

Develop multiplex electrochemical biosensor arrays for cancer diagnosis based on protease activity profiling

by

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AN ABSTRACT OF A DISSERTATION

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## Abstract

Proteases are critically involved in the progression of cancer, cardiovascular disease, and neurodegeneration, where dysregulated enzyme activity serves as an important biomarker for early diagnosis and therapeutic monitoring. Electrochemical detection platforms offer significant advantages for protease sensing due to their high sensitivity, compatibility with miniaturization, and multiplexing capability. This dissertation presents the development, optimization, and mechanistic evaluation of a 3×3 gold (Au) microelectrode array (MEA) functionalized with ferrocene (Fc) tagged peptide substrates for sensitive and selective electrochemical detection of cancer-associated proteases. Proteolytic cleavage of surface-immobilized peptide substrates was monitored using AC voltammetry (ACV), where enzymatic activity induces an exponential decay in redox current. The inverse decay time constant ( $1/\tau$ ) was shown to follow heterogeneous Michaelis-Menten kinetics, with  $1/\tau = (k_{\text{cat}}/K_{\text{M}})[\text{Protease}]$ , enabling quantitative assessment of catalytic efficiency. During early investigations with cathepsin B (CB), a critical interference mechanism was identified. The activation reagent dithiothreitol (DTT) that is required to maintain CB in its active form was found to disrupt the Au-thiol binding in the self-assembled monolayer (SAM) linking the peptide substrate to the Au electrode. Residual DTT caused the peptide to desorb from the Au electrode during continuous ACV measurements, generating a false signal decay and masking the true proteolytic activity. By removing DTT via centrifugal filtration and incorporating EDTA to preserve the enzyme activity, accurate CB kinetics were obtained. These findings clarified the roles of DTT and EDTA in enzyme activation and revealed a fundamental limitation of Au-thiol based electrochemical biosensors when exposed to DTT-like protease activating agents at the operating potentials used for Fc redox reaction.

To improve detection under physiologically relevant neutral pH conditions, rational peptide engineering was employed. Guided by systematic fluorogenic assays, incorporation of norleucine (Nle) at the P3 position of the substrate significantly enhanced CB activity at pH 7.3. The optimized hexapeptide substrate,  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Nle-Leu-Gly-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ , demonstrated markedly improved electrochemical response on the Au MEA platform, achieving a limit of detection of  $0.43 \pm 0.08$  nM for active CB at pH 7.3. This minimal site-directed modification strategy validated sequence-dependent cleavage behavior and demonstrated that targeted amino acid substitution can substantially enhance sensitivity and specificity, establishing a generalizable framework for substrate optimization in multiplex protease sensing.

The platform was further evaluated under complex biological conditions using 3% human serum to assess matrix effects and cross-reactivity from other clinically relevant proteases, including MMP-9 and ADAM-17. Introduction of human serum resulted in a pronounced initial signal decay, attributed to surface heterogeneity and nonspecific proteolytic activity. Kinetic analysis revealed that a double exponential model more accurately described the decay behavior, distinguishing a rapid matrix-induced component from a slower proteolytic process. The slower decay component exhibited a linear dependence on serum concentration, while the rapid component saturated at higher concentrations. Investigation of metalloprotease cross reactivity revealed that the activation agent APMA, used for MMP-9 activation, also disrupted the thiol-Au binding in the SAM and introduced electrochemical interference. Similar to the earlier CB studies, removing APMA after activation by centrifugal filtration preserved the enzymatic activity and eliminated surface disruption. In contrast to CB studies, EDTA was found to suppress metalloprotease activity. For multiplex assays, a better unified buffer system still needs to be

developed. In general, the electrochemical proteolysis experiments demonstrated the highest CB activity, measurable MMP-9 activity (even in an EDTA-containing buffer), and the lowest ADAM-17 activity. Importantly, the Nle modified peptide showed no detectable cleavage by MMP-9 or ADAM-17, confirming enhanced specificity toward CB and validating rational substrate engineering as an effective strategy for minimizing the cross reactivity.

Overall, this dissertation establishes critical design principles for Au-S based electrochemical protease biosensors, including careful control of the interference by the activation agents, optimization of operating potential windows, rational peptide sequence engineering for detection in neutral pH buffers, and initial evaluation of matrix effects in complex biological samples. The Au MEA platform demonstrated here provides a scalable, multiplexing framework for profiling extracellular protease activity, representing an advancement in developing an interference-resistant electrochemical technique for early cancer diagnosis and therapeutic monitoring.

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# Table of Contents

|                                                                                                                                                                     |        |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| List of Figures .....                                                                                                                                               | xv     |
| List of Tables .....                                                                                                                                                | xxx    |
| Acknowledgements .....                                                                                                                                              | xxxiii |
| Dedication .....                                                                                                                                                    | xxxvi  |
| Preface.....                                                                                                                                                        | xxxvii |
| Chapter 1 - Introduction.....                                                                                                                                       | 1      |
| 1.1 Background and Motivation .....                                                                                                                                 | 1      |
| 1.2 Biosensors – Principles and Classification .....                                                                                                                | 2      |
| 1.3 Micro and Nano Technologies in Biosensing Applications .....                                                                                                    | 5      |
| 1.4 Proteases as Disease Biomarkers .....                                                                                                                           | 7      |
| 1.5 Protease Detection Strategies.....                                                                                                                              | 10     |
| 1.5.1 Gel-based assays .....                                                                                                                                        | 10     |
| 1.5.2 ELISA and Immunoassays.....                                                                                                                                   | 10     |
| 1.5.3 Fluorescence and Colorimetric Methods .....                                                                                                                   | 11     |
| 1.5.4 Activity-Based Probes.....                                                                                                                                    | 12     |
| 1.5.5 Electrochemical Protease Biosensors .....                                                                                                                     | 13     |
| 1.6 Challenges in Multiplex Protease Detection.....                                                                                                                 | 15     |
| 1.7 Research Objectives and Hypotheses .....                                                                                                                        | 16     |
| 1.8 Gold Microelectrode Array (Au MEA) and the fundamental principles employed in the thesis .....                                                                  | 17     |
| 1.8.1 Au MEA Fabrication.....                                                                                                                                       | 17     |
| 1.8.2 Experimental setup.....                                                                                                                                       | 21     |
| 1.8.3 Michaelis-Menten Model for Electrochemical Data Analysis.....                                                                                                 | 22     |
| 1.8.4 Alternating current voltammetry (ACV) .....                                                                                                                   | 25     |
| Chapter 2 - Mitigating Dithiothreitol Interference to Gold Electrodes in Electrochemical Detection of Cathepsin B Activity Toward Multiplex Protease Analysis ..... | 28     |
| 2.1 Introduction.....                                                                                                                                               | 28     |
| 2.2 Materials and Methods.....                                                                                                                                      | 31     |
| 2.2.1 Au MEA and Au disk electrode functionalization.....                                                                                                           | 32     |

|                                                                                                                                                                              |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 2.2.2 DTT removal after activating cathepsin B .....                                                                                                                         | 33 |
| 2.2.3 Validation of enzyme activity with fluorogenic assays .....                                                                                                            | 34 |
| 2.2.4 Electrochemical experiments .....                                                                                                                                      | 35 |
| 2.3 Results and Discussion .....                                                                                                                                             | 36 |
| 2.3.1 MEA design and principle of electrochemical detection of protease activity .....                                                                                       | 36 |
| 2.3.2 Interference issue from the activation agent DTT .....                                                                                                                 | 37 |
| 2.3.3 Impact of DTT on the redox tag's chemical stability .....                                                                                                              | 38 |
| 2.3.4 Impact of redox potential window of different redox tags.....                                                                                                          | 41 |
| 2.3.5 Optimization of cathepsin B activity detection protocols with fluorogenic assays .....                                                                                 | 45 |
| 2.3.5.1 Effect of the buffer composition and pH value on cathepsin B activity .....                                                                                          | 46 |
| 2.3.5.2 Effect of the DTT and EDTA on the activity of cathepsin B .....                                                                                                      | 47 |
| 2.3.6 Substrate-dependent proteolysis by cathepsin B: Multiplex analysis on a single MEA chip.....                                                                           | 50 |
| 2.4 Conclusion .....                                                                                                                                                         | 52 |
| Chapter 3 - Peptide Substrate Optimization for Sensitive Multiplex Electrochemical Detection of Cathepsin B under Neutral pH Conditions Using Au Microelectrode Arrays ..... | 54 |
| 3.1 Introduction.....                                                                                                                                                        | 54 |
| 3.2 Materials and Methods.....                                                                                                                                               | 58 |
| Cathepsin B activity with fluorogenic assays in different pH conditions.....                                                                                                 | 58 |
| 3.3 Results and Discussion .....                                                                                                                                             | 59 |
| 3.3.1 Functionalization of Au Microelectrode Array with Multiple Peptides .....                                                                                              | 59 |
| 3.3.2 pH-dependent proteolysis of CB.....                                                                                                                                    | 60 |
| 3.3.3 Fluorogenic assays for identifying amino acid sites that enhance CB cleavage at neutral pH.....                                                                        | 67 |
| 3.3.4 Impact of peptide substrate modification on electrochemical detection of CB .....                                                                                      | 71 |
| 3.3.5 Assessment of the LOD in measuring activated CB with H-32-Fc in neutral conditions .....                                                                               | 77 |
| 3.4 Conclusion .....                                                                                                                                                         | 79 |
| Chapter 4 - Electrochemical Response from Human Serum on Au-MEA and evaluating the cross reactivity from MMP-9 and ADAM-17 like proteases on cathepsin B detection .....     | 80 |
| 4.1 Introduction.....                                                                                                                                                        | 80 |

|                                                                                                                                 |     |
|---------------------------------------------------------------------------------------------------------------------------------|-----|
| 4.2 Materials and Methods.....                                                                                                  | 83  |
| 4.2.1 Activating MMP-9 .....                                                                                                    | 83  |
| 4.2.2 Preparation of active ADAM-17 samples.....                                                                                | 84  |
| 4.2.3 Validation of enzyme activity with fluorogenic assays .....                                                               | 84  |
| 4.3 Results and Discussion .....                                                                                                | 85  |
| 4.3.1 Sensor platform stability in the presence of Human Serum .....                                                            | 85  |
| 4.3.2 Electrochemical assessment of effective cathepsin B activity spiked in Human serum<br>and modelling kinetic response..... | 87  |
| 4.3.3 Linear response of the sensor platform to increasing Human serum concentration. ...                                       | 91  |
| 4.3.4 Nonspecific decay due to other proteases like MMP-9 and ADAM-17 in Human<br>serum.....                                    | 93  |
| 4.3.4.1 Possible interference to the Au/SAM platform from other activation agents.....                                          | 93  |
| 4.3.4.2 APMA interference to the Au/SAM platform .....                                                                          | 95  |
| 4.3.5 Validation of the activity of MMP-9 and ADAM-17 proteases .....                                                           | 99  |
| 4.3.5.1 MMP-9 activity .....                                                                                                    | 100 |
| 4.3.5.2 ADAM-17 activity .....                                                                                                  | 103 |
| 4.3.5.3 Optimization of FRET Assay Conditions for Long-Term Evaluation of MMP-9<br>and ADAM-17 Activity .....                   | 105 |
| 4.3.6 Electrochemical proteolysis by MMP-9 and ADAM-17 using Au MEA.....                                                        | 107 |
| 4.4 Conclusion .....                                                                                                            | 112 |
| Chapter 5 - Conclusion and Future Perspective.....                                                                              | 113 |
| 5.1 Study I: Overcoming Activation-Agent Interference in Cathepsin B Detection .....                                            | 113 |
| 5.2 Study II: Rational Peptide Engineering for Enhanced Cathepsin B Detection at<br>Physiological pH.....                       | 114 |
| 5.3 Study III: Human Serum Matrix Effects and Protease Cross-Reactivity on the Au-MEA<br>Platform .....                         | 115 |
| 5.4 Design Principles Need to be Considered in the Future .....                                                                 | 116 |
| 5.5 Future Perspectives .....                                                                                                   | 116 |
| 5.6 Final Remarks .....                                                                                                         | 118 |
| References.....                                                                                                                 | 119 |
| Appendix A - Supplementary Information for Chapter 2 .....                                                                      | 131 |

|                                                                                                                                                |     |
|------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| A.1 Materials and Instruments.....                                                                                                             | 131 |
| A.2 Design and selection of peptide-Fc substrates for cathepsin B.....                                                                         | 132 |
| A.3 Peptide Synthesis and Characterization .....                                                                                               | 133 |
| A.4 Au electrode surface functionalization .....                                                                                               | 145 |
| A.5 Nonspecific decay observed upon the addition of dithiothreitol (DTT).....                                                                  | 146 |
| A.6 Surface Coverage Determination.....                                                                                                        | 146 |
| A.7 Impact of DTT on AEF and peptide H-15-AEF chemical stability.....                                                                          | 147 |
| A.8 Impact of self-assembled monolayer stability and SAM formation protocol.....                                                               | 149 |
| A.9 Impact of redox potential window of ferrocene (relatively higher potential window)....                                                     | 150 |
| A.10 Investigating any interference from alternative activation agents (TCEP, Cysteine)....                                                    | 152 |
| A.11 Investigating the influence of buffer on fluorescence intensity from AMC .....                                                            | 153 |
| A.12 Optimization of DTT removal by ultrafiltration at different temperatures.....                                                             | 153 |
| A.13 Representative CV curves for H-6-AEF, H-3-AEF, H-15-AEF functionalized on Au<br>MEA in 25 mM MES, 50 $\mu$ M EDTA, pH= 5 .....            | 156 |
| A.14 Representative ACV curves for H-6-AEF functionalized on Au-MEA in 25 mM MES,<br>50 $\mu$ M EDTA, pH= 5 before and after proteolysis ..... | 157 |
| A.15 Representative blank curves (normalized) for H-3-AEF functionalized on Au MEA ..                                                          | 157 |
| Appendix B - Supplementary Information for Chapter 3 .....                                                                                     | 158 |
| B.1 Materials and Instruments .....                                                                                                            | 158 |
| B.2 Utilized Peptide-Fc substrates for cathepsin B .....                                                                                       | 159 |
| B.3 Peptide Synthesis and Characterization .....                                                                                               | 160 |
| B.4 Structural Modeling and Molecule Docking.....                                                                                              | 164 |
| B.5 Obtaining multiplex ACV signals from Ivium-Soft v2.561 software.....                                                                       | 166 |
| B.6 Homogeneous Michaelis-Menten Model for Fluorescence Data Analysis.....                                                                     | 166 |
| B.7 Assessment of the Limit of Detection in Measuring CB at pH = 5.0 with H-3-Fc, H-6-Fc,<br>and H-15-Fc on Au MEA .....                       | 168 |
| B.8 Cathepsin B: Active Site Representation .....                                                                                              | 169 |
| B.9 Fluorogenic assays with Z-peptide-AMC substrates to monitor cathepsin B activity, in<br>different pH conditions .....                      | 170 |
| B.10 Proteolysis of H-32-Fc peptide substrate with CB and cleavage site determination .....                                                    | 171 |

|                                                                                                                                                 |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| B.11 Representative CV curves for H-32-Fc functionalized on Au MEA in 25 mM MES, 50 $\mu$ M EDTA, pH = 7.3 .....                                | 172 |
| B.12 Representative ACV curves for H-32-Fc functionalized on Au-MEA in 25 mM MES, 50 $\mu$ M EDTA, pH = 7.3, before and after proteolysis ..... | 173 |
| B.13 Molecular docking of cathepsin B protein and Nle-Leu-Gly-Phe-Val-Ala peptide substrate .....                                               | 174 |
| Appendix C - Supplementary Information for Chapter 4 .....                                                                                      | 175 |
| C.1 Materials and Instruments .....                                                                                                             | 175 |
| C.2 Confirmation of APMA removal using 10k Amicon Ultra filter .....                                                                            | 176 |
| C.3 Investigation of Apparent Signal Saturation in FRET Assays and Long-Term Activity of MMP-9 .....                                            | 177 |
| C.4 Investigation of the cleavage sites of H-3-Fc, H-6-Fc, H-15-Fc and H-32-Fc peptide substrates by MMP-9 using analytical HPLC .....          | 178 |
| C.5 Investigation of the cleavage sites of H-3-Fc, H-6-Fc, H-15-Fc and H-32-Fc peptide substrates by MMP-9 using HRMS .....                     | 180 |
| C.6 Investigation of the cleavage sites of H-3-Fc, H-6-Fc, H-15-Fc and H-32-Fc peptide substrates by ADAM-17 using HRMS .....                   | 183 |

