Supporting Information

Electrochemical Protease Biosensor Based on Enhanced AC Voltammetry Using Carbon Nanofiber Nanoelectrode Arrays

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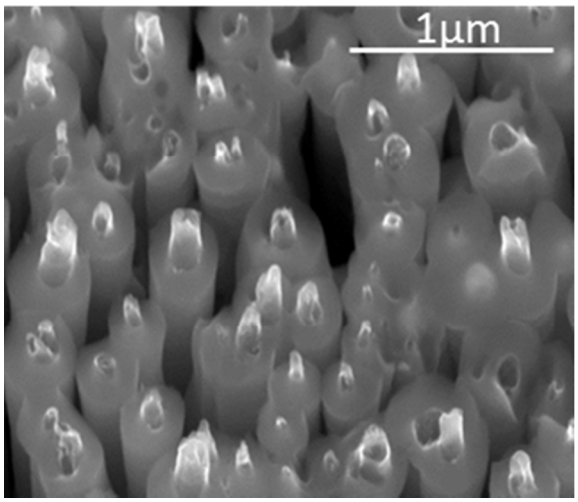

Figure S1. Field-emission scanning electron microscopy image at 45° perspective view shows that VACNFs of ~150 nm in average diameter are encapsulated in SiO_2 matrix leaving only the tips exposed.

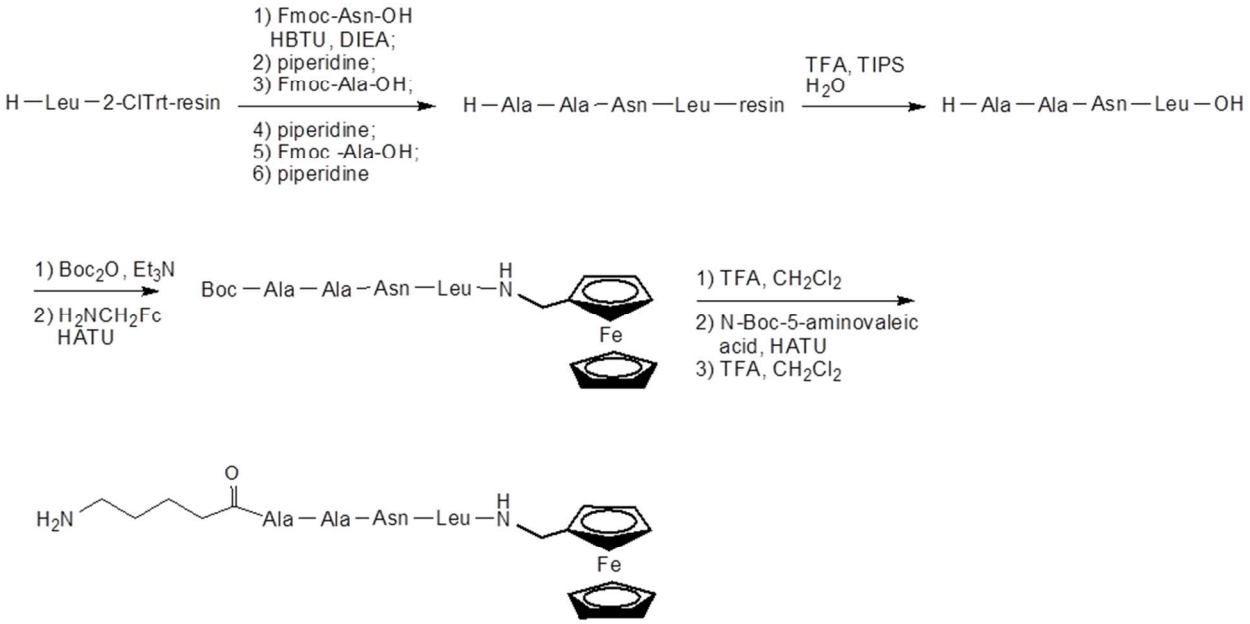

Figure S2. Synthesis of H_2N-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc for legumain detection.

Experimental Details for Figure S2: Synthesis of H_2N-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc for legumain detection

Synthesis of tetrapeptide H-Ala-Ala-Asn-Leu-OH (Figure S2) using a CEM microwave peptide synthesizer. Leucine chlorotrityl resin (0.54 mmol/g; 200 mesh) was purchased from Peptide International. The standard coupling procedure is followed. To 1 g (0.54 mmol) of resin was added a solution of Fmoc-amino acid (1.62 mmol, 3 equiv.) and HBTU (1.46 mmol, 2.7 equiv.) in dry DMF (13 mL) containing 4.2 % diisopropylethyl amine. The mixture was subjected to microwave irradiation (25 W, 5 min, 75 ^{o}C) with stirring. The reaction mixture was filtered and washed with DMF (10 mL each, 5 times). The standard procedure for the removal of Fmoc protecting group is followed. A solution of 20 mL of 20% piperidine in DMF was added to the above resin and subjected to microwave irradiation (50 W, 3 min, 75 ^{o}C). The reaction mixture was filtered and washed with DMF (10 ml each, 5 times). The standard procedure for cleavage of the peptide from resin is followed. The above resin was washed with dichloromethane (20 mL) and mixed with 20 mL of a cleavage cocktail solution consists of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), and 2.5% water. The mixture was irradiated under a microwave reactor (20 W, 38 ^{o}C) for 18 min. The reaction mixture was filtered into a 100 mL flask and diluted with 100 mL of cold hexane:ether (1:1) to precipitate out the desired peptide. The solid peptide was collected by centrifugation (2500 rpm) and washed three times with cold hexane:ether (1:1) to give H-Ala-Ala-Asn-Leu-OH as white solids (95 mg). MS calcd for $C_{16}H_{30}N_5O_6$ $(M+H)^+$ 388.2, found 388.4.

Synthesis of Boc-Ala-Ala-Asn-Leu-OH. To a solution of H-Ala-Ala-Asn-Leu-OH (50 mg, 0.13 mmol) were added triethylamine (36 μL, 0.26 mmol) and dioxane:water (1:1) (10 mL), and the resulting solution was stirred for 10 min. To it, di-*t*-butyl dicarbonate (Boc_2O) (56 mg, 0.26 mmol) was added and the solution stirred for 12 h at r.t. The reaction solution was concentrated to dryness yielding Boc-Ala-Ala-Asn-Leu-OH as a white solid (60 mg; 96% yield). MS calcd for $C_{21}H_{37}N_5NaO_8$ $(M+Na)^+$ 510.3, found 510.4.

