 1H), 4.31 (dd, J = 7.5, 5.2 Hz, 1H), 4.18 – 4.08 (m, 1H), 3.65 (d, J = 5.7 Hz, 2H), 3.55 (d, J = 5.9 Hz, 2H), 3.06 (m, 5H), 2.82 (dd, J = 12.4, 5.1 Hz, 1H), 2.58 (d, J = 12.4 Hz, 1H), 2.04 (t, J = 7.4 Hz, 2H), 1.61 (m, 1H), 1.49 (m, 3H), 1.39 (s, 9H), 1.32 (m, 5H), 1.24 (s, 5H).
Figure S5. $^1$H-NMR of 21 at 400 MHz in DMSO.

3: Compound 21 (100 mg) was dissolved in the DCM (2m) at 0 °C, then 1 mL HCl in 1 mL dioxane was added and stirred for 30 min at rt, then the solvents were removed with toluene in vacuo to give 3 in quantitative yield. $^1$H NMR (400 MHz, D$_2$O) $\delta = 4.57 – 4.51$ (t, 1H), 4.35 (m, 1H), 3.88 (s, 1H), 3.82 (s, 1H), 3.29– 3.24 (m, 1H), 3.08 – 3.18 (m, 4H), 2.93 (dd, $J = 13.0$, 4.9 Hz, 1H), 2.71 (d, $J = 13.1$ Hz, 1H), 2.18 (t, $J = 7.0$ Hz, 2H), 1.66 – 1.53 (m, 4H), 1.46 – 1.42 (m, 4H), 1.37-1.32 (m, 2H), 1.25 (s, 4H). $^{13}$C NMR (100 MHz, D$_2$O) $\delta = 176.6$, 170.7, 167.7, 165.4, 62.2, 60.3, 55.4, 42.5, 40.6, 39.8, 39.4, 39.3, 35.6, 28.3, 27.9, 27.7, 25.7, 25.3.
Figure S6. $^1$H-NMR of 3 at 400 MHz in D$_2$O.

Figure S7. $^{13}$C-NMR of 3 at 100 MHz in D$_2$O.
ii. Synthesis of GG-Lipid 12

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\text{Boc-Gly-Gly-OH (1.2 eq) was dissolved in DCM, then added EDCI (1.2 eq), HOBt (1.2 eq), and stirred at 0 °C for 30 min. Then TEA (2 eq) and alkylamine were added and reaction mixture was stirring at r.t for 34 h. The reaction mixture was washed with water and the organic was dried over Na}_2\text{SO}_4. \text{The solvent was removed in vacuo, then purified by flash column chromatography (DCM:MeOH =50:1 to 40:1). Yield 55%.} \]

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\begin{align*}
1^\text{H NMR} &\ (400 \text{ MHz, CDCl}_3) \delta = 6.95 (s, 1H), 6.40 (s, 1H), 5.28 (t, J = 5.7 Hz, 1H), 3.86 (d, J = 5.6 Hz, 2H), 3.75 (d, J = 5.7 Hz, 2H), 3.16 (dd, J = 13.3, 7.0 Hz, 2H), 1.45 – 1.41 (m, 2H), 1.38 (s, 9H), 1.19 (s, 14H), 0.81 (t, J = 6.9 Hz, 3H). \\
13^\text{C NMR} &\ (100 \text{ MHz, CDCl}_3) \delta = 169.0, 167.5, 155.3, 79.6, 43.4, 42.1, 38.7, 30.9, 28.5(2C), 28.4, 28.3 (2C), 27.3, 25.9, 21.7, 13.1.
\end{align*}
\]

Figure S8. \(^1\text{H-NMR of 22 at 400 MHz in CDCl}_3\).
Compound 22 was dissolved in the 50% TFA/DCM at 0 °C, stirred 30 min at rt, then removed the solvents with toluene in vacuo. $^1$H NMR (400 MHz, MeOD) δ = 3.91 (s, 2H), 3.75 (s, 2H), 3.20 (t, J = 7.1 Hz, 2H), 1.52-1.49 (t, J = 7.2 Hz, 2H), 1.30 (s, 14H), 0.91 (t, J = 6.6 Hz, 3H). $^{13}$C NMR (100 MHz, MeOD) δ = 169.5, 166.5, 41.8, 40.1, 39.1, 31.6, 29.3, 29.0, 26.6, 22.3, 13.0.