## List of Figures

- Figure 1.1 Primary components of biosensors. Biosensors detect targets of biological importance, including rare cells, nucleic acids, mechanical forces, etc. The biosensor recognizes the target in a variety of ways (e.g., affinity interactions) and converts this signal via a transducer element into a measurable signal. These transducers are often optical, electrochemical, or mechanical. Reprinted (adapted) with permission from WILEY Advanced Functional Materials. 2021, 31, 2104126. Copyright 2021 John Wiley and Sons. 3
- Figure 1.2 Schematic diagram of an ideal, single-crystalline SAM of alkane thiolates supported on a gold surface with a (111) texture. The anatomy and characteristics of the SAM are highlighted. Reprinted (adapted) with permission from ACS Chemical Reviews. 2005, 105 (4), 1103-1169. Copyright 2005 American Chemical Society. .... 6
- Figure 1.3 Proteases affect CSC functions and their niches. Proteases degrade niche ECM and influence several CSC properties, including their retention in the niche, invasion, self-renewal, stemness, immune evasion, and therapy resistance. Proteases either support (+) or inhibit (–) CSC-related processes. Reprinted (adapted) with permission from Journal of Histochemistry & Cytochemistry 2021, 69(12) 775–794. Copyright 2021 Sage Publications. .... 8
- Figure 1.4 Schematic illustrations of sandwich ELISA. (a) Capture antibody bound or precoated into each well of a 96-well plate. (b) Target antigen or protease (purple) binds to the capture antibody precoated into each well of the 96-well plate. (c) A horseradish peroxidase (HRP) enzyme-conjugated antibody is added that binds to the target antigen or protease bound to the capture antibody, thus forming a “sandwich” followed by addition of the substrate that undergoes enzymatic hydrolysis for detection. Reprinted (adapted) with permission from Springer Nature 2023, (2612) 1–17. Copyright 2023 Springer Nature. .... 11
- Figure 1.5 Principles of FRET-based protease sensors. (a) FRET sensors based on organic FRET donors and acceptors. (b) FRET sensors based on nanomaterials as FRET acceptors and FRET donor-labelled protease substrates. (c) FRET sensors based on nanomaterials as FRET donors and FRET acceptor-labelled protease substrates. Reprinted (adapted) with

|                                                                                                                                                                                                                                                                                                                                                                                                                             |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| permission from Analyst RCS 2017, (142) 1867-1881. Copyright Creative Commons Attribution 3.0 Unported License. ....                                                                                                                                                                                                                                                                                                        | 12 |
| Figure 1.6 General design of an activity-based probe (ABP). Reprinted (adapted) with permission from Methods on Enzymology 2019, (618) 357-387. Copyright 2019 Elsevier. ....                                                                                                                                                                                                                                               | 13 |
| Figure 1.7 Schematic illustration of the Fc-peptide-based electrochemical biosensor for the detection of trypsin and thrombin activity. Reprinted (adapted) with permission from International Journal of Electrochemical Science 2023, 18 (12) 1452-3981. Copyright 2023 Elsevier Creative Commons CC-BY license. ....                                                                                                     | 14 |
| Figure 1.8 Schematic of Electrochemical Proteolytic Beacon (EPB) for detection of matrix metalloproteinase: (A) self-assembling electrochemical FC-peptide conjugate on the gold substrate; (B) Cleavage of EPB in the presence of MMP-7. Reprinted (adapted) with permission from Journal of the American Chemical Society 2006, 128 (38) 12382-3. Copyright 2006 American Chemical Society. ....                          | 15 |
| Figure 1.9 (A) Fabrication workflow for wafer-scale MEA fabrication. (B) Top-view and side-view diagrams illustrating the structure of the MEA. Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier. ....                                                                                                                                                    | 19 |
| Figure 1.10 SEM images of different regions of a MEA: (A) An exposed Au surface, (B) The top surface of the PECVD SiO <sub>2</sub> layer, and (C) The boundary between the top SiO <sub>2</sub> layer and the exposed Au surface. Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier. ....                                                                  | 20 |
| Figure 1.11 Optical micrographs of a fabricated Au MEA chip. (A) Au MEA chip with 9 contact pads (1.0 mm × 1.0 mm) at the bottom and a magnified optical image of the 3 × 3 Au MEA with nine microelectrodes (200 μm × 200 μm). Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier. ....                                                                    | 20 |
| Figure 1.12 Experimental setup. (A) Box diagram of multiplexed experimental setup. (B) Photograph of the Ivium n-Stat potentiostat. (C) Photograph of the breakout box, electrochemical cell, and copper base plate. (D) Photograph of the electrochemical cell fitted with the copper base plate. Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier. .... | 22 |

Figure 1.13 Optical image of an Au MEA chip with 9 contact pads ( $1.0\text{ mm} \times 1.0\text{ mm}$ ) at the left bottom and schematic illustration of the proteolysis sensing mechanism, including major components of the peptide/Au sensor, representative ACV curves during proteolysis, and the kinetic proteolysis curves under varied protease activities. .... 23

Figure 1.14 (A) In alternating current voltammetry (ACV), an incrementally rising base potential with a superimposed sinusoidal wave is employed. (B) Here, the magnitude of the faradaic response depends on the chosen frequency of the applied AC potential. (C) The final signal is the measured amplitude of the AC current at each incremental potential step. Reprinted (adapted) with permission from Sensors & Diagnostics RCS 2024, (142) 1867-1881.

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Figure 2.1 (A) Optical image of an Au MEA chip with 9 contact pads ( $1.0\text{ mm} \times 1.0\text{ mm}$ ) at the bottom and schematic illustration of the  $3 \times 3$  Au MEA with nine microelectrodes ( $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ ). (B) Schematic illustration of the proteolysis sensing mechanism, including major components of the peptide/Au sensor, representative ACV curves during proteolysis, and the kinetic proteolysis curves under varied protease activities. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd..... 37

Figure 2.2 (A) Schematic illustrations of the functionalization schemes of AMF (AEF), MCMB, and H-15-AEF peptide covalently attached to the Au electrodes via the thiol/Au-based SAM. The kinetic curve of the normalized peak current over time, during continuously repeated ACV cycling (B) for AEF and AMF, and (C) for AMF or AEF -tagged H-15 peptide. (D) The similar measurements with MCMB functionalized on SAM-covered Au disk electrodes. The measurements were done in the presence and absence of DTT ( $20\text{ }\mu\text{M}$ ) in  $0.5\text{X PB}$  at  $36\text{ }^{\circ}\text{C}$ . Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd..... 40

Figure 2.3. (A) Selected ACV curves showing no MCMB peak during continuous ACV cycling at the high Fc redox potential region ( $-0.40$  to  $0.15\text{ V vs MSE}$ ) for MCMB/SAM/Au with DTT. (B) Periodic ACV probing of MCMB/SAM/Au at the low potential ( $-0.85$  to  $-0.40\text{ V vs MSE}$ ) during continuous ACV cycling simulating the conditions of AMF and AEF measurements at higher potentials ( $-0.40$  to  $0.15\text{ V vs MSE}$ ) in the presence of  $20\text{ }\mu\text{M}$  DTT. (C) Similar experiment as panel (A) but without DTT. (D) in the high stability of the ACV peak current in similar experiments as panel (B) in the absence of DTT. (E) Comparison of

the normalized peak current of MCMB/SAM/Au derived from panel B (with DDT, red curve) and panel D (without DDT, green curve). The latter shows a high stability similar to the control experiment of continuous ACV all at the low potentials (-0.40 to 0.15 V vs MSE) in the presence of 20  $\mu$ M DTT (blue curve). All measurements were done in 0.5X PB at 36 °C on Au disk electrodes. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. .... 44

Figure 2.4 (A) Validation of CB activity with FRET assay (100  $\mu$ M Z-L-R-AMC substrate at RT) using 3.45 nM of activated and non-activated CB in two buffers (25 mM MES at pH = 5.0 and 0.5X PB at pH = 7.4, respectively). (B) Comparison of kinetic proteolysis curves by 7.0 nM of CB using FRET assay (100  $\mu$ M Z-L-R-AMC substrate at RT) to investigate the effect of DTT and EDTA on the activity of CB. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. .... 47

Figure 2.5 Illustrative model for in vitro activation and processing of human pro-cathepsin B. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. .... 48

Figure 2.6 (A) Schematic representation of how peptides were fictionalized on a single microelectrode array chip utilizing three microelectrodes for each peptide. (B) Kinetic curves of normalized peak current over time with DTT-removed (at 20 °C) CB on H-6-AEF, H-3-AEF, and H-15-AEF functionalized on SAM-covered Au MEA. EDTA was not incorporated during the DTT removal process and in the proteolysis buffer. (C) Representative normalized kinetic proteolysis curves for an Au MEA modified with three different peptide substrates, H-6-AEF, H-3-AEF, and H-15-AEF, which were proteolyzed by 11.03 nM activated CB (with DTT removed after activation) in 25 mM MES, pH = 5.0, with 50  $\mu$ M EDTA. The protease sample was added after the 10<sup>th</sup> ACV cycle to obtain proteolysis curves. Blanks were run without CB enzyme. The proteolysis curves are normalized to the peak current in the first ACV cycle after CB addition and fitted with Equation (2). (D) A bar graph comparing the  $1/\tau$  values for the faulty blank (without DTT removal) and the corrected blank (after employing the DTT removal protocol) for H-3-AEF. Additionally, the graph includes fitted and blank-subtracted  $1/\tau$  values for three peptide substrates, confirming substrate-dependent proteolysis after resolving the interference issue.

Figure 3.1 An optical image of an Au MEA chip with 9 contact pads (1.0 mm × 1.0 mm) at the bottom and a schematic illustration of the 3 x 3 Au MEA with nine microelectrodes (200 μm × 200 μm), which were fictionalized with three types of hexapeptide substrates with three microelectrodes for each peptide. .... 60

Figure 3.2 (A-C) Representative normalized kinetic proteolysis curves for an Au MEA modified with three different hexapeptide substrates: A: H-6-Fc, B: H-3-Fc, and C: H-15-Fc, which were proteolyzed by 11.03 nM activated CB (with DTT removed after activation) in 25 mM MES, pH = 5.0/ 6.3/ 7.3 with 50 μM EDTA. The protease sample was added after the 10<sup>th</sup> ACV cycle to obtain proteolysis curves. Blanks were run without CB enzyme. The proteolysis curves are normalized to the peak current in the first ACV cycle after CB addition and fitted with Equation 1.6 ( $y = A * \exp(-x / \tau) + b * x + c$ ). (D) The measurement of the activity of 3.45 nM activated CB by a fluorogenic assay with Z-Leu-Arg-AMC substrate (100 μM) at pH = 7.3, pH = 6.3, and pH = 5.0, respectively. (E) Line plot showing the pH-dependent proteolytic activity of CB using three peptide substrates: H-15-Fc (green squares), H-6-Fc (red circles), H-3-Fc (blue triangles), and Z-Leu-Arg-AMC (black squares). Activity is expressed as  $1 / \tau \times 10^{-4}$  over a pH range of 5.0 to 7.3. Data are presented as mean ± SD from independent replicates (n=3). Maximal cleavage observed at pH 5.0 for all substrates. .... 63

Figure 3.3 (A-C) The representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the three hexapeptide-Fc substrates H-3-Fc, H-6-Fc, and H-15-Fc, respectively in activated CB (DTT removed after activation), concentrations of 0, 6.01, 11.03, 22.04, 35.00 and 52.7 nM in 25 mM MES, 50 μM EDTA (pH = 7.3). (D-F) The linear calibration curves of CB protease activity in the activated CB with the hexapeptide-Fc substrates H-3-Fc, H-6-Fc, and H-15-Fc, respectively. .... 65

Figure 3.4 (A) The activity of 3.45 nM CB represented by the fluorescence intensity after mixing for 40 min ( $F_t = 40\text{min}$ ) with each of the Z-peptide-AMC substrates (100 μM concentration) at pH 7.3 (gold bars), pH 6.3 (blue bars) and pH 5.0 (red bars) from Fig. B.6. (B) The table summary of the optimal pH conditions (pH = 7.3, 6.3 and 5.0) for each

substrate assessed by the fluorescence data and preferred amino acid residues (P1, P2, P3) for cathepsin B adjacent to the peptide cleavage site (red arrow). ..... 70

Figure 3.5 (A) The original H-6-Fc hexapeptide substrate ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Gly-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ ) and the modified H-32-Fc hexapeptide substrate ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Nle-Leu-Gly-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ ) with the Pro amino acid at P3 position replaced by a Nle. (B) The representative normalized kinetic proteolysis curves for an Au MEA modified with H-32-Fc, which was proteolyzed by 11.03 nM activated CB (with DTT removed after activation) in 25 mM MES, pH = 5.0, 6.3, and 7.3 with 50  $\mu\text{M}$  EDTA. The protease sample was added after 10<sup>th</sup> ACV cycle to obtain proteolysis curves. Blanks were run without CB enzyme. The proteolysis curves are normalized to the peak current in the first ACV cycle after CB addition and fitted with Equation 1.6 ( $y = A * \exp(-x / \tau) + b * x + c$ ). (C) Summary of the pH-dependent proteolytic activity of CB using four hexapeptide substrates on Au MEA in comparison with the Z-Leu-Arg-AMC fluorogenic peptide substrate. The activity is expressed as  $1 / \tau \times 10^{-4}$  over a pH from 5.0 to 7.3. Data are presented as mean  $\pm$  SD from independent replicates (n=3). Maximal cleavage observed at pH 5.0 for all substrates. (D) Illustrative representation of catalytic triad (Cys29, His199, Asn219) of active human cathepsin B and possible salt bridge formation between His110 and Asp22. (E) Docking pose of the peptide substrate (Nle-Leu-Gly-Phe-Val-Ala) within the active-site pocket of active cathepsin B (PDB ID: 1CSB), molecular surface (gray) while peptide is displayed as a stick model with a semi-transparent mesh representation (blue). ..... 72

Figure 3.6 (A) Representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the peptide-Fc substrate H-32-Fc at activated CB (DTT removed after activation) concentrations of 0, 1.5, 5.0, 7.5, 11.03, 14.5, and 20.0 nM in 25 mM MES, 50  $\mu\text{M}$  EDTA (pH = 7.3). (B) Linear calibration curve of CB protease activity in the activated CB with the peptide-Fc substrate H-32-Fc. The error bars represent the standard deviation of three replicates for each measurement. (C) A line plot containing the summary of LODs of CB using different peptide substrates at pH 5.0 and 7.3. .... 77

Figure 4.1. Stability of the Au/SAM/AEF sensor platform in the presence of human serum. (A) Normalized peak current as a function of time for the Au/SAM/AEF interface in 25 mM MES pH = 7.3, 50  $\mu\text{M}$  EDTA at 36  $^{\circ}\text{C}$  (brown) and after addition of human serum (orange).

Human serum was introduced at ~11 min (dashed line). The AEF layer was immobilized without a protease-cleavable peptide to isolate nonspecific interfacial effects. (B) Background-subtracted ACV curves recorded at different cycles following serum addition (HS added at the 11th cycle). The peak shape and potential remain largely unchanged, with only a modest reduction in peak current over 60 cycles, confirming the electrochemical stability of the interface in complex biological media. .... 86

Figure 4.2 Electrochemical monitoring of peptide cleavage in 3% human serum and CB-spiked serum. Normalized current decay as a function of time for Au-MEA electrodes functionalized with (A) H-3-Fc, (B) H-6-Fc, (C) H-15-Fc, and (D) H-32-Fc upon exposure to 3% human serum (HS) and 3% HS spiked with 11.03 nM cathepsin B (CB). H-3-Fc, H-6-Fc, and H-15-Fc exhibit a pronounced initial rapid decay followed by a slower phase, whereas H-32-Fc shows a more gradual decay profile. Enhanced signal decay in CB-spiked samples confirms effective CB activity in serum. Solid lines represent double-exponential fits used for kinetic modeling. .... 88

Figure 4.3 Extracted kinetic parameters from double-exponential fitting of electrochemical decay profiles. (A) Slow decay component ( $1/\tau_2$ ) and (B) fast decay component ( $1/\tau_1$ ) obtained from double-exponential modeling of current decay curves for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc in 3% human serum (HS) and 3% HS spiked with 11.03 nM CB. Bars represent apparent proteolytic activity in HS, CB-spiked HS, and the corresponding activity difference. .... 90

Figure 4.4 Linear response of peptide-functionalized Au-MEA sensors to increasing human serum concentration. (A, D, G, J) Normalized current decay profiles  $i_p/i_{p0}$  for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc, respectively, upon exposure to increasing human serum (HS) concentrations (0.5-6%). (B, E, H, K) Slow decay rate constants ( $1/\tau_2$ ) extracted from double-exponential fitting plotted against H.S concentration. (C, F, I, L) Fast decay rate constants ( $1/\tau_1$ ) as a function of H.S concentration. .... 92

Figure 4.5 Electrochemical evaluation of APMA-induced interference and SAM degradation on Au disk electrodes. (A) Cyclic voltammograms recorded in 1:1  $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$  (1.0 mM in 0.1 M  $KNO_3$ ) at 36 °C for a freshly prepared mixed SAM on a Au disk electrode (black trace), after 15 ACV cycles in 20  $\mu$ M APMA (red trace), and after 30 ACV cycles in APMA (green trace). The intact SAM suppresses Ferri/ferrocyanide redox peaks,

confirming effective surface blocking. After exposure to APMA, progressively pronounced redox peaks appear, indicating loss of monolayer integrity and formation of pinholes. (B) Alternating current voltammograms (ACV) recorded during the first round of potential cycling (-0.4 to 0.15 V vs. MSE, 50 mV amplitude, 300 Hz) in 0.5X phosphate buffer containing 20  $\mu$ M APMA. An emerging redox feature near -0.3 V vs. MSE develops and increases over successive cycles, suggesting formation of electroactive sites upon APMA exposure. (C) ACV response during a second round of cycling in APMA following initial surface damage. A pronounced and continuously increasing redox peak at  $\sim$ -0.3 V vs. MSE is observed over 15 cycles, consistent with progressive SAM degradation and enhanced exposure of the underlying gold surface. .... 96

Figure 4.6 Electrochemical response of APMA (1 mM) at a clean Au disk electrode. Cyclic voltammograms recorded at a clean Au disk electrode in 0.5X phosphate buffer (PB) (black trace) and in 0.5X PB containing 1 mM APMA (red trace), scanned from -0.6 V to 0.15 V vs. MSE at 100 mV s<sup>-1</sup>. .... 98

Figure 4.7 FRET-based validation of MMP-9 activity under different activation and buffer conditions. Fluorescence intensity as a function of time for cleavage of the FRET peptide substrate (Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub>, 20  $\mu$ M) by MMP-9 (35.4 nM) under various conditions. Measurements were performed in 50 mM TCNB buffer (pH 7.5) and 25 mM MES buffer (pH 7.3) with or without 50  $\mu$ M EDTA, using vendor-purified (APMA removed) MMP-9, APMA-activated MMP-9, and APMA-activated followed by removal in-house using a 10k Amicon Ultra filter. .... 102

Figure 4.8 Time-dependent fluorescence response of the peptide substrate (Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub>, 20  $\mu$ M) upon cleavage by ADAM-17 (35.4 nM) under different buffer conditions. Reactions were carried out in Tris buffer (pH 9.0) and 25 mM MES buffer (pH 7.3). A significantly higher fluorescence intensity and faster signal saturation were observed in Tris buffer compared to MES buffer, indicating enhanced enzymatic activity and/or substrate turnover at alkaline pH. .... 104

Figure 4.9 (A) FRET-based long-term activity measurement of MMP-9 (10 nM) with 100  $\mu$ M substrate in a total volume of 100  $\mu$ L (90  $\mu$ L substrate + 10  $\mu$ L enzyme) using the ProMega system (Ex = 365 nm, Em = 410-460 nm). (B) FRET-based long-term activity measurement