Synthesis of Boc-Ala-Ala-Asn-Leu-NHCH$_2$-Fc. To a solution of Boc-Ala-Ala-Asn-Leu-OH (60 mg, 0.12 mmol) in DMF (2 mL) under argon was added HATU (47 mg, 0.12 mmol) and the solution was stirred for 10 min. To it, aminomethylferrocene (26 mg, 0.12 mmol) was added, and the solution was stirred at r.t. for 2 h, filtered, and separated on a HPLC using a preparative column (Phenomenex-Jupiter C18) and eluting with 40% acetonitrile/water to 80 % acetonitrile/water over 40 min with a 10 ml/min flow rate. The fractions containing the desired

product were combined and lyophilized to yield Boc-HN-Ala-Ala-Asn-Leu-$NHCH_2$-Fc as a yellow solid (40 mg; 48% yield). MS calcd for $C_{32}H_{48}FeN_6NaO_7$ $(M+Na)^+$ 707.3, found 707.5.

Synthesis of H_2N-Ala-Ala-Asn-Leu-$NHCH_2$-Fc. Boc-Ala-Ala-Asn-Leu-$NHCH_2$-Fc (18 mg, 26 μmol) was dissolved in 2 mL of 10% TFA in dichloromethane and stirred at r.t. for 30 min, concentrated gently on a rotovap to give a residue. The residue was dissolved in deionized water (2 mL), frozen, and lyophilized to give H-Ala-Ala-Asn-Leu-$NHCH_2$-Fc as a green solid (15 mg; 100% yield). MS calcd for $C_{27}H_{40}FeN_6NaO_5$ $(M+Na)^+$ 607.2, found 607.3.

Synthesis of Boc-HN-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc. To a solution of *N*-Boc-5-aminovaleric acid (5.6 mg, 26 μmol) in DMF (2 mL) was added HATU (9 mg, 24 μmol), and the resulting solution was stirred at r.t. for 10 min. This solution was cannulated to tetrapeptide H-Ala-Ala-Asn-Leu-$NHCH_2$-Fc (10 mg, 17 μmol), and the resulting solution was stirred for 2 h at r.t., filtered, and separated on a HPLC using a preparative column (Phenomenex-Jupiter C18) and eluting with 40% acetonitrile/water to 80 % acetonitrile/water over 40 min with a 10 ml/min flow rate. The fractions containing the desired product were combined and lyophilized to yield Boc-HN-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc as a yellow solid (6 mg; 45% yield). MS calcd for $C_{37}H_{57}FeN_7NaO_8$ $(M+Na)^+$ 806.4, found 806.5.

HPLC method: (gradient elution), λ=254 nm

Synthesis of H_2N-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc. Boc-HN-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc (6 mg, 8 μmol) was dissolved in 1 mL of 10% TFA in dichloromethane and stirred at r.t. for 30 min. The reaction solution was concentrated gently on a rotary evaporator to give a residue which was dissolved in deionized water (2 mL), frozen and lyophilized to give H_2N-$(CH_2)_4$CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc as a green solid (4 mg; 75% yield). MS calcd for $C_{32}H_{50}FeN_7O_6$ $(M+H)^+$ 684.3, found 684.4.

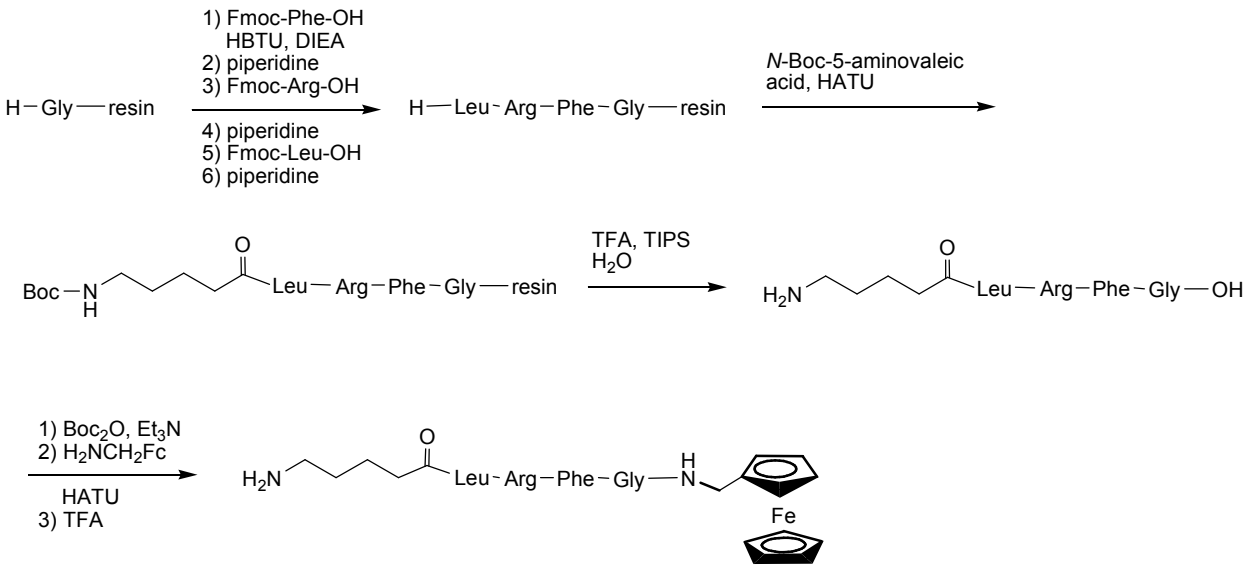

Figure S3. Synthesis of H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc for cathepsin B detection.

Experimental Details for Figure S3: Synthesis of H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc for cathepsin B detection

Synthesis of tetrapeptide H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-OH (Figure S3) using a CEM microwave peptide synthesizer. The Glycine chlorotrityl resin (0.48 mmol/g; 200 mesh) was purchased from peptide international. The procedures for coupling, removal of Fmoc protecting group, and cleavage from the resin were identical to that described above for the synthesis of legumain substrate. From 1.2 g (0.57 mmol) of Glycine chlorotritylresin, 0.32 g (94% yield) of H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-OH was obtained as a white solid. MS calcd for $C_{28}H_{47}N_8O_6$ $(M+H)^+$ 591.4, found 591.2.

Synthesis of Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-OH. A solution of sodium bicarbonate (86 mg, 1.2 mmol) and H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-OH (0.300 g, 0.5 mmol) in 30 mL of dioxane:water (1:1) was stirred at r.t. for 10 min. To it, di-*t*-butyl dicarbonate (Boc_2O) (0.22 g, 1.2 mmol) was added, and the solution stirred for 12 h, concentrated to dryness yielding a white solid (0.35 g; 99% yield). MS calcd for $C_{33}H_{55}N_8O_8$ $(M+H)^+$ 691.4, found 691.7.