Figure S9. $^{13}$C-NMR of 22 at 100 MHz in CDCl3.

Figure S10. $^1$H-NMR of 12 at 400 MHz in MeOD.
Figure S11. $^{13}$C-NMR of 12 at 100 MHz in MeOD.

iii. Synthesis of GG-PEG2000 13

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\begin{array}{c}
\text{Boc-Gly-Gly} \quad \xrightarrow{\text{DCC, DCM}} \quad \text{Boc-Gly-Gly-O-Su} \quad \xrightarrow{\text{PEG2000-NH$_2$}} \quad \text{H$_2$NGG-PEG2000}
\\
\text{BocHNGG-PEG2000} \quad \xrightarrow{\text{50% TFA/DCM}} \quad \text{H$_2$NGG-PEG2000}
\end{array}
\]

23: Boc-Gly-Gly-OH (1 g, 4.3 mmol), N-hydroxysuccinimide (0.5 g, 1 eq) and dicyclohexycarbodiimide (0.887 g, 1 eq) were added at 0 °C. Then the reaction mixture was stirred for 2 h at 0 °C and for 20 h at r.t. After the precipitated dicyclohexylurea was filtered and wash with DCM, organic phase was evaporated under reduced pressure. The residue was recrystallized from hot isopropyl alcohol. Yield 75%. $^1$H NMR (400 MHz, CDCl$_3$) $\delta =$ 6.95 (s, 1H), 5.30 (s, 1H), 4.41 (d, $J = 5.6$ Hz, 2H), 3.86 (d, $J = 5.1$ Hz, 2H), 2.84 (s, 4H), 1.44 (s, 9H).
24: After PEG2000-NH$_2$ (0.1 g, 0.05 mmol) was dissolved in DCM, compound 23 (0.033 g, 2 eq) and TEA (14 µL, 2 eq) were added to the solution. The reaction was stirred at r.t overnight. The solution was concentrated under reduced pressure, purified by flash column chromatography to give 22. (DCM: MeOH = 30:1, 25:1, 20:1). Yield 85%. $^1$H NMR (400 MHz, MeOD) $\delta$ = 3.89 (s, 2H), 3.77 (s, 2H), 3.65(s, PEG), 3.38 (s, 3H), 1.48 (s, 9H).

Figure S12. $^1$H-NMR of 23 at 400 MHz in CDCl$_3$.

Figure S13. $^1$H-NMR of 24 at 400 MHz in MeOD.
**13**: Compound **24** was dissolved in the 50% TFA/DCM at 0 °C, stirred 30 min at r.t, then removed the solvents with toluene in vacuo to give **13**. $^1$H NMR (400 MHz, D$_2$O) δ = 3.91 (s, 2H), 3.82 (s, 2H), 3.63 (s, PEG), 3.31 (s, 3H).

**Figure S14.** $^1$H-NMR of **13** at 400 MHz in D$_2$O.

**IV. Sortase-mediated ligation studies**

i. Sortase-mediated cleavage of **1**, **5** and **17**

Dried peptide resin (0.6 μmol) was swollen in 100 μl reaction buffer (Tris-HCl 0.3 M pH 7.5, 150 mM NaCl, 5 Mm CaCl$_2$, and 0.5 mM mercaptoethanol) for 30 min. Then 20 μl SrtA (100 μM dissolved in buffer) and 80 μl buffer was added to reach total 200 μL. After the reaction was kept at 37 °C for 20 h, 20 μL of the reaction mixture was withdrawn and quenched with the same volume of 0.1% of TFA. The aliquots were then analyzed by HPLC using a RP C18 column and the fractions were analyzed by MALDI-TOF MS.