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| of ADAM-17 (5 nM) with 100 $\mu$ M substrate in a total volume of 100 $\mu$ L (90 $\mu$ L substrate + 10 $\mu$ L enzyme) using the ProMega system (Ex = 365 nm, Em = 410-460 nm). .....                                                                                                                                                                                                                                                                                                                                                                                              | 106 |
| Figure 4.10 Electrochemical proteolysis of surface-immobilized peptide substrates by MMP-9, ADAM-17 and cathepsin B on the Au MEA platform. Normalized peak current ( $i_p/i_{po}$ ) versus time obtained by ACV measurements for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc functionalized on Au MEAs in 25 mM MES buffer (pH 7.3) containing 50 $\mu$ M EDTA at 36 $^{\circ}$ C. (A) Response after addition of 30 nM active MMP-9 (APMA removed before measurement). (B) Response after addition of 98 nM active ADAM-17. (C) Response after addition of 11 nM active cathepsin B. .... | 108 |
| Figure 4.11 Proteolytic activity of MMP-9 (A), ADAM-17 (B), and cathepsin B (C) toward peptide substrates H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc, expressed as the calculated $1/\tau$ values ( $10^{-4} \text{ s}^{-1}$ ) obtained from electrochemical decay measurements. Bars represent the blank response, activity in the presence of the protease, and blank-subtracted activity for each peptide substrate. ....                                                                                                                                                               | 109 |
| Appendix Figure A.1 Evolution of normalized ACV peak current over time for the peptide substrate, H-15-AMF ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH}_2\text{-Fc}$ ) functionalized on SAM-covered Au MEA. The stability was checked during continuous ACV cycling in presence and absence of DTT (20 $\mu$ M) in 0.5X PB at 36 $^{\circ}$ C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                                                                       | 146 |
| Appendix Figure A.2 Cyclic voltammograms of (A) AEF (B) MCMB (C) H-15-AEF on SAM-covered Au disk electrode (2.0 mm in diameter) in 0.5X PB at 36 $^{\circ}$ C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd..                                                                                                                                                                                                                                                                                                             | 146 |
| Appendix Figure A.3 HPLC chromatograms of (A) 5.0 mM amino ethyl ferrocene, (B) 5.0 mM dithiothreitol, and (C) 5.0 mM dithiothreitol and 5.0 mM AEF incubated for 1 hour at 36 $^{\circ}$ C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                                                                                                                                                                                                                                                           | 147 |
| Appendix Figure A.4 HPLC chromatograms of (A) 5.0 mM dithiothreitol, (B) 5.0 mM H-15-AEF, and (C) 5.0 mM dithiothreitol and 5.0 mM H-15-AEF incubated for 1 hour at 36 $^{\circ}$ C.                                                                                                                                                                                                                                                                                                                                                                                                 |     |

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Appendix Figure A.5 (A) The kinetic curve of normalized peak current over time for AEF functionalized on SAM-covered Au disk electrodes using different protocols. Blue: SAM functionalization was done in water for 50 min and AEF was coupled using EDC/Sulfo NHS chemistry. Green: SAM functionalization was done in anhydrous ethanol for 12 hours and AEF was coupled using EDC/Sulfo NHS chemistry followed by  $\text{NH}_2\text{OH}$  treatment. The stability was checked with continuous ACV cycling in 0.5X PB at 36 °C. (B) The stability was checked after adding 20  $\mu\text{M}$  DTT during continuous ACV cycling in 0.5X PB at 36 °C to see stability improvement upon addition of DTT after using the improved SAM formation protocol-2. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. .... 149

Appendix Figure A.6 The schematic representation shows how the experimental ACV scans were performed: (A) Periodic ACV probing of MCMB/SAM/Au at (-0.85 to -0.40 V vs MSE) during continuous ACV cycling simulating the conditions of AMF and AEF measurements at higher potentials (-0.40 to 0.15 V vs MSE) in presence and absence of 20  $\mu\text{M}$  DTT in Figure 2.4; (B) Periodic ACV probing of AEF/SAM/Au at (-0.45 to 0.15 V vs MSE) during continuous ACV cycling simulating the conditions of MCMB measurements at lower potentials (-0.85 to -0.35 V vs MSE) in presence and absence of 20  $\mu\text{M}$  DTT in Figure 2.5. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. .... 150

Appendix Figure A.7 (A) Periodic ACV probing of AEF/SAM/Au at the high potential window (-0.45 to 0.15 V vs MSE) during continuous ACV cycling simulating the conditions of MCMB measurements at lower potentials (-0.85 to -0.35 V vs MSE) in the presence of 20  $\mu\text{M}$  DTT. (B) Selected ACV curves showing no AEF peak during continuous ACV cycling at the MCMB redox potential region (-0.85 to -0.35 V vs MSE) in the presence of DTT. (C) No decay in ACV peak current in similar experiments as panel (A) in the absence of DTT, confirming that ACV at high potentials (-0.40 to 0.15 V vs MSE) in the presence of DTT caused the signal decay. (D) Selected ACV curves showing no AEF peak during continuous ACV cycling at the MCMB redox potential region (-0.35 to -0.85 V vs MSE) without DTT. (E) Comparison of the rapid decay in normalized peak current of AEF/SAM/Au in 20  $\mu\text{M}$

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| DTT during continuous ACV cycling at high potentials (-0.45 V to 0.15 V vs MSE, blue curve) to the much slower decay shown by periodical probing at this potential range during continuous ACV cycling at the low potentials (-0.85 V to -0.35 V vs MSE). All measurements are carried out in 0.5X PB at 36 °C on Au disk electrodes. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                                                                | 151 |
| Appendix Figure A.8 The kinetic curve of the normalized peak current over time during continuously repeated ACV cycling for H-3-AEF, H-6-AEF, and H-15-AEF. (A) TCEP (12.5 µM) was added at the 10 <sup>th</sup> cycle. (B) Cysteine (12.5 µM) was added at the 10 <sup>th</sup> cycle, functionalized on the SAM-covered Au MEA. The measurements were in 0.5X PB at 36 °C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                         | 152 |
| Appendix Figure A.9 Investigating the influence of the buffer (0.5X PB (pH = 7.4) and 25 mM MES (pH = 5.0)) on fluorescence intensity from amino methyl coumarin (AMC) fluorophore utilized in FRET assay in Figure 2.4 panel (A). Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                                                                                                                                                                   | 153 |
| Appendix Figure A.10 Evolution of normalized peak current over time for (A) Addition of DTT removed (at 4°C) sample on H-6-AEF, H-3-AEF and H-15-AEF functionalized SAM-covered Au MEA (B) Addition of DTT removed sample (at 25 °C) on H-6-AEF, H-3-AEF and H-15-AEF functionalized on SAM-covered Au MEA. All electrochemical measurements were done in 25 mM MES buffer containing 50.0 µM EDTA, pH=5.0 at 36 °C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. .... | 156 |
| Appendix Figure A.11 Representative CV curves for H-6-AEF, H-3-AEF, H-15-AEF functionalized on SAM-covered Au MEA in 25 mM MES, 50 µM EDTA, pH = 5.0. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                                                                                                                                                                                                                                                | 156 |
| Appendix Figure A.12 Representative ACV curves for H-6-AEF functionalized on SAM-covered Au MEA in 25 mM MES, 50 µM EDTA, pH = 5.0. (A) Raw data and linear baselines (dashed lines) of ACV obtained before (black) and after (red) proteolysis. (B) The processed ACV curves after baseline subtraction for before (black) and after (red)                                                                                                                                                                                        |     |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| proteolysis. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | 157 |
| Appendix Figure A.13 Evolution of normalized ACV peak current over time for the peptide substrate, H-3-AEF functionalized on SAM-covered Au MEA. The blank run after incorporating DTT removal protocol (blue curve) and blank run without DTT removal (green curve) in 25 mM MES (pH = 5.0), 50 $\mu$ M EDTA for comparison. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd. ....                                                                                                                                                                         | 157 |
| Appendix Figure B.1 Analytical HPLC chromatogram of the H-3-Fc peptide substrate showing a single major peak at a retention time of 34.6 minutes, confirming the purity and identity of the synthesized peptide. ....                                                                                                                                                                                                                                                                                                                                                                                               | 163 |
| Appendix Figure B.2 High-resolution mass spectrum (HRMS) of the intact H-32-Fc peptide substrate, confirming its expected molecular weight and verifying the identity and purity of the peptide before enzymatic cleavage. ....                                                                                                                                                                                                                                                                                                                                                                                     | 164 |
| Appendix Figure B.3 Representative multiplex alternating current voltammetry (ACV) data collected from nine independent microelectrodes on the Au microelectrode array (MEA) chip. The H-3-Fc, H-6-Fc, and H-15-Fc were immobilized on separate electrodes, enabling simultaneous measurement of their electrochemical responses. ACV experiments were performed using an IVIUM n-Stat potentiostat with IviumSoft v2.561, and the resulting voltammograms illustrate the reproducible and well-defined ferrocene redox peaks from each modified electrode. ....                                                    | 166 |
| Appendix Figure B.4 (A-C) The representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the hexapeptide-Fc substrates H-3-Fc, H-6-Fc and H-15-Fc, respectively in activated CB ( DTT removed after activation), concentrations of 0, 1.22, 6.05, 11.03, 15.04 and 34.72 nM in 25 mM MES, 50 $\mu$ M EDTA (pH = 5.0). (D-F) The linear calibration curves of CB protease activity in the activated CB with the three hexapeptide-Fc substrates H-3-Fc, H-6-Fc, and H-15-Fc. The error bars represent the standard deviation of three replicates for each measurement. .... | 168 |
| Appendix Figure B.5 (A) Illustrative representation of catalytic triad (Cys29, His199, Asn219) of active human cathepsin B and possible salt bridge formation between His110 and Asp22 (B) Surface view of the active site cleft of active human cathepsin B showing its S3, S2, S1, S1'                                                                                                                                                                                                                                                                                                                            |     |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |     |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| and S2' subsites that interact with substrate's P3, P2, P1, P1' and P2' residues respectively.<br>.....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 169 |
| Appendix Figure B.6 Z-Peptide–AMC (100 $\mu$ M) substrates that can monitor CB (3.45 nM) activity, illustrated by fluorescence intensity in different pH conditions. Cathepsin B activity with (A) Z-Lys-Arg–AMC, (B) Z-Leu-Arg-AMC, (C) Z-Nle-Arg-AMC, (D) Z-Leu-Lys-Arg-AMC, and (E) Z-Nle-Lys-Arg-AMC substrates at pH 7.3, pH 6.3, and pH 5.0 in 25 mM MES buffer was evaluated over 40 mins. ....                                                                                                                                                                                                                                                                                                                           | 170 |
| Appendix Figure B.7 Proteolysis analysis by analytical HPLC for H-32-Fc after mixing with cathepsin B for different proteolysis times. The intact peptide appeared at 34.6 min retention time, and two new peaks appeared at 19.5 min and 31.1 min retention time, corresponding to the cleaved products.....                                                                                                                                                                                                                                                                                                                                                                                                                    | 171 |
| Appendix Figure B.8 High-resolution mass spectrum (HRMS) of the cleaved crude products generated from H-32-Fc following proteolysis by CB in 25 mM MES buffer (pH 7.3) containing 50 $\mu$ M EDTA. The observed product ions correspond to the expected fragments produced by cleavage at the Gly–Phe bond, confirming the specific proteolytic site within the peptide sequence. ....                                                                                                                                                                                                                                                                                                                                           | 171 |
| Appendix Figure B.9 Representative CV curves of H-32-Fc functionalized on SAM-covered Au MEA in 25 mM MES, 50 $\mu$ M EDTA, pH = 7.3. ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 172 |
| Appendix Figure B.10 Representative ACV curves for H-32-Fc functionalized on SAM-covered Au MEA in 25 mM MES, 50 $\mu$ M EDTA, pH = 7.3. (A) Raw data and linear baselines (dashed lines) of ACV obtained before (brown) and after (green) proteolysis. (B) The processed ACV curves after baseline subtraction before (brown) and after (green) proteolysis. ....                                                                                                                                                                                                                                                                                                                                                               | 173 |
| Appendix Figure B.11 Molecular docking of the peptide substrate Nle–Leu–Gly–Phe–Val–Ala with Cathepsin B. (A) Docking pose of the peptide within the active-site pocket of the 1CSB, shown as a surface representation (gray), with the peptide displayed as sticks. The substrate occupies the catalytic groove and aligns along the S subsites, positioning the putative scissile bond (Gly–Phe) proximal to the catalytic residue Cys29. (B) Surface representation of the 1CSB (gray), highlighting shape complementarity of the substrate-binding pocket; the peptide is shown as sticks with a semi-transparent mesh (blue). (C) Docking pose of the peptide within the active-site pocket of AlphaFold2 modeled cathepsin |     |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| B, shown as a surface representation (sky blue), with the peptide displayed as sticks and adopting a similar binding orientation along the S subsites. (D) Surface representation of AlphaFold2 modeled cathepsin B (sky blue), highlighting shape complementarity of the substrate-binding pocket, with the peptide shown as sticks and a semi-transparent mesh (blue). .....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 174 |
| Appendix Figure C.1 HPLC analysis confirming APMA removal using a 10k Amicon Ultra centrifugal filter. (A) Analytical HPLC chromatogram of 1.29 mM APMA (50 $\mu$ L injection) showing a distinct peak at 19.60 min under a 0–95% acetonitrile gradient (60 min, 1.0 mL min <sup>-1</sup> , detection at 214 nm), establishing the retention time of APMA. (B) Chromatogram of 0.073 mM APMA (50 $\mu$ L injection), corresponding to the concentration used after MMP-9 activation. A clear peak is observed at 19.74 min with an intensity of approximately 700 mV, confirming detectability at lower concentrations. (C) Chromatogram of the same APMA sample after dilution (to 500 $\mu$ L) and centrifugal filtration using a 10k Amicon Ultra filter (14,000 g, 5 min; final volume 57 $\mu$ L). The APMA peak at ~19.7 min is nearly eliminated, with only a trace signal (~35 mV) remaining, demonstrating efficient removal of APMA by the filtration process. .... | 176 |
| Appendix Figure C.2 Effect of substrate-to-enzyme ratio on MMP-9 FRET kinetics and signal saturation behavior. Fluorescence intensity as a function of time for cleavage of the FRET substrate (Mca-K-P-L-G-L-Dpa-A-R-NH <sub>2</sub> , 40 $\mu$ M) by MMP-9 at different enzyme concentrations (2.0 nM, 4.0 nM, and 35.4 nM). ....                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | 177 |
| Appendix Figure C.3 Analytical HPLC chromatograms of peptide substrates after incubation with proteases to identify cleavage sites responsible for the observed electrochemical signal decay. Peptides H-3-Fc (A), H-6-Fc (B), H-15-Fc (C), and H-32-Fc (D) (1 mM) were incubated with MMP-9 (15 nM) in TCNB buffer (pH 7.5). Aliquots collected at the initial time point and after 2 hours of incubation were analyzed by analytical HPLC. Newly emerged peaks corresponding to proteolytic fragments are indicated in the chromatograms, allowing identification of cleavage products for each peptide substrate. Chromatograms for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc are shown in panels A-D, respectively.....                                                                                                                                                                                                                                                        | 178 |
| Appendix Figure C.4 Analytical HPLC chromatograms of peptide substrates after incubation with proteases to identify cleavage sites responsible for the observed electrochemical signal decay. Peptides H-3-Fc (A), H-6-Fc (B), H-15-Fc (C), and H-32-Fc (D) (1 mM) were                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |     |

incubated with ADAM-17 (30 nM) in Tris buffer (pH 9.0). Aliquots collected at the initial time point and after 2 hours of incubation and 20 hours were analyzed by analytical HPLC. Newly emerged peaks corresponding to proteolytic fragments are indicated in the chromatograms, allowing identification of cleavage products for each peptide substrate. Chromatograms for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc are shown in panels A-D, respectively. .... 179

## List of Tables

|                                                                                                                                                                                                |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 3.1 The average $1/\tau$ values obtained for H-3-Fc, H-6-Fc, and H-15-Fc at pH 5.0, 6.3, and 7.3 with 11.03 nM CB.....                                                                   | 61  |
| Table 3.2 Fitting parameters and detection limits of electrochemical detection of cathepsin B using hexapeptide substrates. ....                                                               | 64  |
| Table 4.1 Calculated $1/\tau_1$ and $1/\tau_2$ values for 3% HS added and CB spiked 3% HS added proteolysis decay curves. ....                                                                 | 89  |
| Table 4.2 Different peptide substrates used in this study and their cleavage sites with different proteases.....                                                                               | 111 |
|                                                                                                                                                                                                |     |
| Appendix Table A.1 Peptide sequences screened for cathepsin B in this study and their cleavage sites highlighted. ....                                                                         | 133 |
| Appendix Table A.2 The time taken to reach 50 $\mu$ L from 500 $\mu$ L using 3k and 10k Amicon ultracentrifugation filters at different temperatures (4 $^{\circ}$ C and 20 $^{\circ}$ C)..... | 154 |
| Appendix Table B.1 Peptide sequences screened for cathepsin B in previous and this study, with the cleavage sites between the purple and cyan highlighted amino acids. ....                    | 160 |

## **List of Abbreviations**

|         |                                           |
|---------|-------------------------------------------|
| ACV     | Alternating Current Voltammetry           |
| Au      | Gold                                      |
| MEA     | Microelectrode Array                      |
| PECVD   | Plasma-Enhanced Chemical Vapor Deposition |
| Fc      | Ferrocene                                 |
| AMF     | Amino Methyl Ferrocene                    |
| AEF     | Amino Ethyl Ferrocene                     |
| CB      | Cathepsin B                               |
| DTT     | Dithiothreitol                            |
| EDTA    | Ethylenediaminetetraacetic Acid           |
| SAM     | Self-Assembled Monolayer                  |
| LOD     | Limit of Detection                        |
| MMP-9   | Matrix Metalloprotease-9                  |
| ADAM-17 | A Disintegrin and Metalloprotease-17      |
| APMA    | 4-Aminophenylmercuric Acetate             |
| FRET    | Fluorescence Resonance Energy Transfer    |
| CV      | Cyclic Voltammetry                        |
| EIS     | Electrochemical Impedance Spectroscopy    |
| PB      | Phosphate Buffer                          |
| HS      | Human Serum                               |
| MES     | 2-(N-morpholino)ethane sulfonic acid      |
| AMC     | Aminomethylcoumarin                       |

|      |                                        |
|------|----------------------------------------|
| HPLC | High Performance Liquid Chromatography |
| HRMS | High Resolution Mass Spectrometry      |
| SEM  | Scanning Electron Microscopy           |

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## **Dedication**

“To my beautiful wife, Nathali.”