Synthesis of Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc. A solution of Boc-$NH(CH_2)_4$CO-Leu-Arg-Phe-Gly-OH (60 mg, 0.09 mmol) and HATU (47 mg, 0.12 mmol) in DMF (2 mL) was stirred at r.t. for 10 min. To it, aminomethylferrocene (26 mg, 0.12 mmol) was added, and the solution was stirred for 2 h, filtered, and separated on a HPLC using a preparative column (Phenomenex-Jupiter C18) and eluting with 40% acetonitrile/water to 80 % acetonitrile/water over 40 min with a 10 ml/min flow rate. The fractions containing the desired product were combined and lyophilized to yield Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc as a yellow solid (40 mg; 50% yield). MS calcd for $C_{44}H_{66}FeN_9O_7$ $(M+H)^+$ 888.4, found 888.6.

Synthesis of H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc. A solution of Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc (50 mg; 56 μmol) in 2 mL of 10% TFA in dichloromethane was stirred at r.t. for 30 min, concentrated gently on a rotary evaporator, dissolved in deionized water (2 mL), frozen, and lyophilized to give H_2N-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc as a green solid (43 mg; 98% yield). MS calcd for $C_{39}H_{58}FeN_9O_5$ $(M+H)^+$ 788.4, found 788.5.

Verification of the inactivity of the guanidine NH function of arginine residue of Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc. The following reaction has been carried out to verify that the guanidine NH function of arginine residue of Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc does not form amide bond with the carboxylic function on carbon nanofibers. To a 1 mL aqueous solution of Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc (3 mg, 3.4 μmol), benzoic acid (0.4 mg, 3.4 μmol), and *N*-hydroxysuccinimide (0.4 mg, 3.4 μmol) was added with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.66 mg, 3.4 μmol), and the resulting solution was stirred at room temperature for 2 hours. Mass spectrometry analysis showed that no reaction took place between the NH group of arginine residue and the carboxylic acid moiety of benzoic acid. Boc-HN-$(CH_2)_4$CO-Leu-Arg-Phe-Gly-$NHCH_2$-Fc remained unchanged. The test suggested that the NH moieties in the side chain of arginine residue does not react with the carboxylic acid function of carbon nanofibers. The most likely route for the peptide to be attached to the VACNF NEA is by forming the amide bond through the $–NH_2$ group at the distal end of the linker (as shown in Figure S3).

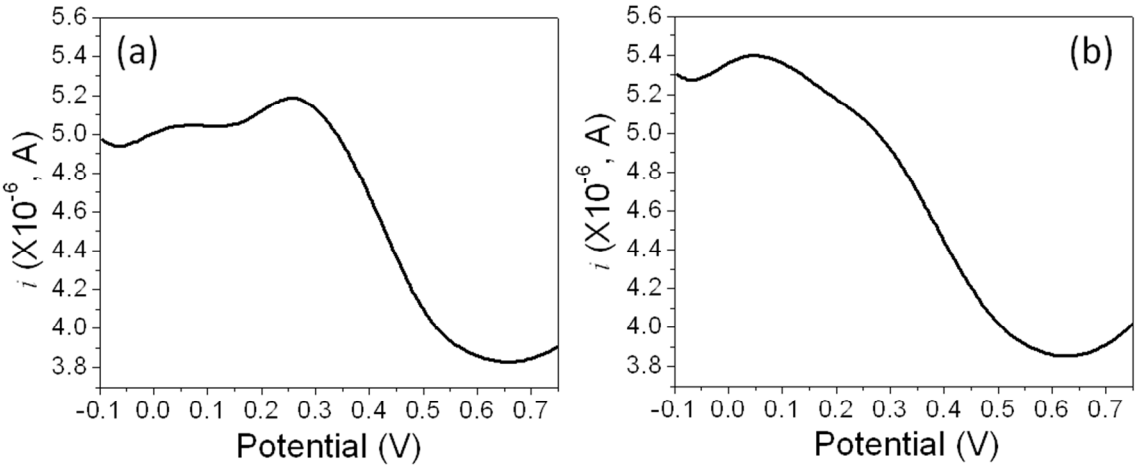

Figure S4. The ACV of H_2N-$(CH_2)_4$-CO-Leu-Arg-Phe-Gly-NH-CH_2-Fc immobilized on the GCE (a) from the initial measurement and (b) from the measurement after 20 minutes. The AC frequency was 30 Hz and the amplitude was 25 mV. The measurement was done in 250 μL of 25 mM MES solution (pH 5.0).

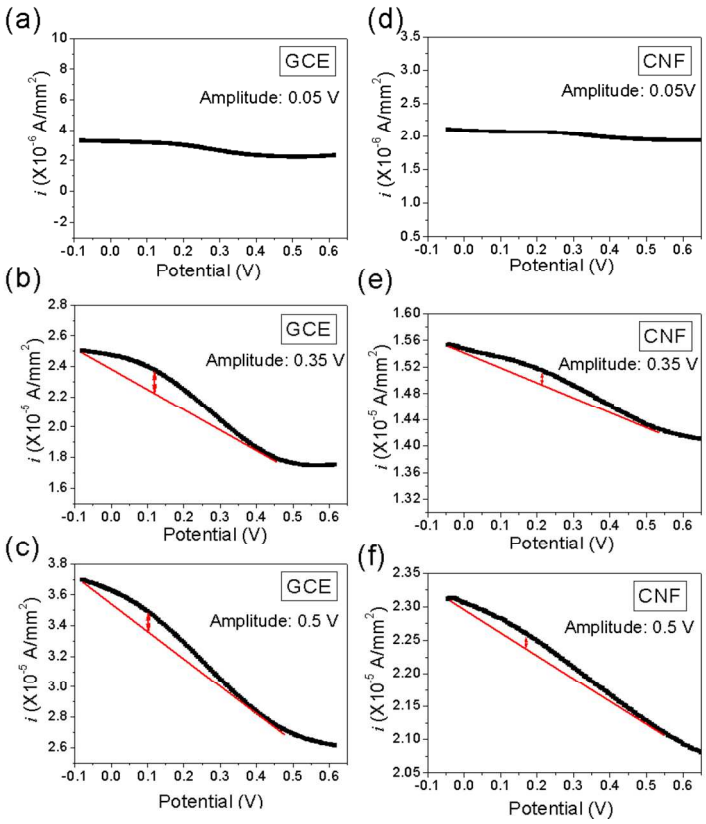

Figure S5. Comparison of AC voltammograms (ACVs) of H_2N-$(CH_2)_4$-CO-Ala-Ala-Asn-Leu-NH-CH_2-Fc immobilized on a macroscopic glassy carbon electrode (GCE) (a–c) and a VACNF NEA (d–f) at the AC voltage amplitude of 0.05, 0.35 and 0.5 V. All the measurements were done in 500 µL of 50 mM MES (pH 5.0) and 250 mM NaCl. Sinusoidal waves with fixed frequency of 40 Hz for GCE and frequency of 1750 Hz for CNF NEA were superimposed on a DC staircase ramp from -0.05 to 0.65 V at a scan rate of 10 mV/s. The measured average AC current at each point was normalized by the 7.1 mm^2 geometric surface area defined by the 3-mm i.d. O-ring. The real CNF surface area is ~100 times less.