In large scale reaction: 10 mg of dry peptide PEGA resin **1**, **5** and **17** was treated with SrtA for 20 h. After filtration and isolation by HPLC. The yield of product **2**, NH$_2$-BK-Acp-LPET-CO$_2$H and DNS-LPET-CO$_2$H was 87.5% (1.1 mg), 73.2% (2.3 mg) and 80% (1.2 mg), respectively.
Figure S15. HPLC profile of NH$_2$-BK-Acp-LPET-CO$_2$H and DNS-LPET-CO$_2$H cleaved from PEGA resin by SrtA, monitored with UV at 220 nm. A. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 40% ACN in H$_2$O in 50 min; flow rate: 1 mL/min; R.T.: NH$_2$-BK-Acp-LPET-CO$_2$H (34.03 min); SrtA (36.41 min). B. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 50% ACN in H$_2$O in 30 min; flow rate: 1 mL/min; R.T.: DNS-LPET-CO$_2$H (21.05 min); SrtA (19.92 min).
Figure S16. MALDI-TOF MS of NH$_2$-BK-Acp-LPET-CO$_2$H and DNS-LPET-CO$_2$H. A. NH$_2$-BK-Acp-LPET-CO$_2$H, calcd : 1613.8, observed 1614.9 [M+H]$^+$. B. DNS-LPET-CO$_2$H, calcd : 691.8, observed 714.2 [M+Na]$^+$, 730.2 [M+K]$^+$.

ii. Sortase-mediated ligation of 1 and 5 with polyglycine nucleophiles in one-pot

Dried peptide resin (0.6 µmol) was swollen in 100 µL reaction buffer (Tris-HCl 0.3 M pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$, and 0.5 mM mercaptoethanol) for 30min. Then 20 µL SrtA (100 µM dissolved in buffer) and 5 eq polyglycine nucleophiles were added. The final volume was 200ul. After the reaction was kept at 37 °C for 20 h, 20 µL of the reaction mixture was withdrawn and quenched with the same volume of 0.1% of TFA. The aliquots were then analyzed by HPLC using a RP C18 column and the fractions were analyzed by MALDI-TOF MS.

In large scale reaction: 10 mg of dry peptide PEGA resin 1 was treated with SrtA and 3 (5 eq) for 20 h. 1.5 mg of product 4 (yield 68%) was afforded after isolation by semi preparative HPLC. For reaction of peptide PEGA resin 5 with 3, 12 and 13, 10 mg of dry peptide PEGA resin 5 was treated with SrtA and 3 (5 eq), 12 (5 eq) and 13 (5 eq) for 20 h respectively. After filtration and isolation by HPLC, the yield of product 14, 15 and 16 was 71% (2.9 mg), 61% (2.5 mg) and 65% (4.8 mg) respectively. For reaction of peptide PEGA resin 5 with ACE inhibitory peptides, ACE inhibitory peptides (5 eq) and SrtA were added to 10 mg peptide PEGA resin 5 and reacted for 20 h. The product was isolated by HPLC. The yield of produc of 6, 7, 8, 9, 10 and 11 was 57% (2.4 mg), 53% (2.5 mg), 52% (2.3 mg), 63% (3.2 mg), 64% (3.5 mg) and 68% (4.3 mg) respectively.
Figure S17. HPLC profile of ligation of BK peptide with ACE inhibitory peptides in one-pot on PEGA resin, monitored with UV at 220 nm. A. Ligation of BK peptide with NH$_2$-GGIKW-CO$_2$H. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 60% ACN in H$_2$O in 50 min; flow rate: 1 mL/min; R.T.: 6 (27.01 min); B. Ligation of BK peptide with NH$_2$-GGFQKVVA-CO$_2$H. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 60% ACN in H$_2$O in 40 min; flow rate: 1 mL/min; R.T.: 7 (21.24 min); C. Ligation of BK peptide with NH$_2$-GGGALPHA-CO$_2$H. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 60% ACN in H$_2$O in 60 min; flow rate: 1 mL/min; R.T.: 8 (26.78 min); D. Ligation of BK peptide with NH$_2$-GGGAVPYPQR-CO$_2$H. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 40% ACN in H$_2$O in 50 min; flow rate: 1 mL/min; R.T.: 9 (33.06 min); E. Ligation of BK peptide with NH$_2$-GGGALKAWSVAR-CO$_2$H. HPLC conditions: 10% ACN in H$_2$O (both containing 0.1% TFA) to 60% ACN in H$_2$O in 60 min; flow rate: 1 mL/min; R.T.: 10 (29.04 min); F. Ligation of BK peptide with NH$_2$-GGGIHPFAQTQSPLVYP-CO$_2$H. HPLC conditions: 5% ACN in H$_2$O (both containing 0.1% TFA) to 95% ACN in H$_2$O in 10 min; flow rate: 1 mL/min; R.T.: 11 (9.64 min).
A