## Preface

The dissertation comprises of the following chapters.

**Chapter 1** provides the background and motivation for the study, beginning with fundamental principles of biosensors and their classification. It reviews the role of nanomaterials in biosensing applications and discusses proteases as disease biomarkers along with conventional and emerging detection strategies, including electrochemical approaches. The chapter further outlines challenges in multiplex protease detection, introduces the Au MEA fabrication and experimental setup, explains the Michaelis-Menten model and alternating current voltammetry used for data analysis, and concludes with the research objectives, hypotheses, and scope of the thesis.

**Chapter 2** addresses interference from the activation agent dithiothreitol (DTT) during electrochemical detection of CB. The study investigates the impact of DTT on gold electrodes and redox tag stability, optimizes buffer conditions, and validates enzyme activity using fluorogenic assays. The chapter demonstrates strategies to mitigate activation agent interference and establishes a reliable protocol for demonstrating multiplex protease analysis on a single Au MEA chip.

**Chapter 3** focuses on rational peptide substrate optimization to enhance sensitive detection of CB under neutral pH conditions. Through fluorogenic screening and electrochemical evaluation, amino acid modifications were identified to improve cleavage efficiency at physiological pH. The optimized H-32-Fc substrate enabled improved electrochemical performance and lower limits of detection, advancing the platform toward physiologically relevant conditions.

**Chapter 4** evaluates the electrochemical response of the Au MEA platform in the presence of human serum and investigates potential cross-reactivity from other proteases, including MMP-9 and ADAM-17. The study examines sensor stability in complex matrices, models kinetic responses to CB spiked in serum, and assesses linearity with increasing serum concentration. It further investigates nonspecific decay mechanisms, activation agent interference, and validates protease activity using fluorogenic and electrochemical assays. The findings highlight matrix effects, buffer-dependent enzyme activity, and improved specificity of the optimized peptide substrate.

**Chapter 5** summarizes the major findings of the three studies, discusses key design principles for future multiplex electrochemical protease detection, outlines limitations, and presents future perspectives for advancing the Au MEA biosensing platform toward practical biomedical applications.

# Chapter 1 - Introduction

## 1.1 Background and Motivation

Proteases constitute a large and diverse class of enzymes that regulate numerous physiological processes through selective cleavage of peptide bonds in protein substrates <sup>1</sup>. They play essential roles in protein turnover, cell signaling, immune response, tissue remodeling, and apoptosis. In normal physiology, protease activity is tightly regulated at multiple levels, including zymogen activation, endogenous inhibitors, compartmentalization, and pH dependence <sup>2</sup>. Dysregulation of protease activity, however, is implicated in a wide range of pathological conditions such as cancer, inflammation, neurodegenerative diseases, and cardiovascular disorders <sup>3</sup>. For example, cysteine proteases such as cathepsins and zinc-dependent matrix metalloproteinases (MMPs) are known to contribute to extracellular matrix degradation, angiogenesis, and tumor invasion when aberrantly activated, highlighting their critical role in disease progression <sup>4</sup>.

While protease expression levels are often used as biomarkers in cancer diagnostics, increasing evidence suggests that protease activity, rather than total protein abundance, is a more reliable indicator of disease state and aggressiveness <sup>5</sup>. Proteases are frequently expressed in inactive proenzyme forms and require specific biochemical conditions, such as acidic pH, cofactor availability, or removal of inhibitory domains, to become catalytically active <sup>6</sup>. Consequently, elevated expression does not necessarily correlate with enzymatic function. For instance, cathepsin B may be present in both normal and cancerous tissues, but its extracellular and acidic microenvironment-activated form is closely associated with tumor invasion and metastasis <sup>7</sup>. Similarly, MMPs are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs), making activity-based measurements essential for accurate functional assessment <sup>8</sup>. These observations

underscore the need for analytical tools capable of directly probing protease activity rather than expression alone.

Conventional protease detection methods used in research and clinical settings include ELISA <sup>9</sup>, zymography <sup>10</sup>, fluorescence-based assays <sup>11</sup>, and mass spectrometry-based proteomics <sup>12</sup>. While these techniques provide valuable information, they suffer from several inherent limitations. Immunoassays such as ELISA primarily measure total protein levels and fail to distinguish between active and inactive enzyme forms. Gel-based methods, including zymography, are labor-intensive and unsuitable for rapid or point-of-care analysis. Fluorescence assays, although sensitive, often require complex labeling strategies, bulky instrumentation, and are prone to signal interference in complex biological matrices. Moreover, most traditional assays are limited to single-analyte detection, making them inadequate for studying the coordinated action of multiple proteases involved in cancer progression.

## **1.2 Biosensors – Principles and Classification**

A biosensor is an analytical device that combines a biological recognition element with a physicochemical transducer to produce a measurable signal proportional to the concentration or activity of a target analyte <sup>13</sup>. According to the widely accepted IUPAC definition <sup>14</sup>, biosensors rely on specific biochemical interaction-such as enzyme catalysis, antigen-antibody binding, or nucleic acid hybridization, which is subsequently converted into an electrical, optical, or mechanical signal. The fundamental working principle of a biosensor involves selective recognition of the analyte by the bioreceptor, followed by transduction of this interaction into a quantifiable signal that can be processed and displayed <sup>15</sup>. Due to their high specificity, rapid response, and potential for miniaturization, biosensors have become indispensable tools in medical diagnostics, environmental monitoring, food safety, and biodefense applications.

A typical biosensor consists of three key components: a bioreceptor, a transducer, and a signal processing unit <sup>15</sup>. The bioreceptor is responsible for selective recognition of the target analyte and may include enzymes, antibodies, nucleic acids, cells, or synthetic biomimetic receptors. Primary components of biosensors are shown in **Fig. 1.1**. In protease biosensors, peptide substrates are commonly employed as bioreceptors due to their tunable specificity and compatibility with activity-based detection <sup>16</sup>. The transducer converts the biorecognition event into a measurable signal, such as a change in current, potential, light intensity, or mass. Finally, the signal processing unit amplifies, filters, and converts the raw signal into a readable output. The performance of a biosensor is defined by parameters such as sensitivity, selectivity, response time, and limit of detection, which are critically dependent on the efficient integration of these components <sup>15</sup>.

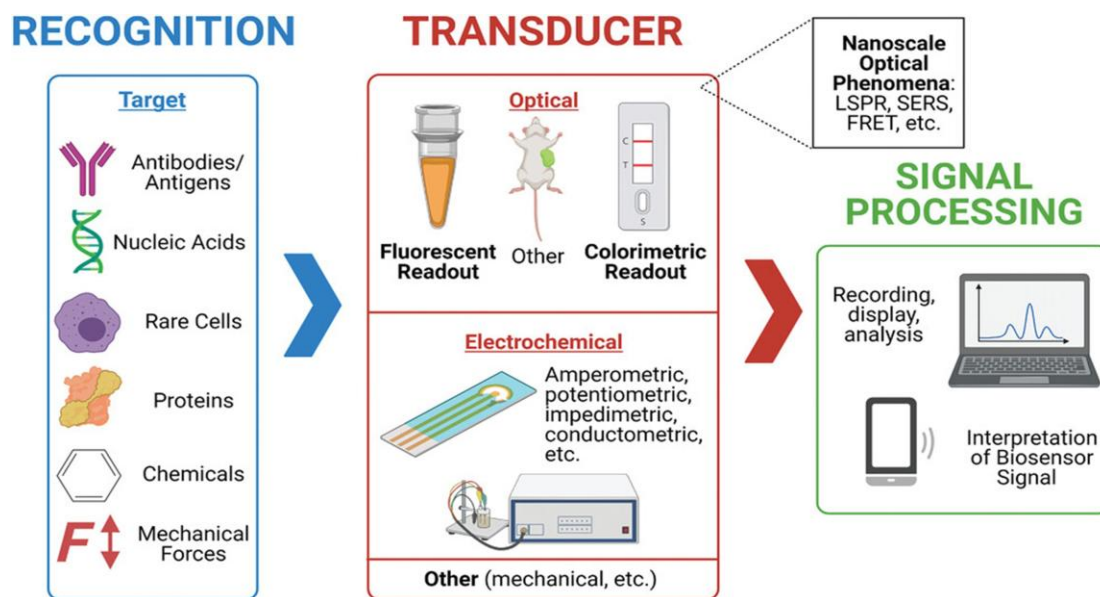


**Figure 1.1** Primary components of biosensors. Biosensors detect targets of biological importance, including rare cells, nucleic acids, mechanical forces, etc. The biosensor recognizes the target in a variety of ways (e.g., affinity interactions) and converts this signal via a transducer element into a measurable signal. These transducers are often optical, electrochemical, or mechanical. Reprinted (adapted) with permission from WILEY Advanced Functional Materials. 2021, 31, 2104126. Copyright 2021 John Wiley and Sons.

Biosensors can be broadly classified based on the nature of their transduction mechanism. Among the most widely studied categories are optical, electrochemical, and piezoelectric biosensors, each offering distinct advantages and limitations depending on the target application<sup>15</sup>.

Optical biosensors detect changes in optical properties such as absorbance, fluorescence, luminescence, or refractive index resulting from a biorecognition event. Common examples include fluorescence-based assays, surface plasmon resonance (SPR), and Forster resonance energy transfer (FRET) sensors<sup>17</sup>. Optical biosensors offer high sensitivity and real-time monitoring capabilities and have been extensively applied to protease activity measurements. However, they often require bulky instrumentation, complex labeling strategies, and sophisticated optical alignment, which limit their portability and suitability for point-of-care applications<sup>17</sup>.

Piezoelectric biosensors, such as quartz crystal microbalance (QCM) devices, detect changes in mass at the sensor surface through shifts in resonance frequency<sup>18</sup>. These label-free sensors enable direct monitoring of biomolecular interactions and have been used for studying protein-protein interactions and enzymatic reactions<sup>19</sup>. Despite their high sensitivity, piezoelectric biosensors are susceptible to environmental factors such as temperature and viscosity changes and are less amenable to multiplexed detection in complex biological samples.

Electrochemical biosensors operate by measuring changes in electrical parameters, such as current, potential, or impedance, induced by the interaction between the analyte and the bioreceptor. These biosensors are particularly attractive due to their inherent sensitivity, low power requirements, and compatibility with miniaturized and multiplexed platforms<sup>20</sup>. Techniques such as amperometry, voltammetry, and electrochemical impedance spectroscopy are commonly employed. Electrochemical biosensors have demonstrated exceptional performance in detecting

enzymatic activity, including protease-mediated peptide cleavage, making them highly suitable for clinical diagnostics and real-time monitoring <sup>21</sup>.

Electrochemical biosensors offer several advantages that make them particularly well-suited for point-of-care diagnostics. They provide high sensitivity and low detection limits while requiring minimal sample volumes and simple instrumentation. Their compatibility with microfabrication techniques allows integration into portable, low-cost, and multiplexed devices <sup>22</sup>. Importantly, electrochemical readouts are less affected by optical interference from turbid or colored biological samples, enabling reliable operation in clinically relevant matrices <sup>23</sup>. These attributes, combined with their ability to directly probe enzyme activity under physiologically relevant conditions, position electrochemical biosensors as a powerful platform for next-generation diagnostic technologies, including multiplex protease activity sensing.

### **1.3 Micro and Nano Technologies in Biosensing Applications**

Nanomaterials have become integral components in modern biosensor design due to their unique physicochemical properties, which differ significantly from their bulk counterparts. High surface to volume ratios, tunable electronic properties, and enhanced catalytic activity enable nanomaterials to substantially improve biosensor sensitivity, response time, and limit of detection <sup>24</sup>. In biosensing applications, nanomaterials facilitate efficient immobilization of bioreceptors, promote rapid electron transfer, and enhance signal transduction <sup>23</sup>. These advantages are particularly beneficial for enzyme-based and activity-based biosensors, where subtle biochemical changes must be detected against complex biological backgrounds. As a result, nanomaterial-enabled biosensors have demonstrated superior analytical performance in detecting low-abundance biomarkers relevant to disease diagnosis, including cancer-associated proteases <sup>25</sup>.

Among various nanomaterials, gold nanostructures have been extensively employed in biosensor development owing to their excellent electrical conductivity, chemical stability, and biocompatibility. Gold nanoparticles, nanowires, and nanostructured electrodes provide versatile platforms for biomolecule immobilization while maintaining biological activity<sup>26</sup>.

Apart from the above nano scale technologies, gold microelectrode arrays offer distinct advantages for multiplex biosensing by enabling spatially resolved detection of multiple analytes on a single device<sup>27</sup>. Their well-defined geometry and reproducible fabrication allow precise control over electrode surface area and electrochemical behavior<sup>28</sup>. Furthermore, gold surfaces support the formation of ordered self-assembled monolayers, facilitating the development of robust and reproducible biosensing interfaces<sup>29, 30</sup>. Schematic diagram of an ideal, single-crystalline SAM of alkane thiolates supported on a gold surface with a (111) texture is shown in **Fig. 1.2**. These characteristics make gold microelectrode arrays especially attractive for electrochemical detection of protease activity using peptide-based substrates<sup>31</sup>.

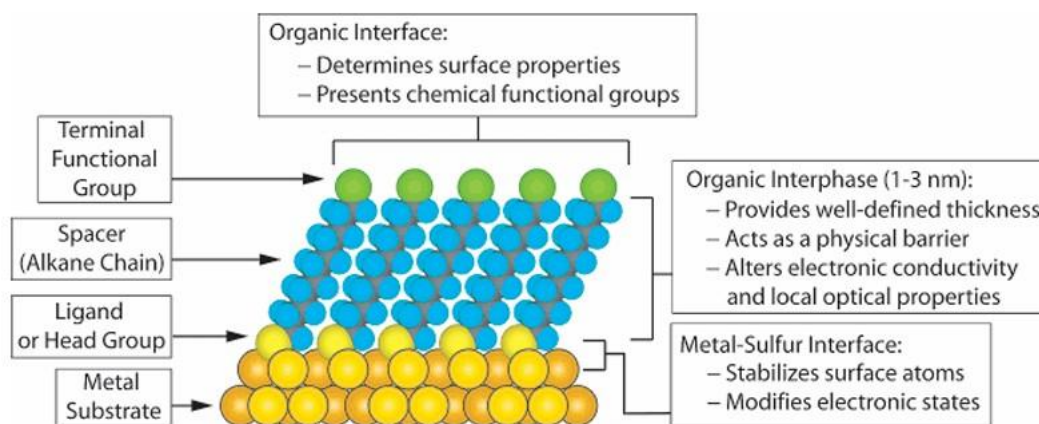


**Figure 1.2** Schematic diagram of an ideal, single-crystalline SAM of alkane thiolates supported on a gold surface with a (111) texture. The anatomy and characteristics of the SAM are highlighted. Reprinted (adapted) with permission from ACS Chemical Reviews. 2005, 105 (4), 1103-1169. Copyright 2005 American Chemical Society.

Effective surface functionalization is critical for achieving high stability in nanomaterial-based biosensors. Gold nanostructures are commonly functionalized using thiol-based self-assembled monolayers, which enable strong and well-oriented attachment of biomolecules through Au-S (40-50 kcal/mol) bonds <sup>32</sup>. Peptide substrates, antibodies, and other bioreceptors can be immobilized either directly or via linker molecules to optimize accessibility and enzymatic activity. Additionally, surface passivation strategies, such as the incorporation of polyethylene glycol or mixed monolayers, are often employed to reduce nonspecific adsorption and improve signal-to-noise ratios <sup>32</sup>.

### 1.4 Proteases as Disease Biomarkers

Protease activity is tightly controlled through zymogen activation, endogenous inhibitors, subcellular localization, and environmental factors such as pH. Dysregulation of these control mechanisms can lead to aberrant proteolytic activity, contributing to the onset and progression of numerous diseases <sup>3</sup>. Owing to their central role in pathological remodeling and signaling pathways, proteases have emerged as important functional biomarkers for disease diagnosis and prognosis <sup>33</sup>.

Cancer progression is characterized by extensive remodeling of the extracellular matrix, altered cell-cell interactions, and dysregulated signaling pathways, all of which are heavily influenced by protease activity. Tumor cells and associated stromal cells secrete a variety of proteases that facilitate invasion, metastasis, angiogenesis, and immune evasion <sup>34</sup>. Importantly, the coordinated action of multiple proteases <sup>34</sup>, rather than the activity of a single enzyme, drives malignant transformation and tumor progression <sup>35</sup>. This complexity highlights the need for multiplexed analytical approaches capable of simultaneously monitoring the activity of key cancer-associated proteases within biologically relevant environments. **Fig. 1.3** shows how

proteases regulate cancer stem cell (CSC) properties and remodel their extracellular matrix (ECM), including their retention in the niche, invasion, self-renewal, stemness, immune evasion, and therapy resistance. Proteases either support (+) or inhibit (–) CSC-related processes <sup>36</sup>.