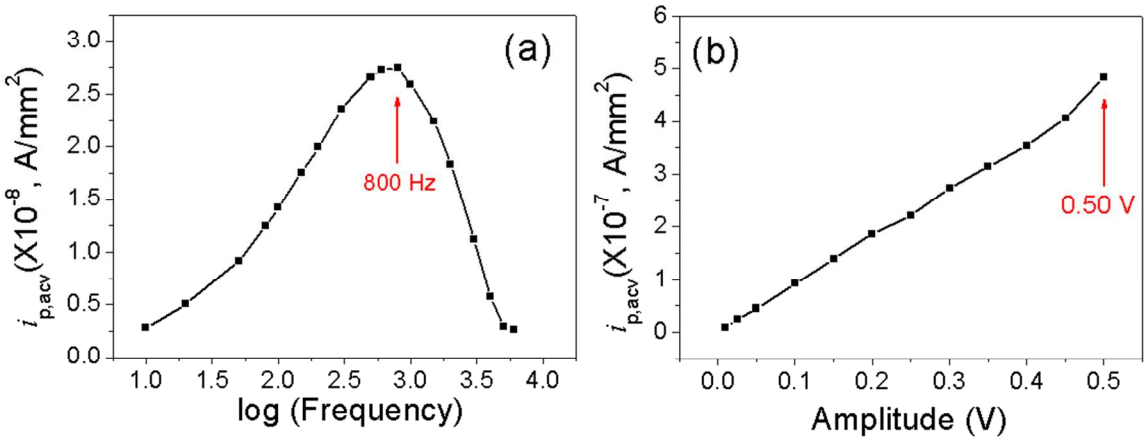

Figure S6. (a) Background-corrected ACV peak current density $i_{p,acv}$ (normalized to the 7.1 mm^2 geometric electrode area) of H_2N-$(CH_2)_4$-CO-Leu-Arg-Phe-Gly-NH-CH_2-Fc immobilized on a VACNF NEA plotted against the logarithm of the frequency. (b) Background-corrected ACV peak current density $i_{p,acv}$ (normalized to the 7.1 mm^2 geometric electrode area) of H_2N-$(CH_2)_4$-CO-Leu-Arg-Phe-Gly-NH-CH_2-Fc immobilized on a VACNF NEA plotted against the amplitude. All the measurements were done in 250 µL of 25 mM MES (pH 5.0). Note: The real CNF surface area is ~100 times less than the geometric surface area defined by the O-ring.

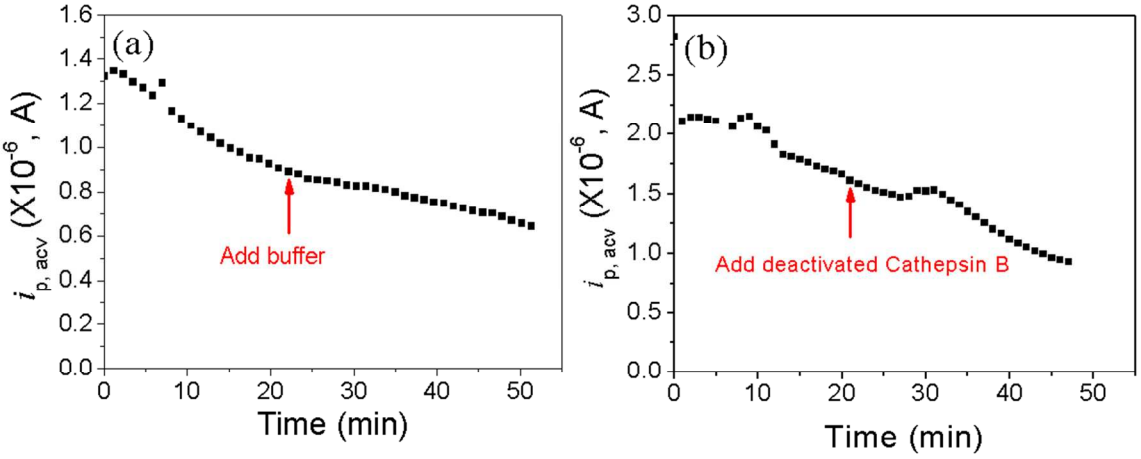

Figure S7. Negative control experiments. The change of the peak current ($i_{p,acv}$) of H_2N-$(CH_2)_4$-CO-Leu-Arg-Phe-Gly-NH-CH_2-Fc immobilized on a VACNF NEA in continuously repeated ACV measurements while (a) 25 µL of activation buffer consisting of 5 mM DTT and 25 mM MES (pH 5.0) and (b) the same buffer containing 9.8 ng μL^{-1} (338 nM) deactivated cathepsin B were added into the electrochemical cell containing 250 µL of 25 mM MES (pH 5.0). All ACV measurements were carried out at f = 800 Hz and AC voltage amplitude V_0 = 150 mV.