\[ [M+H]^+ \]

6

B

\[ [M+H]^+ \]

7
Figure S18. MALDI-TOF MS of ligation product of ACE inhibitory peptides. A. 6, calcd: 2155.5, observed 2156.4 [M+H]⁺; B. 7, calcd: 2400.8, observed 2401.5 [M+H]⁺; C. 8, calcd: 2274.6, observed 2275.8 [M+H]⁺; D. 9, calcd: 2596.9, observed 2598.1 [M+H]⁺; E. 10, calcd: 2768.2, observed 2769.4 [M+H]⁺; F. 11, calcd: 3267.7, observed 3268.6 [M+H]⁺.
iii. SrtA-mediated ligation of 17 with protein in one-pot

Dried peptide resin 17 (0.6 µmol) was swollen in 100 µL reaction buffer (Tris-HCl 0.3 M pH 7.5, 150 mM NaCl, 5 mM CaCl2, and 0.5 mM mercaptoethanol) for 30 min. Then 20 µL SrtA (100 µM dissolved in buffer) and 5 eq insulin were added. The final volume was 200 µL. After the reaction was kept at 37 °C for 20 h, 20 µL of the reaction mixture was withdrawn and quenched with the same volume of 0.1% of TFA. The aliquots were then analyzed by HPLC using a RP C18 column and the fractions were analyzed by MALDI-TOF MS.

In large scale reaction: 10 mg of dry peptide PEGA resin 17 was treated with SrtA and insulin (5 eq) for 20 h. 3.1 mg of product 18 (yield 71%) was afforded after isolation by semi preparative HPLC.

In another procedure, 10 equiv. of dried peptide resin 17 (1.8 µmol) was swollen in 100 µL reaction buffer (Tris-HCl 0.3 M pH 7.5, 150 mM NaCl, 5 mM CaCl2, and 0.5 mM mercaptoethanol) for 30 min. Then 20 µL SrtA (100 µM dissolved in buffer) and 0.18 µmol insulin were added. The final volume was 200 µL. After the reaction was kept at 37 °C for 20 h, 20 µL of the reaction mixture was withdrawn and quenched with the same volume of 0.1% of TFA. The aliquots were then analyzed by HPLC using a RP C18 column and the fractions were analyzed by MALDI-TOF MS. The conversion yield is 46% based on the HPLC analysis.
Figure S20. SrtA-mediated *in situ* N-terminal modification of insulin using PEGA resin 17 in one-pot. Reaction condition: dried peptide resin 17 (1.8 µmol), 10 uM SrtA, 0.3 M Tris buffer (pH 7.5) containing 150 mM NaCl, 5 mM CaCl$_2$, and 0.5 mM mercaptoethanol and 1 equiv. of insulin.