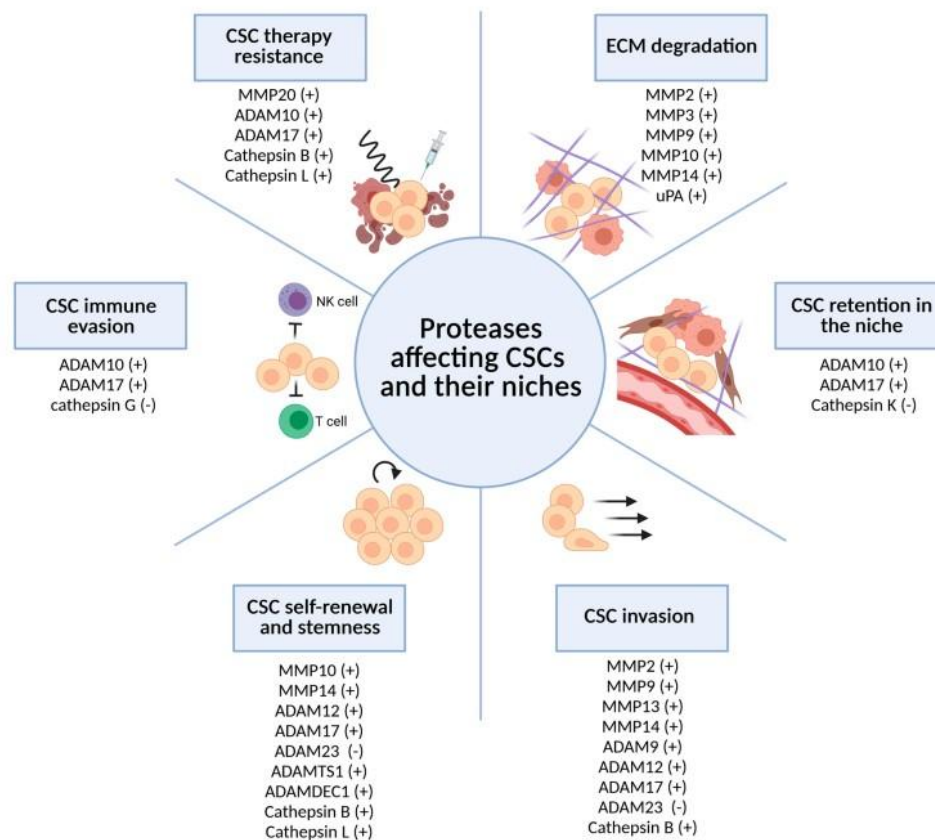


**Figure 1.3** Proteases affect CSC functions and their niches. Proteases degrade niche ECM and influence several CSC properties, including their retention in the niche, invasion, self-renewal, stemness, immune evasion, and therapy resistance. Proteases either support (+) or inhibit (–) CSC-related processes. Reprinted (adapted) with permission from Journal of Histochemistry & Cytochemistry 2021, 69(12) 775–794. Copyright 2021 Sage Publications.

Cathepsins are lysosomal cysteine proteases that play essential roles in intracellular protein turnover under normal physiological conditions. In cancer, several cathepsins, particularly cathepsins B, L, and S, are overexpressed, mis-localized, and secreted into the extracellular space, where they contribute to tumor invasion and metastasis <sup>37</sup>. CB has been extensively studied due to

its ability to degrade extracellular matrix components and activate other proteases. Although cathepsins exhibit optimal activity under acidic conditions, their activity in the mildly acidic tumor microenvironment has been shown to be sufficient to promote pathological proteolysis <sup>38</sup>. These features make Cathepsins attractive targets for activity-based biosensing strategies, especially those designed to operate under variable pH conditions <sup>39</sup>.

Matrix metalloproteinases are a family of zinc-dependent endopeptidases responsible for the degradation of extracellular matrix proteins and the regulation of cell signaling. MMPs such as MMP-2 and MMP-9 are frequently associated with tumor invasion, metastasis, and angiogenesis <sup>40</sup>. Unlike cathepsins, MMPs typically exhibit optimal activity at neutral to slightly basic pH and are regulated by tissue inhibitors of metalloproteinases (TIMPs) <sup>41</sup>. The balance between MMP activity and inhibition is a critical determinant of disease progression <sup>42</sup>. As a result, direct measurement of MMP activity rather than expression alone is essential for accurately assessing their pathological significance in cancer.

A Disintegrin and Metalloproteinase (ADAM) family proteases are membrane-anchored enzymes involved in ectodomain shedding of cell surface proteins, thereby modulating signaling pathways related to growth, inflammation, and cell migration. ADAM-10 and ADAM-17 have been implicated in cancer progression through their role in releasing growth factors and cytokines such as tumor necrosis factor- $\alpha$  and epidermal growth factor ligands. Dysregulated ADAM activity has been linked to increased tumor aggressiveness and resistance to therapy <sup>43</sup>.

The tumor microenvironment is characterized by distinct physicochemical conditions, including hypoxia, elevated protease expression, and extracellular acidification <sup>44</sup>. These conditions profoundly influence protease activity and substrate specificity. For example, cathepsins exhibit enhanced activity in acidic environments, whereas MMPs and ADAM proteases

are more active near physiological pH <sup>45</sup>. This pH-dependent behavior complicates multiplex protease detection, as optimal activity conditions differ among protease classes. Consequently, biosensing platforms designed to assess protease activity must account for microenvironmental factors such as pH to provide biologically meaningful measurements. Understanding and exploiting these dependencies is crucial for developing accurate diagnostic tools capable of profiling complex proteolytic networks in cancer.

## **1.5 Protease Detection Strategies**

### **1.5.1 Gel-based assays**

Gel-based techniques, such as zymography and SDS-PAGE based protease assays, have long been used to evaluate protease activity by visualizing substrate degradation in polyacrylamide gels <sup>46</sup>. These methods allow qualitative or semi-quantitative assessment of enzyme activity and can provide information on molecular weight and isoform distribution <sup>47</sup>. Despite their widespread use, gel-based assays are labor-intensive, time-consuming, and poorly suited for high-throughput or real-time analysis. Moreover, they often require extensive sample preparation and lack the sensitivity required for detecting low abundance proteases in complex biological samples.

### **1.5.2 ELISA and Immunoassays**

Immunoassays such as enzyme-linked immunosorbent assays (ELISA) are widely employed in clinical diagnostics due to their high specificity and ease of standardization <sup>48</sup>. These assays rely on antibody-antigen interactions to quantify protease expression levels in biological samples, including plant-based samples <sup>49</sup>. Schematic illustrations of sandwich ELISA are shown in **Fig. 1.4** <sup>50</sup>. However, ELISA and related immunoassays do not distinguish between active and inactive enzyme forms, limiting their ability to provide functional insight into proteolytic activity.

Additionally, antibody cross-reactivity and the need for multiple antibodies hinder their applicability in multiplex protease analysis <sup>51</sup>.

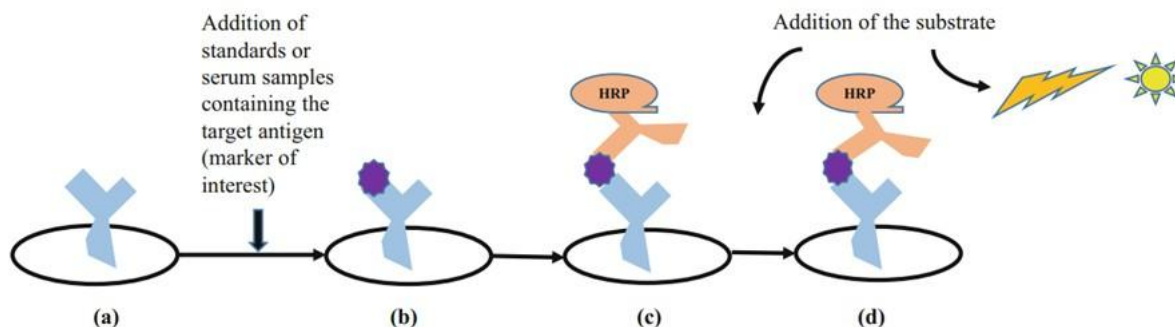


**Figure 1.4** Schematic illustrations of sandwich ELISA. (a) Capture antibody bound or precoated into each well of a 96-well plate. (b) Target antigen or protease (purple) binds to the capture antibody precoated into each well of the 96-well plate. (c) A horseradish peroxidase (HRP) enzyme-conjugated antibody is added that binds to the target antigen or protease bound to the capture antibody, thus forming a “sandwich” followed by addition of the substrate that undergoes enzymatic hydrolysis for detection. Reprinted (adapted) with permission from Springer Nature 2023, (2612) 1–17. Copyright 2023 Springer Nature.

### 1.5.3 Fluorescence and Colorimetric Methods

Fluorescence and colorimetric-based assays are commonly used to monitor protease activity through substrate cleavage induced signal changes. Techniques such as Forster resonance energy transfer (FRET) and quenched fluorogenic peptides offer high sensitivity and real-time monitoring capabilities <sup>11</sup>. Principles of FRET-based protease sensors <sup>52</sup> are shown in **Fig. 1.5**. Nevertheless, these methods often require expensive optical instrumentation, complex labeling strategies, and are susceptible to photobleaching and background interference from biological matrices. Colorimetric assays, while simpler, typically suffer from lower sensitivity and limited dynamic range <sup>52</sup>.

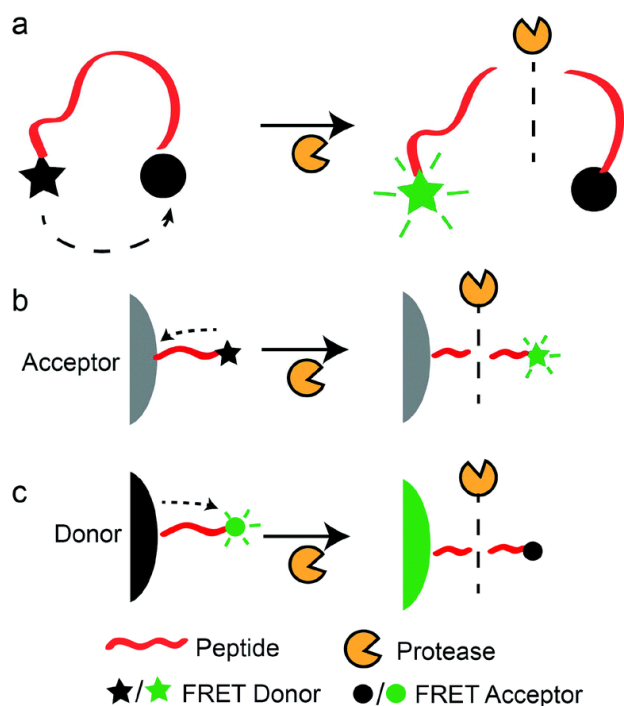


**Figure 1.5** Principles of FRET-based protease sensors. (a) FRET sensors based on organic FRET donors and acceptors. (b) FRET sensors based on nanomaterials as FRET acceptors and FRET donor-labelled protease substrates. (c) FRET sensors based on nanomaterials as FRET donors and FRET acceptor-labelled protease substrates. Reprinted (adapted) with permission from Analyst RCS 2017, (142) 1867-1881. Copyright Creative Commons Attribution 3.0 Unported License.

Collectively, traditional protease detection techniques are constrained by long assay times, limited sensitivity, and poor compatibility with multiplex analysis. Most importantly, many of these methods primarily measure protease abundance rather than enzymatic activity, which can lead to misleading conclusions in disease diagnostics. These limitations underscore the need for alternative detection strategies capable of rapid, sensitive, and activity-based protease profiling.

#### 1.5.4 Activity-Based Probes

Activity-based probes (ABPs) are designed to covalently bind to the active site of proteases, enabling selective labeling of catalytically active enzymes. These probes have been widely used for imaging and proteomic studies and provide valuable insights into protease activity in complex biological systems<sup>53</sup>. **Fig. 1.6** shows the general design of an activity-based probe.

However, ABPs often involve irreversible binding, complex synthesis, and limited reusability, which restrict their applicability in real-time and multiplexed biosensing platforms.

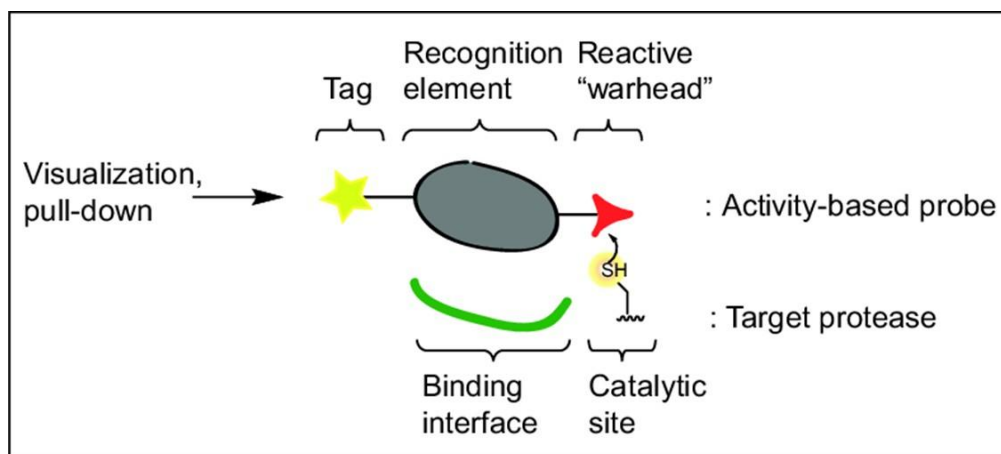


**Figure 1.6** General design of an activity-based probe (ABP). Reprinted (adapted) with permission from *Methods on Enzymology* 2019, (618) 357-387. Copyright 2019 Elsevier.

### 1.5.5 Electrochemical Protease Biosensors

Electrochemical protease biosensors convert protease-mediated cleavage events into electrical signals, offering high sensitivity, rapid response, and compatibility with miniaturized platforms. These sensors typically employ peptide substrates immobilized on conductive surfaces, where enzymatic cleavage modulates electron transfer or interfacial properties<sup>54</sup>. **Fig. 1.7** shows a schematic illustration of the Fc-peptide-based electrochemical biosensor for the detection of trypsin and thrombin activity<sup>54</sup>.

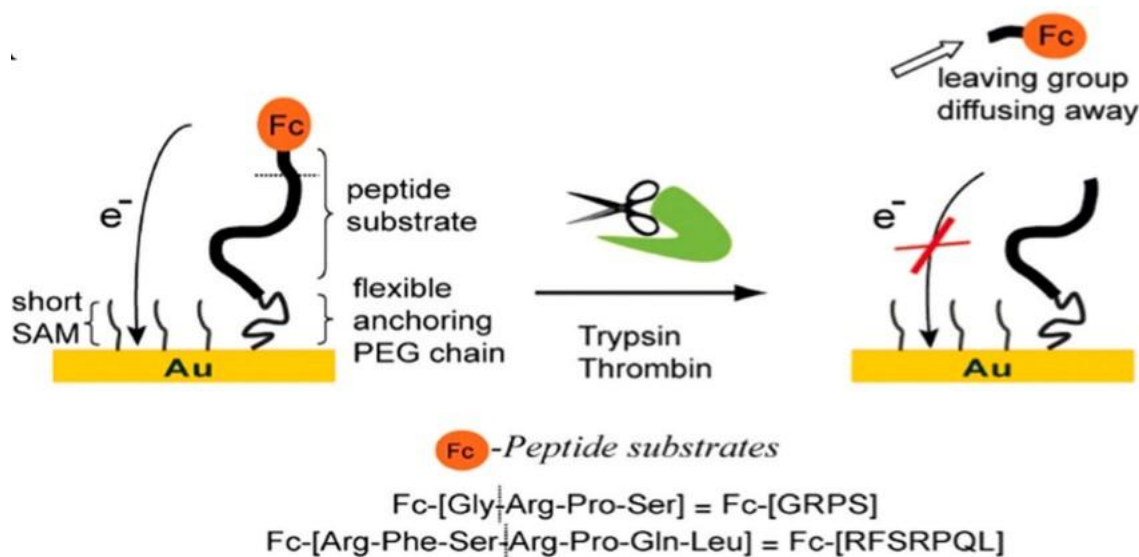


**Figure 1.7** Schematic illustration of the Fc-peptide-based electrochemical biosensor for the detection of trypsin and thrombin activity. Reprinted (adapted) with permission from International Journal of Electrochemical Science 2023, 18 (12) 1452-3981. Copyright 2023 Elsevier Creative Commons CC-BY license.

Peptide substrate-based electrochemical sensors based upon the specificity of protease-substrate interactions to generate measurable signals after enzymatic cleavage. These sensors are particularly attractive for activity-based detection because peptide sequences can be rationally designed to target specific proteases <sup>54</sup>. **Fig. 1.8** shows a schematic illustration of an electrochemical proteolytic beacon (EPB) for the detection of matrix metalloproteinase (MMP-7). Here, it shows a self-assembling electrochemical FC-peptide conjugate on the gold substrate and cleavage of EPB in the presence of MMP-7 <sup>16</sup>. Advances in peptide engineering and surface immobilization have enabled improved selectivity and sensitivity. Nonetheless, overlapping substrate specificity among protease families remains a significant challenge, especially in multiplex detection scenarios. Electrochemical approaches are particularly well suited for activity-based protease detection in complex biological samples due to their robustness and low susceptibility to optical interference.

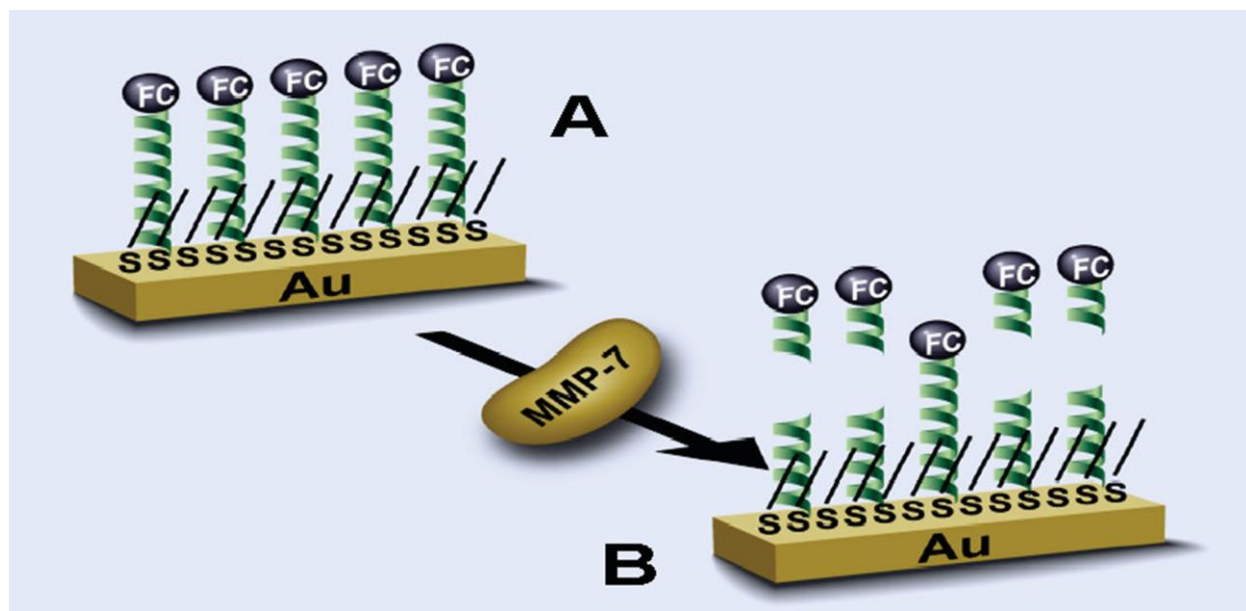


**Figure 1.8** Schematic of Electrochemical Proteolytic Beacon (EPB) for detection of matrix metalloproteinase: (A) self-assembling electrochemical FC-peptide conjugate on the gold substrate; (B) Cleavage of EPB in the presence of MMP-7. Reprinted (adapted) with permission from Journal of the American Chemical Society 2006, 128 (38) 12382-3. Copyright 2006 American Chemical Society.