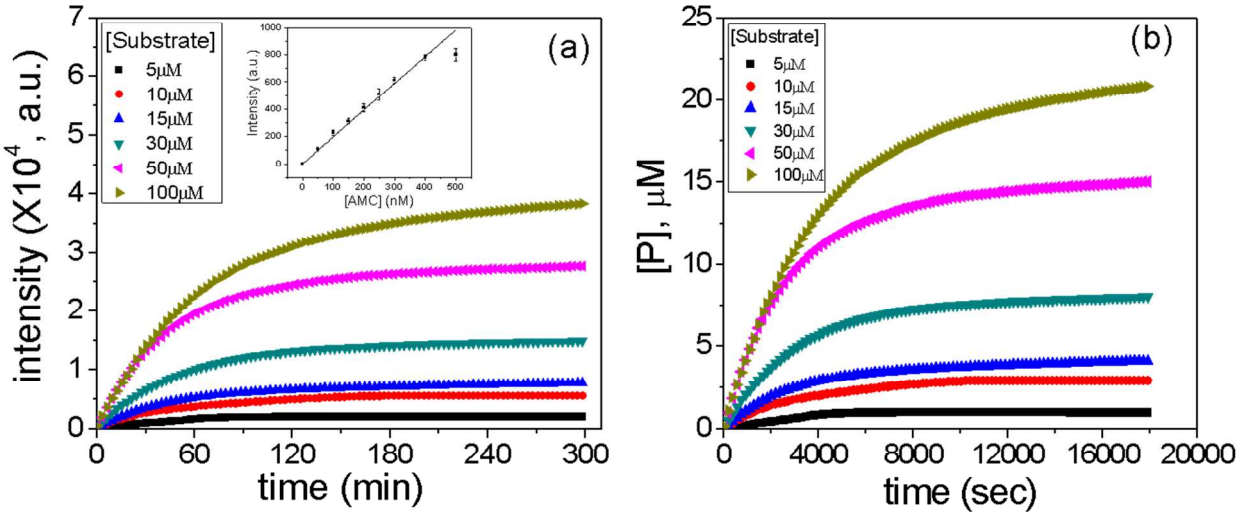

Figure S8. (a) The kinetic curves of the fluorescence intensity of 0.1 ng μL^{-1} (3.45 nM) cathespin B reacting with different concentrations of substrate Z-Leu-Arg-AMC in 25 mM MES (pH 5.0). The cleavage between Arg and AMC released free AMC products which were strongly fluorescent and gave the increased fluorescence intensity. Inset: calibration curve of the fluorometer. (Excitation wavelength: 365 nm, emission wavelength: 410–460 nm) (b) The calibrated concentration of free AMC product during cathepsin B cleavage of various concentrations of substrate Z-Leu-Arg-AMC.

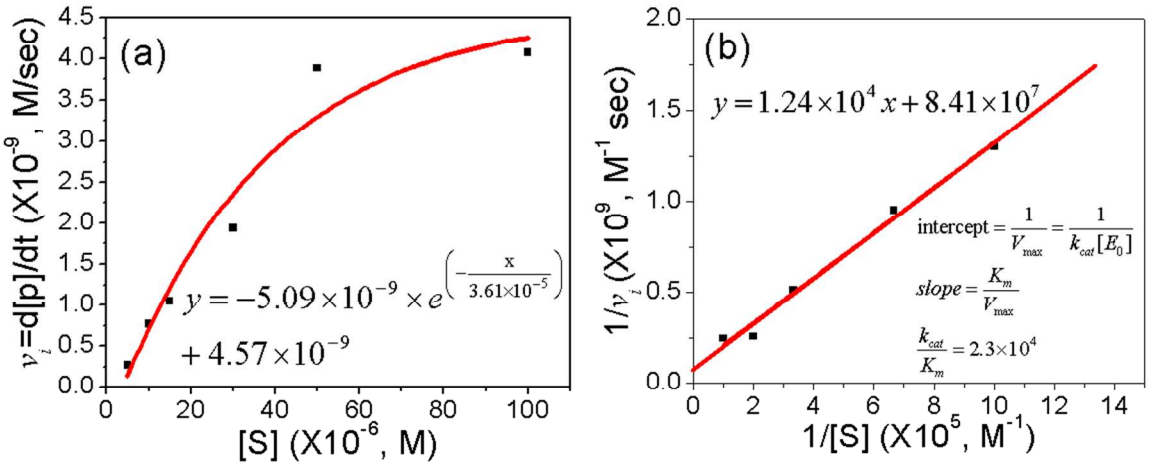

Figure S9. (a) The initial proteolysis reaction rate v_i of cathepsin B plotted against the starting concentration of the substrate Z-Leu-Arg-AMC. The reaction rate was calculated from the slope of the tangent of the initial segment of the curves shown in Figure S8b. (b) Replot of the data presented in (a) and derivation of various catalytic constants. The value of k_{cat}/K_m for cathepsin B was calculated as 2.3 x 10^4 $M^{-1}s^{-1}$.

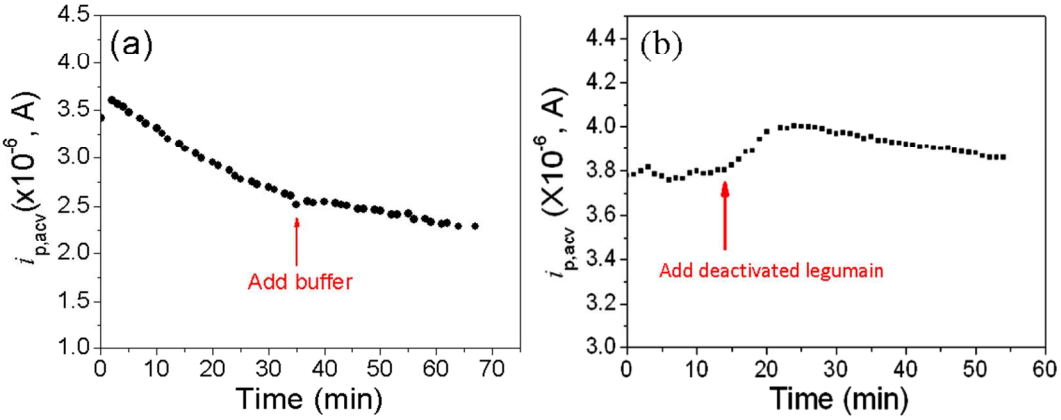

Figure S10. Negative control experiments for legumain proteolysis. The change of the peak current ($i_{p,acv}$) of H_2N-$(CH_2)_4$-CO-Ala-Ala-Asn-Leu-NH-CH_2-Fc immobilized on a VACNF NEA in continuously repeated ACV measurements (a) while 11 μL of activation buffer consisting of 50 mM CH_3COONa (pH = 4.0, adjusted by adding acetic acid) and 100 mM NaCl was added into the electrochemical cell containing 250 μL of 50 mM MES (pH 5.0) and 250 mM NaCl; (b) similar experiments by including 90.9 ng μL^{-1} (1.90 μM) deactivated legumain in the activation buffer. All ACV measurements were carried out at f = 1750 Hz and AC voltage amplitude V_0 = 150 mV.