Recent efforts have focused on developing multiplexed protease sensors capable of simultaneously monitoring multiple enzymes using microelectrode arrays and distinct peptide substrates<sup>28</sup>. Microelectrode arrays and nanostructured platforms have emerged as promising tools for achieving multiplex detection with high sensitivity and minimal sample consumption<sup>55</sup>. Despite these advances and challenges related to specificity, pH compatibility continues to limit widespread implementation.

## 1.6 Challenges in Multiplex Protease Detection

Multiplex protease detection presents several fundamental challenges that complicate sensor design and data interpretation. One major issue is overlapping substrate specificity, as many proteases recognize similar peptide motifs, leading to potential cross-reactivity. Additionally, different proteases exhibit distinct optimal pH conditions, reflecting their biological roles and

subcellular localization. This disparity complicates the development of unified assay conditions for simultaneous detection. Signal instability of surface-immobilized substrates further hinders accurate multiplex measurements. Achieving long-term stability, reproducibility, and reliable quantification remains a critical barrier to practical multiplex protease biosensing.

## **1.7 Research Objectives and Hypotheses**

The primary objective of this thesis is to develop a multiplex electrochemical biosensing platform for activity-based detection of cancer-associated proteases and validate the sensor platform by demonstrating substrate-dependent proteolysis as an initiative. Our previous work demonstrated that nanoelectrode arrays (NEAs) constructed from vertically aligned carbon nanofibers (VACNFs) and functionalized with ferrocene-labeled peptides can effectively measure protease activity of CB, while hexapeptides provided optimal performance at 36 °C compared to tetra- and octapeptide substrates<sup>25</sup>. Depending on these previous findings, this work aims to further improve and modify the peptide substrates with high specificity toward selected protease CB and check the cross-reactivity with matrix metalloproteinases and ADAM proteases. A central hypothesis of this research is that rational peptide design can overcome challenges associated with substrate cross-reactivity and pH-dependent activity by tuning hexa-peptide substrates. The performance of the developed biosensor is systematically evaluated under physiologically relevant pH conditions to ensure biological relevance and diagnostic potential.

## **1.8 Gold Microelectrode Array (Au MEA) and the fundamental principles employed in the thesis**

### **1.8.1 Au MEA Fabrication**

Photolithography is a cornerstone microfabrication technique used to define microscale patterns on solid substrates with high spatial precision. It enables the controlled transfer of geometric patterns from a photomask onto a photosensitive resist layer, which subsequently serves as a template for material deposition, etching, or lift-off processes. In the fabrication of gold microelectrode arrays (Au-MEAs), photolithography plays a critical role in defining electrode geometry, interconnects, and exposed sensing areas. Overall, photolithography enables the precise spatial control required to fabricate reproducible and electrically isolated Au microelectrode arrays. The sequential use of patterning, etching, and passivation steps ensures well-defined electrode geometries, stable electrical performance, and compatibility with surface chemistry strategies employed in electrochemical biosensing. The fabrication of the Au MEA chip was performed at the Stanford Nanofabrication Facility (Stanford, CA).

The 4" Si (100) wafer was first oxidized in gaseous H<sub>2</sub>O at 1100 °C for 45 min to form a 550 nm SiO<sub>2</sub> layer on the surface. Then, the wafers were coated with Ti, Au, and Ti of respective thicknesses 20 nm, 100 nm, and 20 nm sequentially by using electron beam evaporation (Innotec ES26C, Battle Ground, WA). The wafers were then spin-coated with an automated spin-coating track (SVG 8400, San Jose, CA) with 1.0 μm SPR3612 positive photoresist (Rohm and Haas Electronic Materials, Marlborough, MA) and exposed on a mask aligner (Karl Suss MA-1, Garching, Germany) with an exposure dose of 80 mJ/ cm<sup>2</sup> (exposure time = 5.3 s) through a chrome photomask (FrontRange Photomask, Lake Havasu City, AZ). After being developed with an automated developing track (SVG 8600, San Jose, CA), a photoresist electrode MEA pattern was

formed on the wafer. The exposed Ti top layer was then etched with 2% aqueous HF solution, the underneath Au layer was removed with Transene TFA Au, and again with 2% HF to remove the bottom layer of Ti. The MEA pattern was retained because of the photoresist, which was stripped off after etching. A 1- $\mu\text{m}$  thick layer of  $\text{SiO}_2$  was deposited over the entire wafer by plasma-enhanced chemical vapor deposition (PE-CVD), Plasma-Therm Shuttle-lock SLR-730-PECVD (St. Petersburg, FL) with 250 sccm 5%  $\text{SiH}_4/\text{He}$ , 800 sccm He, and 1700 sccm  $\text{N}_2\text{O}$  at 350 °C, 1100 mTorr, and 200 W for 15 min. Next, the second photolithography step with the same recipe was processed on the wafer again to expose the contact pad and microelectrode while covering the remaining areas protected by the photoresist. The exposed  $\text{SiO}_2$  was etched with  $\text{CHF}_3$  plasma (2 sccm  $\text{O}_2$  and 45 sccm  $\text{CHF}_3$  at 5.0 mTorr for 8 min) using a reactive ion etcher (RIE, Plasma-Therm Versaline LL-ICP, St. Petersburg, FL), and then the top layer of Ti was etched with 2% aqueous HF to expose the Au surface. Finally, the wafers were coated with a 5.0- $\mu\text{m}$  layer of SPR3612 as a protective layer. A wafer saw (DISCO DAD3240, Tokyo, Japan) was used to dice the wafer into individual chips. Before use, chips were sonicated in acetone for 10 min, followed by rinsing with methanol and isopropanol to remove the protective layer. A workflow of the wafer-scale fabrication process is shown in **Fig. 1.9A**. **Fig. 1.9B** illustrates the top and side views of the MEA structure<sup>27</sup>.

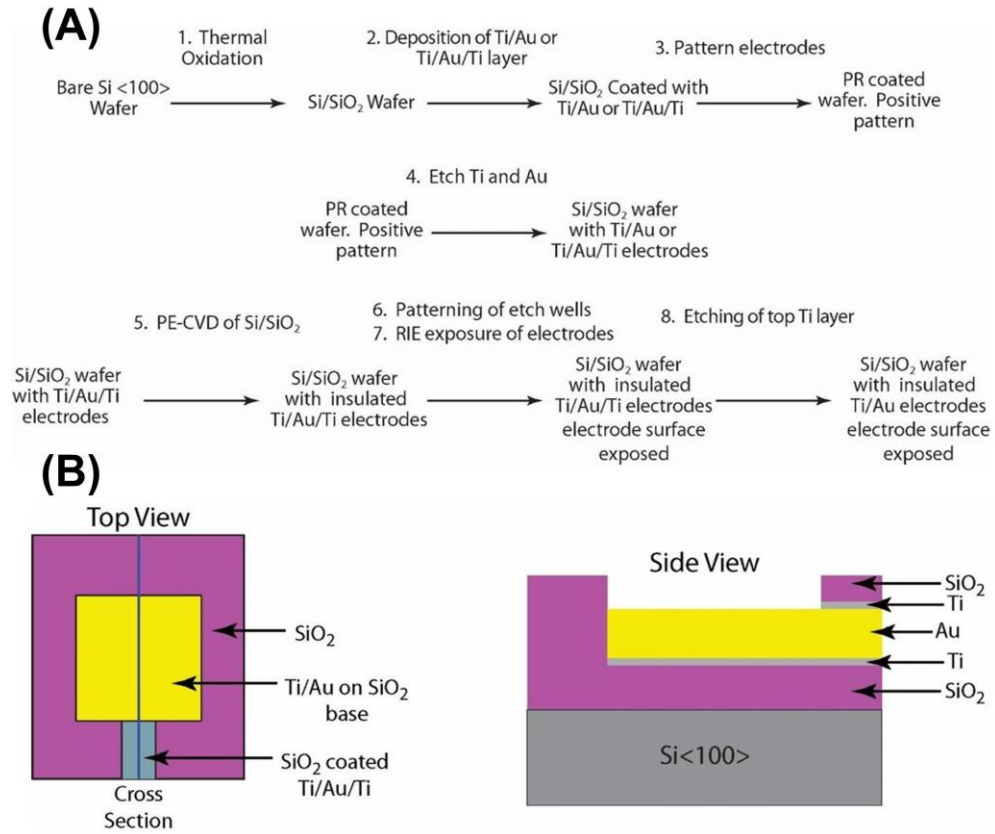


**Figure 1.9** (A) Fabrication workflow for wafer-scale MEA fabrication. (B) Top-view and side-view diagrams illustrating the structure of the MEA. Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier.

**Fig. 1.10** shows the SEM characterization of the fabricated Au MEAs. An exposed Au surface is shown in **Fig. 1.10A**. As shown in **Fig. 1.10B**, the upper SiO<sub>2</sub> surface exhibits noticeably higher roughness compared to the Au regions exposed. **Fig. 1.10C** presents a high-magnification electron micrograph highlighting the interface between the top SiO<sub>2</sub> passivation layer and the exposed Au electrode surface. This helps to protect the buried Au layer that acts as the microelectrode.

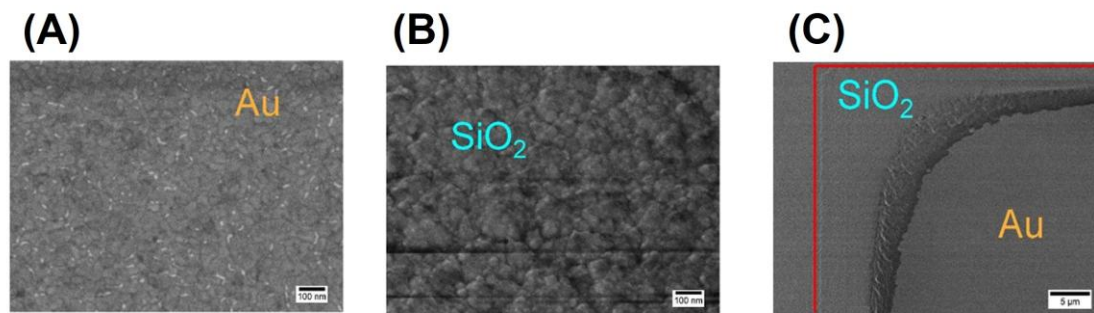


**Figure 1.10** SEM images of different regions of a MEA: (A) An exposed Au surface, (B) The top surface of the PECVD SiO<sub>2</sub> layer, and (C) The boundary between the top SiO<sub>2</sub> layer and the exposed Au surface. Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier.

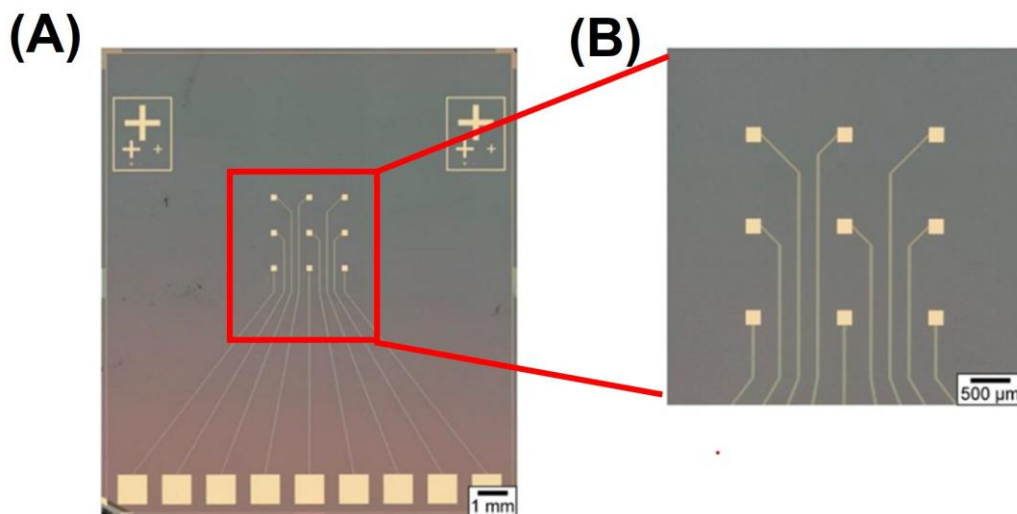


**Figure 1.11** Optical micrographs of a fabricated Au MEA chip. (A) Au MEA chip with 9 contact pads (1.0 mm × 1.0 mm) at the bottom and a magnified optical image of the 3 x 3 Au MEA with nine microelectrodes (200 μm × 200 μm). Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier.

**Fig. 1.11A** shows an optical image of a single MEA chip and a magnified optical image of a 3×3 Au MEA. These nine microelectrodes (200 μm × 200 μm each) are separately connected to contact pads (1.0 mm × 1.0 mm each) via electrical leads, which are buried underneath an insulating SiO<sub>2</sub> film<sup>27</sup>.

### 1.8.2 Experimental setup

The experimental configuration is illustrated in **Fig. 1.12**. A schematic block diagram of the setup is presented in **Fig. 1.12A**. In this setup, the nine output leads from the potentiostat (**Fig. 1.12B**) were first routed through a breakout box and subsequently connected to a custom-built electrochemical cell using a ribbon cable (**Fig. 1.12C**). The sensor chip can be integrated into the custom electrochemical cell (**Fig. 1.12D**), with electrical connections established via pogo pins. All electrochemical measurements were conducted using a shared mercury/mercurous sulfate reference electrode ( $\text{Hg}/\text{Hg}_2\text{SO}_4$ , MSE) containing saturated  $\text{K}_2\text{SO}_4$  as the internal electrolyte, along with a common platinum wire counter electrode. Gold microelectrode arrays (Au MEAs) can be sensitive to chloride ions; therefore, using a commonly employed  $\text{Ag}/\text{AgCl}$  reference electrode would introduce  $\text{Cl}^-$ , potentially altering the gold surface<sup>56</sup> forming  $\text{AuCl}_4^-$  like species in anodic scans and redeposition during cathodic scans<sup>57</sup>. In contrast, the  $\text{Hg}/\text{Hg}_2\text{SO}_4$  (MSE) reference electrode is chloride-free and provides a more stable and reliable environment for this system.

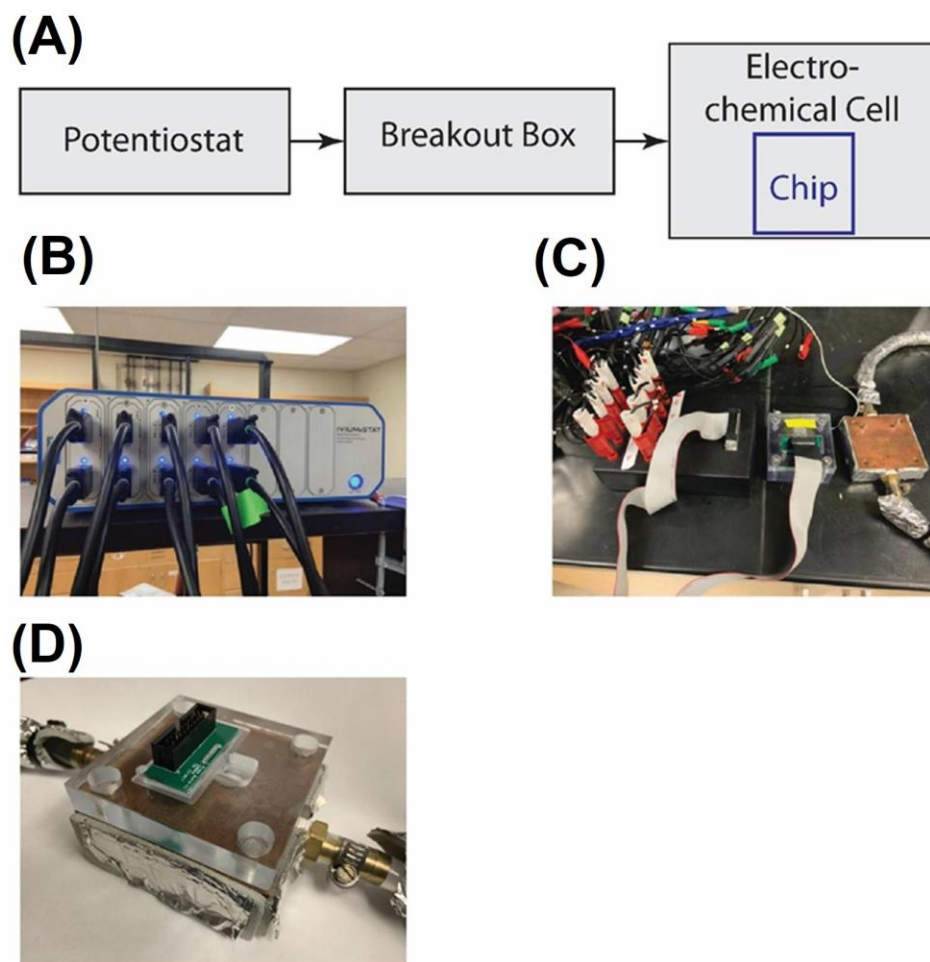


**Figure 1.12** Experimental setup. (A) Box diagram of multiplexed experimental setup. (B) Photograph of the Ivium n-Stat potentiostat. (C) Photograph of the breakout box, electrochemical cell, and copper base plate. (D) Photograph of the electrochemical cell fitted with the copper base plate. Reprinted (adapted) with permission from Biosensors and Bioelectronics 2020, 165 (112330). Copyright 2020 Elsevier.