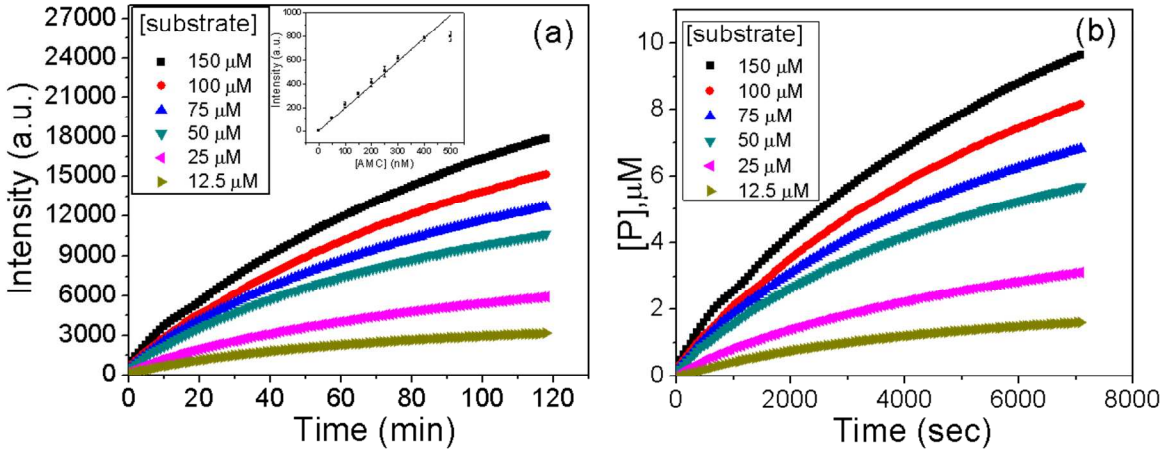

Figure S11. (a) The fluorescence intensity curves of 0.5 ng μL^{-1} (10.2 nM) legumain reacting with different concentration of substrate Z-Ala-Ala-Asn-AMC in the assay buffer consisting of 50 mM MES (pH 5.0) and 250 mM NaCl. Inset: calibration curve of the fluorometer. (excitation wavelength: 365 nm, emission wavelength: 410–460 nm). (b) The calibrated free AMC product concentration during legumain cleavage the substrate Z-Ala-Ala-Asn-AMC.

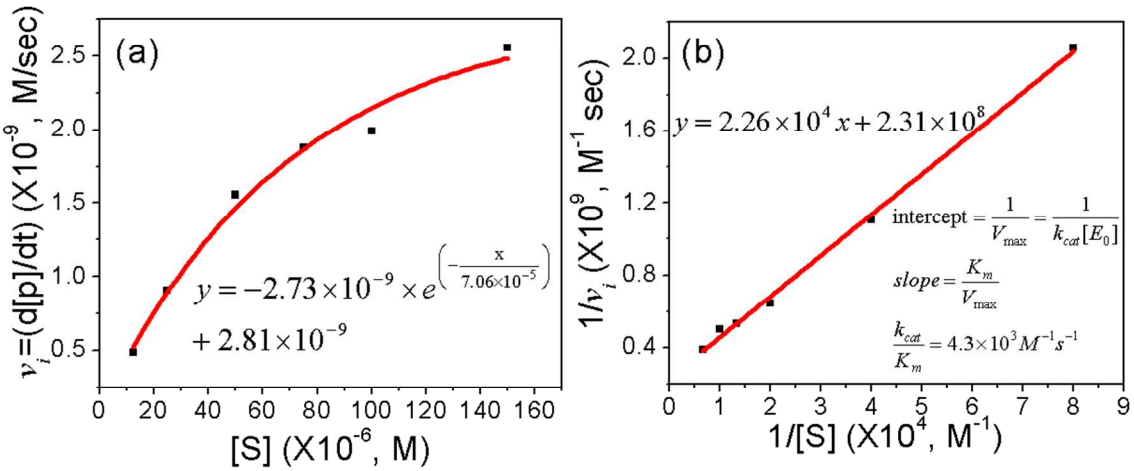

Figure S12. (a) The initial proteolysis reaction rate v_i of legumain plotted against the concentration of the substrate Z-Ala-Ala-Asn-AMC. The reaction rate was calculated from the slope of the tangent of the initial segment of the curves shown in Figure S11b. (b) Replot of the

data presented in (a) and the derivation of various catalytic constants. The value of k_{cat}/K_m for legumain was calculated as 4.3 x 10^3 $M^{-1}s^{-1}$.

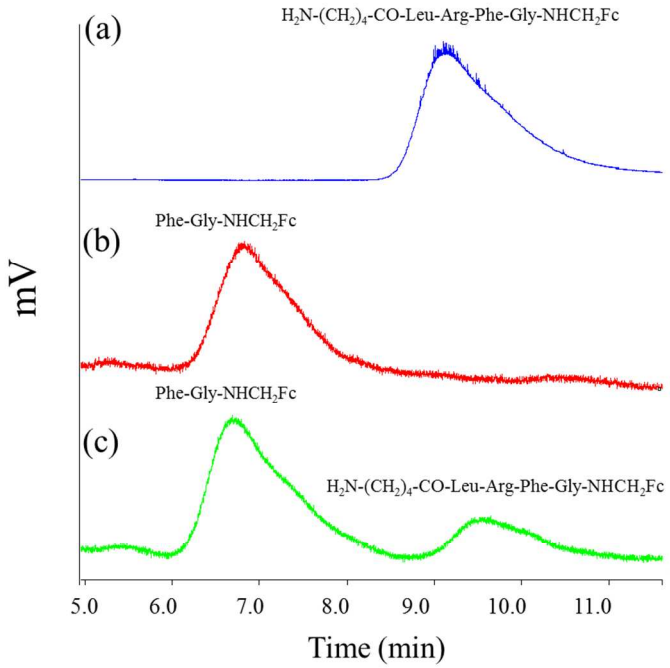

Figure S13. (a) HPLC chart of H_2N-$(CH_2)_4$-CO-Leu-Arg-Phe-Gly-$NHCH_2Fc$ in 25 mM MES (pH 5.0) buffer. The tetrapeptide appeared at 9.4 minute in the HPLC chart and its structure was verified by mass spectrometry analysis. (b) HPLC chart of Phe-Gly-$NHCH_2Fc$ in 25 mM MES (pH 5.0) buffer. The dipeptide appeared at 6.7 minute in the HPLC chart and its structure was verified by mass spectrometry analysis. (c) HPLC chart of 6.35 mM H_2N-$(CH_2)_4$-CO-Leu-Arg-Phe-Gly-$NHCH_2Fc$ in 25 mM MES (pH 5.0) buffer incubated with 4.95 ng μl^{-1} (0.17 μM) ***cathepsin B*** for 2 hours. Absorbance peak at 6.7 minute was collected and lyophilized and mass spectrometry analysis showed it to be Phe-Gly-$NHCH_2Fc$. HPLC Method: a gradient elution from 10% to 50% of acetonitrile in 0.1 M phosphate buffer (pH = 6) over 40 minutes was used with a flow rate of 10 ml/min and detection wavelength λ at 254 nm. The column is Xperchrom AEGIS C18 120A 10 μm (Phenomenex).