### 1.8.3 Michaelis-Menten Model for Electrochemical Data Analysis

The proteolysis sensing mechanism is depicted in **Fig. 1.13**. The ball and stick structures represent the alkane thiols that assemble into a densely packed SAM on the Au surface through Au-S interactions. The surface ratio of -COOH to -OH terminal groups is controlled by forming the SAM in a mixed solution of MHA and MCH (1:10) in anhydrous ethanol. The peptide substrate (hexapeptides with a  $\text{H}_2\text{N}-(\text{CH}_2)_4$ - linker) bearing a distal ferrocene (Fc) redox tag  $[-\text{HN}-(\text{CH}_2)_2-$

Fc] is covalently linked to the -COOH groups of SAM via EDC/Sulfo-NHS mediated amide coupling. Upon applying ACV, the Fc tag undergoes reversible oxidation to Fc<sup>+</sup>, transferring electrons to the electrode as shown by the green arrows.

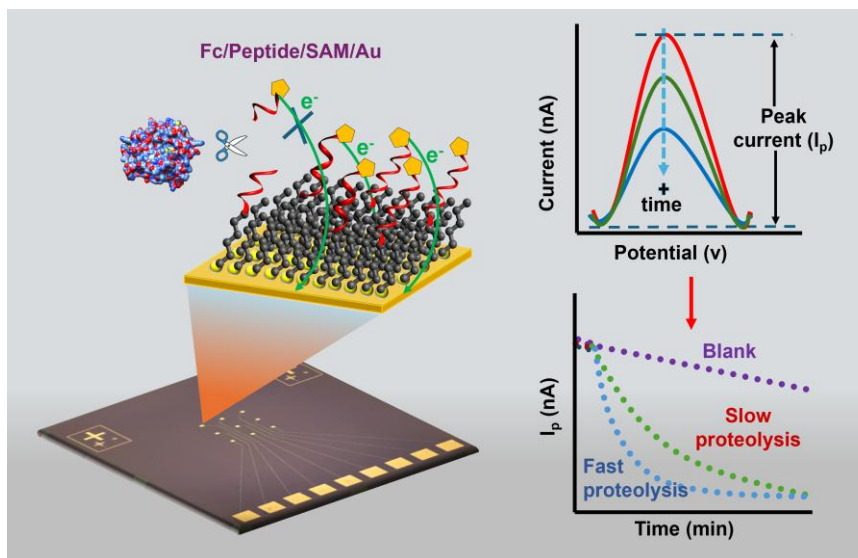
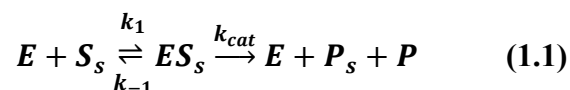


**Figure 1.13** Optical image of an Au MEA chip with 9 contact pads (1.0 mm × 1.0 mm) at the left bottom and schematic illustration of the proteolysis sensing mechanism, including major components of the peptide/Au sensor, representative ACV curves during proteolysis, and the kinetic proteolysis curves under varied protease activities.

As shown in **Fig. 1.13**, the Fc-modified peptide produces a characteristic ACV peak current (*i<sub>p</sub>*) near -0.15 V vs. MSE on top of a sloping background, which is subtracted during analysis. Each ACV scan takes ~1 min and is continuously repeated over time. As proteolysis occurs, the cleaved Fc-peptide fragments diffuse away from the surface, leading to the progressive decrease of the *i<sub>p</sub>* amplitude, as illustrated in **Fig. 1.13**. Previous work <sup>27, 28</sup> established that the decay of *i<sub>p</sub>* aligns with proteolysis kinetics predicted by a heterogeneous Michaelis-Menten model, enabling quantitative, time-resolved measurement of enzyme activity at the electrode interface:



where E is the enzyme,  $S_s$  is the surface-bound peptide-Fc substrate,  $ES_s$  is the enzyme-substrate complex,  $P_s$  is the cleaved peptide fragment bound at the surface, P is the mobile peptide fragment containing Fc to be diffused into the bulk solution, and  $k_1$ ,  $k_{-1}$ , and  $k_{cat}$  are the rate constants of the respective reactions. Based on the steady-state assumption, proteolysis kinetics can be described as an exponential decay function<sup>27, 28</sup> and explained here.

Using the steady-state assumption, the reaction rate can be expressed as:

$$v = -\frac{d\Gamma_{ss}}{dt} = \frac{k_{cat}}{K_M + [E]_0} [E]_0 \Gamma_{ss} \quad (1.2)$$

Where  $\Gamma_{ss}$  is the surface concentration of peptide-Fc substrate,  $K_M$  is the Michaelis-Menten constant  $K_M = (k_{cat} + k_{-1})/k_1$ , and  $[E]_0$  is the active enzyme concentration. The capacitive baseline was subtracted from the raw ACV curves to extract the faradaic ACV peak current,  $i_p$ , which decreases with the proteolysis time. Since  $i_p$  is proportional to the Fc-moiety density, the reaction rate can be written as:

$$-\frac{d(i_p/i_{p0})}{dt} = \frac{k_{cat}}{K_M + [E]_0} [E]_0 \left( \frac{i_p}{i_{p0}} \right) \quad (1.3)$$

Assume  $K_M \gg [E]_0$ , we can derive

$$-\frac{d(i_p/i_{p0})}{dt} \approx \frac{k_{cat}}{K_M} [E]_0 \left( \frac{i_p}{i_{p0}} \right) \quad (1.4)$$

After integrating the equation,

$$\frac{i_p}{i_{p0}} = \exp\left(-\frac{k_{cat}}{K_M} [E]_0 t\right) = \exp[-t/\tau] \quad (1.5)$$

The value of  $1/\tau$  represents the activity of the target protease toward the specific peptide–Fc substrate and directly reflects the decay rate of the peak current ( $i_p$ ). The baseline value decreases linearly with the time before the injection of the enzyme, which can be attributed to the inherent instability of the Fc moiety. To compensate for this, a linear term was added to equation 1.5.

$$i_p = a (\exp[-t/\tau]) + bt + c \quad (1.6)$$

$i_p$  is redefined as

$$i_{p0} = a + c \quad (1.7)$$

Equation 1.6 was used to fit the proteolysis data and derive the  $1/\tau$  value for each proteolysis kinetics measurement.

The  $1/\tau$  value for each proteolysis kinetics measurement represents the activity of the target protease on the specific peptide substrate, following:

$$\frac{1}{\tau} = \left( \frac{k_{cat}}{K_M} \right) [E_0] \quad (1.8)$$

#### 1.8.4 Alternating current voltammetry (ACV)

Alternating current voltammetry (ACV) employs a small-amplitude sinusoidal potential superimposed on a linearly or incrementally increasing base (DC) potential<sup>58</sup>, as illustrated in **Fig. 1.14A**<sup>59</sup>. The measured output corresponds to the amplitude of the AC current at each linear or incremental potential step (**Fig. 1.14B**)<sup>59</sup>. As with other voltammetric techniques, the resulting peak is centered at the formal potential of the redox reporter. The ACV experiments done in conjunction with a lock-in amplifier or frequency analyzer offer considerably increased sensitivity. The final signal is the measured amplitude of the AC current at each incremental potential step, as shown in **Fig. 1.14C**<sup>59</sup>.

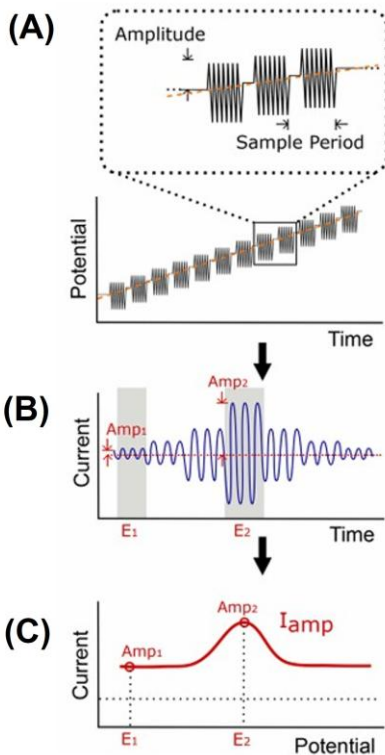


**Figure 1.14** (A) In alternating current voltammetry (ACV), an incrementally rising base potential with a superimposed sinusoidal wave is employed. (B) Here, the magnitude of the faradaic response depends on the chosen frequency of the applied AC potential. (C) The final signal is the measured amplitude of the AC current at each incremental potential step. Reprinted (adapted) with permission from Sensors & Diagnostics RCS 2024, (142) 1867-1881. Copyright Creative Commons Attribution 3.0 Unported License.

The phase relationship between the current and voltage is also of great interest in AC voltammetry. When the AC amplitude is sufficiently small, the electrochemical response remains linear, allowing the current to be decomposed into in-phase (real) and out-of-phase (imaginary) components<sup>60</sup>. The faradaic current arises from reversible oxidation-reduction of electroactive species, while the capacitive current originates from charging and discharging of the electrical double layer. Importantly, these two contributions respond differently to changes in frequency and potential, enabling partial discrimination between surface-bound redox species and background charging currents<sup>61</sup>. This technique can be applied to both bulk and surface-attached redox species

<sup>60</sup>.

For ideal bulk redox species that undergo a fast, reversible electron transfer,

$$i_{p(ACV)} = \frac{n^2 F^2 A \omega^{1/2} D^{1/2} C \Delta E}{4RT} \quad (1.9)$$

Here, In this expression,  $n$  is the number of electrons transferred in the redox reaction,  $F$  is the Faraday constant,  $A$  is the geometric area of the working electrode,  $\omega$  is the angular frequency of the applied AC potential,  $D$  is the diffusion coefficient of the redox species,  $C$  is its bulk concentration,  $\Delta E$  is the amplitude of the sinusoidal AC perturbation,  $R$  is the universal gas constant, and  $T$  is the absolute temperature.

For ideal surface-attached redox species <sup>62, 63</sup>,

$$i_{p(ACV)} = \left(\frac{2}{\pi}\right) \left(\frac{F^2}{4RT}\right) \Gamma_{ss} (2\pi f) \Delta E \quad (1.10)$$

Here, in this expression,  $F$  is the Faraday constant,  $A$  is the geometric area of the working electrode,  $f$  is the frequency of the applied AC potential,  $\Gamma$  is the surface density of the redox species,  $\Delta E$  is the amplitude of the sinusoidal AC perturbation,  $R$  is the universal gas constant, and  $T$  is the absolute temperature.

In this study, alternating current voltammetry (ACV) was employed as a sensitive signal amplification technique to reliably obtain the overall current response associated with the surface coverage of the redox species. A higher current signal was obtained in ACV because the electrons were cycled at each redox tag by the sinusoidal AC wave superimposed on the linear DC potential ramp. For this purpose, the phase-sensitive detection option was not enabled, and the total AC response of the electrode was recorded as the root mean square (RMS) current magnitude, which includes contributions from both faradaic and capacitive components.

## **Chapter 2 - Mitigating Dithiothreitol Interference to Gold Electrodes in Electrochemical Detection of Cathepsin B Activity Toward Multiplex Protease Analysis.**

This work was published in Biosensors and Bioelectronics journal <sup>31</sup>. Copyright © 2025 Elsevier Ltd.

### **2.1 Introduction**

Proteases in normal cells are important in conducting biological processes and maintaining homeostasis. Their main function is to selectively break down proteins at specific sessile bonds <sup>64</sup>, <sup>65</sup>. Many disease conditions, including inflammatory, neurodegenerative disorders, cardiovascular diseases, and cancers, may interrupt the balanced functions of proteases by overexpression and increased activity because of abnormal extracellular and intracellular microenvironments <sup>3</sup>, <sup>35</sup>. They actively participate in cancer progression and promotion by regulating the expression of growth factors and cell signaling pathways, suppressing cell death pathways, and enhancing cell survival mechanisms <sup>35</sup>. Different types of invasive, difficult-to-detect, and hard-to-treat cancers have overexpressed proteases, including MMP-9, MMP-2, cathepsin B, ADAM 17, and uPA <sup>66</sup>, <sup>67</sup>. These proteases have been identified as key targets for drug development, including the design of protease inhibitors, and as prognostic biomarkers for disease diagnosis <sup>68</sup>. Developing new techniques for the direct detection of extracellular proteases is crucial for advancing disease diagnosis.

Various conventional methods are available for quantifying proteases and assessing their activity. These methods generally fall into two main categories: affinity-based and activity-based techniques <sup>69</sup>. In affinity-based techniques, such as enzyme-linked immunosorbent assays

(ELISA), specific antibodies are utilized to selectively bind with target proteases. However, affinity-based methods do not provide information regarding the activity of proteases <sup>70, 71</sup>. Another type of method is activity-based quantification. Such activity assays are of great interest, given the direct relationship between protease activities and diseases. For homogenous assays, fluorescence-based techniques are the most popular due to their high sensitivity and quantitative nature <sup>52</sup>. However, homogeneous assays have limited multiplex sensing capabilities. In contrast, heterogeneous assays can be employed to detect multiple proteases simultaneously, given the microarray technology that is already available <sup>52, 72, 73</sup>. Specifically, activity-based electrochemical biosensors <sup>74</sup> are considered highly sensitive, fast, and cost-effective platforms <sup>52</sup>. Previously, it has demonstrated the ability to measure the activity of proteases such as CB using nanoelectrode arrays (NEAs) fabricated with vertically aligned carbon nanofibers (VACNFs) functionalized with ferrocene redox-tagged peptides <sup>25</sup>. Though the VACNF NEA biosensor platform was successfully demonstrated, the difficulty in scale-up fabrication limited its application. In response to this challenge, a 3×3 gold (Au) microelectrode array (MEA) was recently fabricated, evaluated, and confirmed the capability of detecting CB activity using various peptide substrates <sup>27, 28</sup> due to its potential for rapid multiplex detection of various proteases that lead to early disease diagnosis, treatment monitoring, and inhibitor screening. Hexapeptides with a redox tag (aminomethyl)ferrocene (AMF) at the distal end were attached to the Au MEA via a self-assembled monolayer (SAM) of thiol linkers. The exponential decay of the peak current in continuously repeated alternating current voltammetry (ACV) measurements was attributed to the proteolytic cleavage of the functionalized hexapeptides <sup>27, 28</sup>.

However, while exploring the potential of enhancing this 3×3 Au microelectrode array system into a multiplex detection platform, a significant challenge emerged that people did not

notice in the previous studies <sup>27, 28</sup>. It was discovered that the presence of a reducing agent, dithiothreitol (DTT), an organosulfur compound  $[\text{CH}(\text{OH})\text{CH}_2\text{SH}]_2$ , commonly used to activate CB <sup>75</sup> by reducing its disulfide bridges, introduced a non-specific decay in ACV peak current as shown in **Fig. A.1** in **Appendix A**. This nonspecific decay has the potential to yield false positive results and poses a critical obstacle to achieving the specificity and accuracy required for reliable protease activity detection. Understanding the reasons behind the interference by DTT, and possibly by other activation agents for various proteases <sup>76</sup>, to the thiol/Au biosensor platform and developing mitigating strategies are crucial for detecting the intrinsic activity of proteases as biomarkers.

Activation of recombinant CB with DTT is crucial for proper sensor validation since it is generally synthesized in the non-activated proenzyme form, which shows minimal enzymatic activity, as demonstrated in this chapter <sup>77</sup>. Before tackling the DTT interference, we conducted a series of carefully designed electrochemical experiments using different functionalization schemes with different redox tags, including (aminomethyl)ferrocene (AMF), (2-aminoethyl)ferrocene (AEF), and monocarboxy methylene blue (MCMB). Ferrocene (Fc)-based redox tags and methylene blue (MB) redox tags have distinct inherent redox potential windows, with ferrocene ranging from -0.45 to 0.15 V vs. mercury/mercurous sulfate reference electrode saturated with  $\text{K}_2\text{SO}_4$  (MSE) and methylene blue ranging from -0.80 to -0.45 V vs. MSE. A previous study on Au-S-based DNA sensors has observed a significant signal decay over time, either due to the inherent instability of the redox reporter or the instability of thiol/Au adsorption in the SAMs at potentials below -0.95 V or above +0.48 V (vs. MSE) <sup>78</sup>. In contrast to the Fc reporter, the MB reporter has shown exceptionally high stability, even in blood serum <sup>78</sup>. Another study demonstrated that the MB-based probes form SAMs with more stable electroactivity, able to

generate more robust and readily analyzable data for trypsin protease detection (with no activation agents incorporated), free from the complications of background drift experienced in Fc-based probes <sup>79</sup>. Additionally, the oxidized form of the Fc, i.e., ferrocenium ion ( $\text{Fc}^+$ ), is extremely prone to nucleophilic attack <sup>80, 81</sup>. These studies underscored the importance of investigating the electrochemical stability of thiol-based SAMs at the applied potentials, particularly when using Au-S-based biosensors in the presence of interference agents like DTT.

In this work, we discovered that the main signal decay is due to the combination of the high potential for measuring the Fc signal and the presence of DTT in the electrolyte. A strategy has therefore been developed to remove DTT after activating CB to mitigate the DTT interference. Furthermore, EDTA is incorporated into the buffer solutions to maintain CB in its active state after DTT removal. Using this newly developed protocol, we successfully detected the intrinsic proteolysis activity of CB on an Au MEA platform in 25 mM MES buffer (pH 5.0). This approach allowed us to observe the substrate-dependent proteolysis kinetics that were previously obscured by the nonspecific decay, demonstrating the capability of the Au MEA system for accurate protease activity detection.

## 2.2 Materials and Methods

A detailed description of the materials and instruments in this chapter is given in **Appendix A, Section A.1**. The fabrication of the Au MEA chip was performed at the Stanford Nanofabrication Facility (Stanford, CA), and the fabrication process is discussed in previous studies <sup>27, 28</sup>. More details are provided in **Section 1.8.1**. The design and synthesis of peptide substrates for CB detection are described in **Sections A.2 and A.3 of Appendix A**.