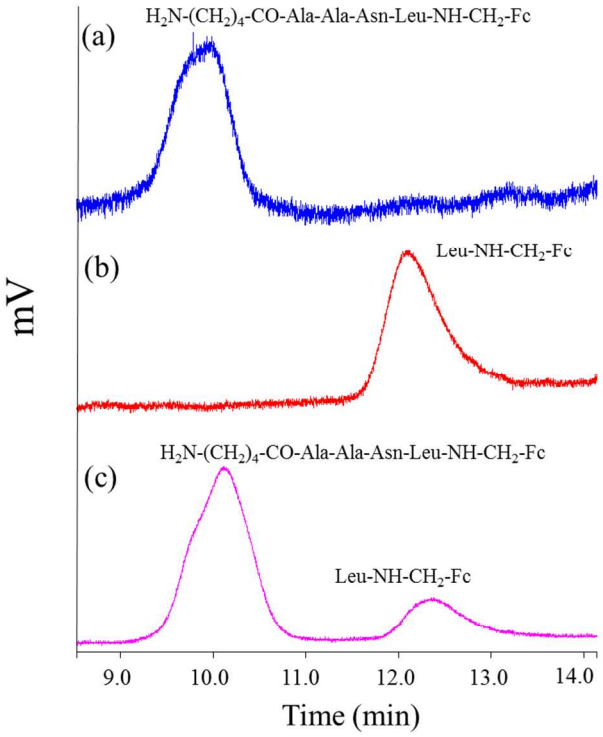

Figure S14. (a) HPLC chart of H_2N-$(CH_2)_4$-CO-Ala-Ala-Asn-Leu-$NHCH_2Fc$ in 50 mM MES (pH 5.0) and 250 mM NaCl. The tetrapeptide appeared at 9.7 minute in the HPLC chart and its structure was verified by mass spectrometry analysis. (b) HPLC chart of Leu-$NHCH_2Fc$ in 50 mM MES (pH 5.0) and 250 mM NaCl. The Leu-$NHCH_2Fc$ appeared at 12.2 min in the HPLC chart and its structure was verified by mass spectrometry analysis. (c) HPLC chart of 100 μM H_2N-$(CH_2)_4$-CO-Ala-Ala-Asn-Leu-$NHCH_2Fc$ in 50 mM MES (pH 5.0) and 250 mM NaCl incubated with 98.7 ng/μL (2.01 μM) ***legumain*** for 2 hours. Absorbance peak at 12.2 min was collected and lyophilized and mass spectrometry analysis showed it to be Leu-$NHCH_2Fc$. HPLC Method: a gradient elution from 10% to 50% of acetonitrile in 0.1 M phosphate buffer (pH=6) over 40 minutes was used with a flow rate of 10 ml/min and detection wavelength λ at 254 nm. The column is Xperchrom AEGIS C18 120A 10 μm (Phenomenex).

Experimental for Figure S13b: Synthesis of H-Phe-Gly-$NHCH_2$-Fc for HPLC analysis. Boc-Gly-OH was coupled with H_2N-CH_2-Fc activated by HATU in DMF to yield Boc-Gly-$NHCH_2$-Fc. The Boc protecting group was removed using 10% TFA in dichloromethane after which the resulting H-Gly-$NHCH_2$Fc was coupled with Boc-Phe-OH using EDC and DMAP as activating reagents in dichloromethane to furnish Boc-Phe-Gly-$NHCH_2$-Fc. Removal of Boc protecting group using 10% TFA in dichloromethane gave H-Phe-Gly-$NHCH_2$-Fc.

Synthesis of Boc-Gly-$NHCH_2$-Fc. To a solution of Boc-Gly-OH (32 mg, 0.19 mmol) in dry DMF under argon was added HATU (77 mg, 0.20 mmol), and the resulting solution was stirred for 5 minutes. To the above solution, NH_2CH_2-Fc (40 mg, 0.185 mmol) was added and the solution stirred at room temperature for 12 hours. The reaction solution was partitioned between 20 mL of deionized water and 20 mL of dichloromethane. Organic layer was removed and aqueous layer was extracted once with 20 mL of dichloromethane. The organic layers were combined and dried ($MgSO_4$), filtered, concentrated, and column chromatographed on silica gel using a mixture of dichloromethane and methanol (30:1) as eluant to give 25 mg (36% yield) of an orange oil. MS calc'd for $C_{18}H_{24}FeN_2NaO_3$ 395.1 $(M+Na)^+$, found to be 395.1.

Synthesis of H-Gly-$NHCH_2$-Fc. To a solution of Boc-Gly-$NHCH_2$-Fc (25 mg, 0.092 mmol) in 4 mL of dichloromethane under argon was added 0.4 mL of trifluoroacetic acid, and the solution was stirred for 1 hour at room temperature. The resulting solution was gently concentrated on a rotary evaporator and placed under high vacuum for 1 hour to yield a green solid. The material is used in the next experiment without purification. MS calc'd for $C_{13}H_{17}FeN_2O$ 273.1 $(M+H)^+$, found to be 273.2.

Synthesis of Boc-Phe-Gly-$NHCH_2$-Fc. A solution of H-Gly-$NHCH_2$-Fc (25 mg, 0.092 mmol)

(obtained from the aforementioned experiment), Boc-Phe-OH (24 mg, 0.092 mmol), 4-(dimethylamino)pyridine (23 mg, 0.184 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (35 mg, 0.184 mmol) in 1 mL of dry DMF under argon was stirred for 1 minute at room temperature. Dry dichloromethane (2 mL) and additional DMAP (until pH of the reaction solution was slightly basic) were added, and the reaction solution stirred at room temperature for 12 hours. The reaction mixture was partitioned between 20 mL of dichloromethane and 20 mL of deionized water, and 2 N hydrochloric acid was added until pH reached about 2. The organic layer was separated using a separatory funnel, and the aqueous layer was extracted four times (20 mL each) with dichloromethane. The combined organic layers were washed with brine, dried (anhydrous Na_2SO_4), filtered, concentrated, and column chromatographed on silica gel using a mixture of dichloromethane and methanol (40:1) as eluant to give Boc-Phe-Gly-$NHCH_2$-Fc as a yellow oil (23 mg, 48% yield). MS calc'd for $C_{27}H_{33}FeN_3NaO_4$ 542.1 $(M+Na)^+$ and found to be 541.9.

Synthesis of H-Phe-Gly-$NHCH_2$-Fc. A solution of Boc-Phe-Gly-$NHCH_2$-Fc (23 mg, 0.044 mmol) in 5 mL of 10% trifluoroacetic acid in dichloromethane was stirred at room temperature for 1 hour. The reaction solution was gently concentrated on a rotary evaporator, then 20 mL of dichloromethane was added and concentrated again. Addition of dichloromethane and subsequent concentration were repeated a total of 3 times in an effort to remove all residual trifluoroacetic acid. The resulting residue was dried under vacuum to give the title compound as a green solid (18 mg, 98% yield). ^1H NMR ($CDCl_3$) δ 7.3 (s, 5 H), 7.12 (s, 2 H), 5.4 (s, 1 H), 4.4 - 4.0 (m, 9 H), 3.85 (m, 2 H), 3.5 (s, 2 H), 3.2 (s, 2 H), 2.85 (s, 2 H); MS calc'd for $C_{22}H_{25}FeN_3NaO_2$ 442.1 $(M+Na)^+$, found to be 442.1.

Experimental for Figure S14b: Synthesis of H-Leu-$NHCH_2$-Fc for HPLC analysis.

To a solution of Fmoc-Leu-OH (126 mg, 0.36 mmol) and HATU (123 mg, 0.33 mmol) in DMF (1 mL) was added NH_2CH_2-Fc (70 mg, 0.33 mmol), and the resulting solution was stirred at room temperature for 30 min. It was diluted with ethyl acetate (100 mL), and the organic layer was washed with water (50 mL x 3) and brine (50 mL), dried ($MgSO_4$), filtered, and concentrated to yield a brown oil, which was purified by silica gel chromatography using 50% EtOAc/hexane as eluant to give Fmoc-Leu-$NHCH_2$-Fc (98 mg, 54% yield) as a yellow solid. MS calc'd for $C_{32}H_{34}FeN_2NaO_3$ 573.2 $(M+Na)^+$, found to be 573.3.

A solution of Fmoc-Leu-$NHCH_2$-Fc (81 mg, 0.15 mmol) in 20% piperidine and DMF (5 mL) was stirred at room temperature for 30 min. DMF was removed under reduced pressure and the resulting crude oil was purified by column chromatography using a mixture of MeOH and EtOAc (5:95) as eluant to give H-Leu-$NHCH_2$-Fc as a yellow solid (33 mg, 67% yield). MS calc'd for $C_{17}H_{24}FeN_2NaO$ 351.1 $(M+Na)^+$, found to be 351.1.

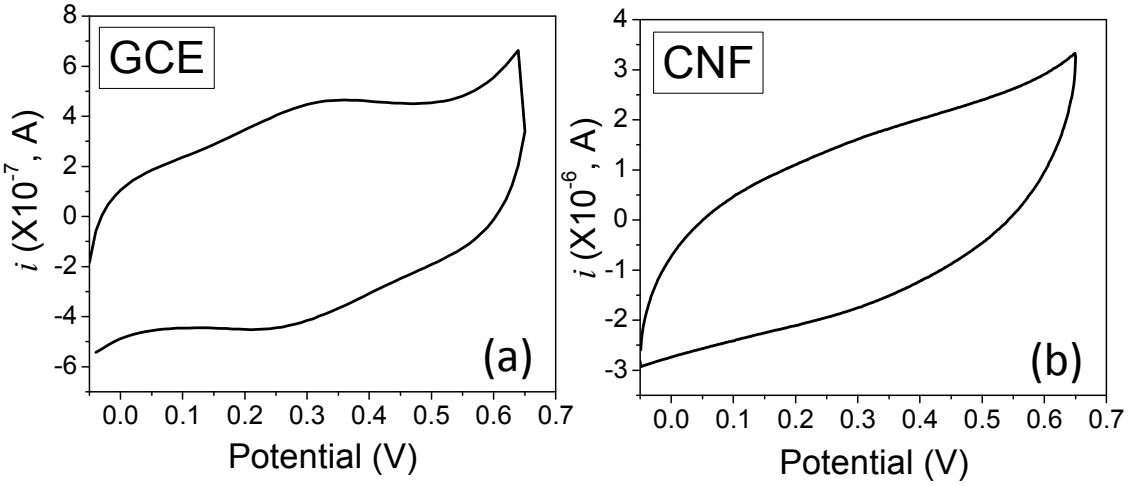

Figure S15. Comparison of cyclic voltammetric measurements of H_2N-$(CH_2)_4$-CO-Ala-Ala-Asn-Leu-$NHCH_2$-Fc immobilized on (a) a macro-GCE and (b) a VACNF NEA. The

measurements were carried out at the scan rate of 50 mV s^{-1} in 1 mL of 1.0 M KCl.

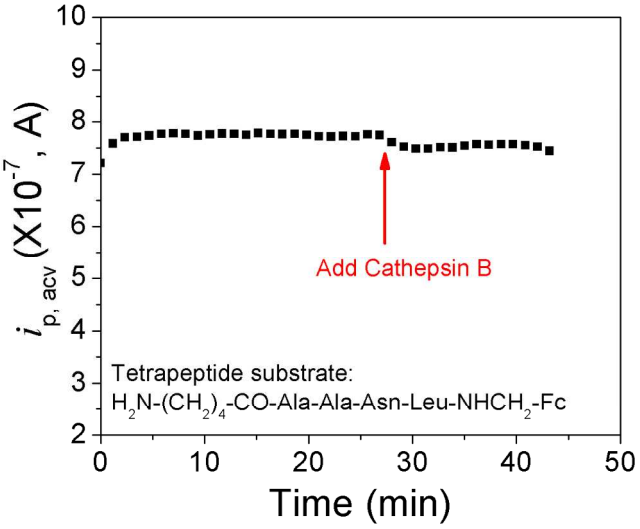

Figure S16. Preliminary specificity testing result. The change of the peak current ($i_{p,acv}$) of H_2N-$(CH_2)_4$-CO-Ala-Ala-Asn-Leu-NH-CH_2-Fc immobilized on a VACNF NEA in continuously repeated ACV measurements while 9.8 ng μL^{-1} (338 nM) activated cathepsin B in 25 µL of activation buffer consisting of 5 mM DTT and 25 mM MES (pH 5.0) was added into the electrochemical cell containing 250 µL of 25 mM MES (pH 5.0).