### 2.2.1 Au MEA and Au disk electrode functionalization

The Au MEA chip was sonicated in acetone for 5 minutes to remove the protective photoresist layer, followed by rinsing with methanol and isopropanol for 30 seconds each. The chip was then washed with deionized water and dried using nitrogen gas. Au disk electrodes (2.0 mm in diameter) were first cleaned mechanically by polishing with 0.05  $\mu\text{m}$  alumina oxide slurry and sonicated in DI water for 10 minutes. Before use, the Au MEA chip and Au disk electrodes were cleaned electrochemically. An Au MEA chip was mounted in a custom electrochemical cell, as described in our previous report <sup>27</sup>. Electrochemical cleaning was done by cyclic voltammetry (CV) in 0.10 M phosphate buffer (PB) (0.04 M  $\text{NaH}_2\text{PO}_4$  and 0.06 M  $\text{Na}_2\text{HPO}_4$ , pH = 7.4), using a MSE reference electrode filled with saturated  $\text{K}_2\text{SO}_4$  solution. The CV was performed between  $-0.60$  V and  $0.70$  V to clean the electrode surface. The cleaned Au MEA was then rinsed with deionized water and dried with nitrogen gas.

Functionalization of Au electrodes was done using two main protocols, and the resulting stability was compared using each protocol during electrochemical cycling. The first protocol (protocol-1) is explained in detail in our previous reports <sup>27, 28</sup> and described in **Section A.4** in **Appendix A**. Briefly in the **protocol-1**, the chip was initially incubated in a solution of 1.0 mM 6-mercapto-1-hexanol and 0.2 mM 6-mercapto-1-hexanoic acid in deionized water for 40 minutes, forming a densely packed self-assembled monolayer (SAM) due to thiol adsorption on the gold surface. The electrode was then incubated with a 2.0 mM peptide-Fc substrate solution, containing 0.2 g/L 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.2 g/L N-hydroxysulfosuccinimide (Sulfo-NHS) as coupling agents, for 2 hours at room temperature and washed with DI water before use. In the improved protocol (**protocol-2**), the Au MEA chip or Au disk was first incubated in a solution of 1.0 mM 6-mercapto-1-hexanol and 0.1 mM 6-mercapto-

1-hexanoic acid in anhydrous ethanol overnight, forming a close-packed SAM on the Au surface via thiol adsorption. This SAM serves as an insulator, reducing background current, and provides a ratio of approximately 1:10 between -COOH and -OH groups at the surface. The electrode was then incubated in a solution of 2.0 mM peptide substrate-Fc redox tags (AMF/AEF) along with 0.3 g/L 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.2 g/L (N-hydroxysulfosuccinimide) (Sulfo-NHS), which act as coupling agents, for 2 hours at room temperature. This facilitated the formation of amide bonds between the peptide's amine group and the carboxylic acid group on the SAM surface. The mixed -COOH and -OH groups on the SAM surface were crucial for lowering the peptide-Fc density on the Au surface, minimizing steric hindrance during subsequent proteolysis measurements. Finally, the functionalized chip was incubated in 10 mM hydroxyl amine solution for 3 hours to cap the remaining active intermediate species (Sulfo-NHS ester), resulting in the conversion of the original carboxyl groups on the SAM surface to a hydroxamic acid, which is less reactive toward further coupling and effectively caps the reactive intermediate. After that, it was rinsed with deionized water for 30 seconds to remove any physically adsorbed molecules.

To functionalize the MCMB redox reporter on Au electrodes, 0.1 mM 6-amino-1-hexane thiol was used in place of 6-mercapto-1-hexanoic acid during SAM formation in the above protocols, and the hydroxyl amine treatment was skipped due to not having active intermediate species on the amine-terminated SAM surface. EDC/NHS coupling was done for 2 hours and rinsed with deionized water for 30 seconds to remove any physically adsorbed molecules.

### **2.2.2 DTT removal after activating cathepsin B**

1.0  $\mu$ L of stock cathepsin B (13030 nM) was activated in 22  $\mu$ L of 1 mM DTT, 25 mM MES (pH = 5.0) for 15 min at 25 °C (23  $\mu$ L total volume). The activated CB sample was then

mixed with 477  $\mu\text{L}$  of 25 mM MES, 50  $\mu\text{M}$  EDTA (pH = 5.0) dilution buffer in an Amicon ultra 10K 0.5 mL filter (total volume = 500  $\mu\text{L}$ ). The device spun down at  $14,000 \times g$  for 5 min in a  $40^\circ$  fixed angle rotor at  $20^\circ\text{C}$  with  $\sim 50 \pm 7$   $\mu\text{L}$  remaining in the vial at the end. After that, another  $450 \pm 7$   $\mu\text{L}$  of dilution buffer was added and spun down to  $\sim 50 \pm 7$   $\mu\text{L}$  using the same procedure. The remaining residue was taken as the final activated CB sample for proteolysis experiments in 25 mM MES buffer (pH = 5.0) containing 50  $\mu\text{M}$  EDTA. The blank was prepared following the same procedure without CB.

### **2.2.3 Validation of enzyme activity with fluorogenic assays**

CB enzyme activity was validated using a fluorogenic assay based on fluorescence resonance energy transfer (FRET) with a GloMax Multi+ Microplate Multimode reader (Promega, Madison, WI) in 96-well plates. The recommended CB substrate, Z-L-R-AMC (100  $\mu\text{M}$ ), was used with the desired enzyme concentration to measure fluorescence increase over time due to the lifting of quenching of the amino methyl coumarin (AMC) fluorophore upon proteolytic cleavage of it from the peptide substrate. The excitation and emission wavelengths were set to 365 nm and 460 nm, respectively. Activated CB (10  $\mu\text{L}$ ) was added to the wells, and fluorescence measurements were initiated immediately after adding 90  $\mu\text{L}$  of substrate solution. The effect of different proteolysis buffers was examined by preparing the substrate in various buffers, including 0.5X PB (2.3 mM  $\text{NaH}_2\text{PO}_4$  and 3.6 mM  $\text{Na}_2\text{HPO}_4$ , pH = 7.4), 25 mM MES (pH = 5.0), and 25 mM MES with 50  $\mu\text{M}$  EDTA (pH = 5.0). Additionally, the activity of activated CB was compared after activating in either 1 mM or 5 mM DTT to assess the impact of DTT on persistent enzyme activity.

## 2.2.4 Electrochemical experiments

Electrochemical experiments were conducted using a custom-designed electrochemical cell mounted on a large copper block for temperature regulation, as described in the previous report<sup>27</sup> and explained in **Section 1.8.2**. Thermostatic silicone liquid was circulated through internal channels to maintain a consistent temperature control at 36°C set at the thermal circulator (Julabo, Allentown, PA). Proteolysis experiments were done in 25 mM MES buffer and 50  $\mu$ M EDTA at pH 5.0. CB activation and DTT removal follow the above-mentioned procedure. After activation, 40 $\pm$ 5  $\mu$ L of the activated CB solution was added to the electrochemical cell containing 710 $\pm$ 5  $\mu$ L of buffer (25 mM MES buffer, 50  $\mu$ M EDTA at pH 5.0) as the electrolyte. The Fc signal on the gold surface was detected using ACV with an AC frequency of 300 Hz and a voltage amplitude of 50 mV, applied over an anodic DC ramp from - 0.40 to 0.15 V vs. MSE. These conditions were optimized empirically by performing ACV vs different frequencies and amplitudes in order to obtain a higher peak current/background ratio. An IVIUM n-Stat potentiostat (Eindhoven, The Netherlands) was used in these experiments, which allowed for simultaneous measurement of up to 10 independent working electrodes with a shared MSE reference electrode and a common platinum coil counter electrode. The MCMB signal on the gold surface was measured using ACV with an AC frequency of 100 Hz and a voltage amplitude of 75 mV, applied over a cathodic DC ramp from -0.45 to -0.8 V vs. MSE. These conditions were optimized using the same method as for the Fc system. These optimized frequencies are well aligned with reported electron-transfer rates for surface-bound ferrocene and methylene blue, with ferrocene exhibiting higher rates ( $k_0 \approx 8 \pm 2 \text{ cm s}^{-1}$ )<sup>82</sup> and methylene blue lower rates ( $k_0 \approx 1.5 \pm 0.5 \text{ cm s}^{-1}$ )<sup>82</sup>, such that Fc is best probed at higher AC frequencies and MB at correspondingly lower frequencies.

## 2.3 Results and Discussion

### 2.3.1 MEA design and principle of electrochemical detection of protease activity

**Fig. 2.1A** shows an optical image of a single MEA chip and an illustrative representation of a 3×3 Au MEA. These nine microelectrodes ( $200\ \mu\text{m} \times 200\ \mu\text{m}$  each) are separately connected to contact pads ( $1.0\ \text{mm} \times 1.0\ \text{mm}$  each) via electrical leads, which are buried underneath an insulating  $\text{SiO}_2$  film. Contact pads are connected to a multichannel IVIUM potentiostat through pogo pins to obtain the electrochemical measurements simultaneously.

The proteolysis sensing mechanism is illustrated in **Fig. 2.1B**. The cylindrical rods represent the long-chain thiol molecules packed in the SAM formed via Au-S interactions on the electrode surface. The ratio of -COOH to -OH terminal groups in the SAM is controlled by incubating Au electrodes in a mixture of 6-mercapto-1-hexanoic acid and 6-mercapto-1-hexanol at a 1:10 ratio in anhydrous ethanol. The specific substrate peptide (containing 6 amino acids and an  $\text{H}_2\text{N}-(\text{CH}_2)_4$ - linker) probes with a redox tag (such as Fc) at the distal end are covalently attached to the -COOH group at the SAM surface via an amide bond. Fc tags can be oxidized into ferrocenium ( $\text{Fc}^+$ ) by transferring electrons to the electrode, as depicted by the red arrows.

The ACV of Fc-tagged peptide on Au electrode generates a peak current ( $i_p$ ) at about -0.15 V vs. MSE on top of a slanted background, which can be subtracted. Each ACV measurement takes about 1 minute and is continuously repeated. As a result of proteolysis and Fc-moieties diffusing away from the electrode surface, the  $i_p$  amplitude in each ACV curve decreases over time, as illustrated in **Fig. 2.1B**. In previous studies <sup>27, 28</sup> we have shown that this  $i_p$  corresponds to the proteolysis kinetics predicted by the heterogeneous Michaelis-Menten enzyme kinetics model also described in **the Introduction section 1.8.3 up to Equation 1.5** :

To obtain the proteolysis kinetics data, the following single exponential function (Equation 2.1) has been employed in this chapter.

$$\frac{i_p}{i_{p^0}} = A \left[ \exp\left(-\frac{t}{\tau}\right) \right] + y_0 \quad (2.1)$$

Where,  $\frac{1}{\tau} = \left(\frac{k_{cat}}{K_M}\right) [E_0]$  which is described in Section 1.8.3.

The value of  $1/\tau$  is defined as the activity of the target protease on the specific peptide substrate and directly corresponds to the decay rate of  $i_p$  in experimental proteolysis curves as described in Section 1.8.3.

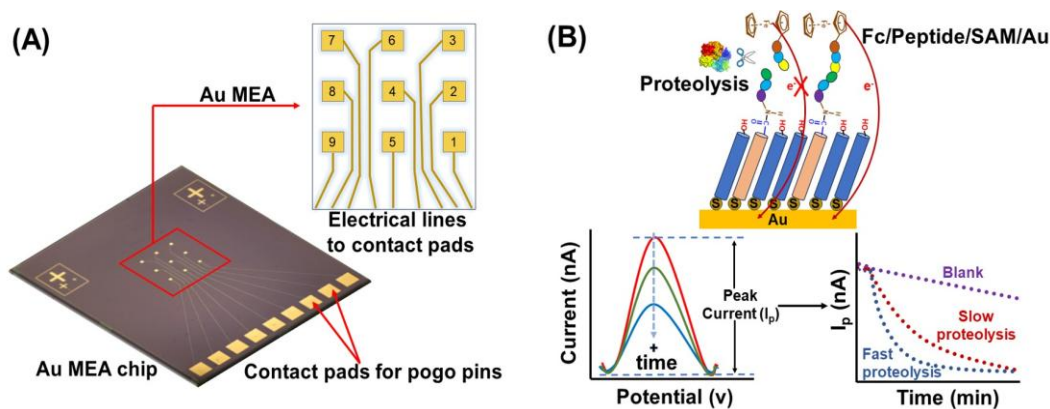


**Figure 2.1** (A) Optical image of an Au MEA chip with 9 contact pads (1.0 mm × 1.0 mm) at the bottom and schematic illustration of the 3 × 3 Au MEA with nine microelectrodes (200 μm × 200 μm). (B) Schematic illustration of the proteolysis sensing mechanism, including major components of the peptide/Au sensor, representative ACV curves during proteolysis, and the kinetic proteolysis curves under varied protease activities. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

### 2.3.2 Interference issue from the activation agent DTT

According to the literature, the optimal activation buffer for CB is 25 mM MES buffer (pH = 5.0) containing 1-5 mM DTT <sup>75, 83</sup> with an incubation period of 15 minutes. This buffer is also recommended by the vendor (R&D Systems Inc.) and was used in our previous studies <sup>27, 28</sup>. However, after the activation process, DTT was introduced into the electrochemical protease assay

together with the activated CB. We observed that DTT caused a rapid, nonspecific current decay in our electrochemical protease sensor during continuous ACV cycling, even though DTT was diluted by 50-fold. **Fig. A.1** in **Appendix A** shows the kinetic curve of normalized ACV peak current ( $i_p/i_{p0}$ ) over time for the peptide substrate H-15-AMF ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH}_2\text{-Fc}$ ) functionalized on a SAM-covered Au MEA during continuous ACV cycling, both in the presence and absence of 20  $\mu\text{M}$  DTT in 0.5X PB at 36°C. The dashed line indicates the point at which DTT is added to the electrochemical cell. In the absence of DTT, the  $i_p$  exhibited a considerable decrease with 52% drop from the initial peak current ( $i_{p0}$ ) after 60 ACV cycles. Upon the addition of DTT, a much larger drop up to 98% was observed. Ideally, the current should be at a constant value, representing the true blank for this assay. This unexpected rapid  $i_p$  drop obscured the true current decay attributed to proteolysis by the active CB. It is critical to understand the cause of this large  $i_p$  drop in the absence of CB to develop the mitigation solutions. A series of experiments was designed for this purpose.

### 2.3.3 Impact of DTT on the redox tag's chemical stability

In our previous studies <sup>27, 28, 84</sup> we utilized in-house synthesized AMF as the redox tag for hexapeptide substrates. Here, we used two additional redox tags, i.e., in-house synthesized AEF and commercial MCMB (from emp Biotech), for comparison with AMF. These redox tags were directly functionalized onto SAM-covered gold electrodes using **protocol-1** in **Appendix A, Section A.4.**, as depicted in the first two functionalization schemes in **Fig. 2.2A**. **Fig. 2.2B** compares the stability of AMF and AEF directly functionalized on a SAM-covered gold disk electrode (2 mm diameter) in 0.5X PB buffer (pH = 7.4) at 36°C, both in the presence and absence of 20  $\mu\text{M}$  DTT. Notably, the AEF tag demonstrated better stability (36%  $i_p$  drop) than AMF (53%  $i_p$  drop) in the absence of DTT, indicating that the longer  $-(\text{CH}_2)_2-$  moiety in AEF to separate Fc

from the amide bond helps to increase the stability of the thiol/Au based electrochemical sensor compared to AMF with a shorter linker  $-\text{CH}_2-$ . A five-membered carbon ring of the ferrocene can be more stable when strong electron-withdrawing groups are not directly attached and placed further away from the ring structure<sup>85</sup>. A detailed description of the AEF synthesis protocol is given in **Appendix A, Section A.3**. However, the addition of DTT led to rapid signal declines for both AEF and AMF tags.

To further validate the stability conferred by AEF, we tested the electrochemical stability of AEF-tagged peptide (H-15-AEF) and AMF-tagged peptide (H-15-AMF) in 0.5X PB buffer (pH = 7.4) at 36°C, following functionalization on SAM-covered Au MEAs. As shown in **Fig. 2.2C**, the H-15-AEF peptide substrate exhibited significantly better stability (25%  $i_p$  drop) compared to the H-15-AMF (53%  $i_p$  drop) peptide substrate. Nevertheless, upon the addition of 20  $\mu\text{M}$  DTT, the peak current signals from both H-15-AEF and H-15-AMF rapidly decreased, indicating that DTT interference persists regardless of the specific Fc-based redox tags.

These findings suggest that DTT interference occurs in systems with Fc redox moieties, regardless of whether the redox tag is directly attached to the SAM or incorporated into peptide substrates. To further investigate this interference, we examined the stability of MCMB, which was directly functionalized onto a SAM-covered Au disk electrode. As shown in **Fig. 2.2D**, MCMB demonstrated remarkable stability (with only  $\sim 5\%$   $i_p$  drop) both in the presence and absence of 20  $\mu\text{M}$  DTT, indicating no interference in this system.

CV characterization was carried out using Au disk electrodes of 2.0 mm diameter functionalized with AEF, MCMB, and H-15-AEF peptide substrate as shown in **Fig. A.2** in **Appendix A**. The potentials of the redox pairs are consistent with the peak potential in ACV, and the peak areas reveal that the surface densities ( $\Gamma_{ss}$ ) for AEF, MCMB, and H-15-AEF were 25.4

pmol/cm<sup>2</sup>, 10.0 pmol/cm<sup>2</sup>, and 5.58 pmol/cm<sup>2</sup>, respectively. This corresponds to average molecular spacings of 2.75 nm, 4.37 nm, and 5.86 nm, respectively. The exact mechanism of DTT interference remains unclear. It may be due to several possibilities: (1) the interactions between DTT and the Fc redox tags, (2) the interaction of DTT with the thiol monolayer, and (3) the higher (relatively positive) potential windows for ACV detection of Fc signal (0.45V to 0.15 V vs. MSE) relative to that for detection of MCMB (-0.85 V to -0.40 V vs. MSE). We have designed a series of specific experiments to tackle these possibilities.

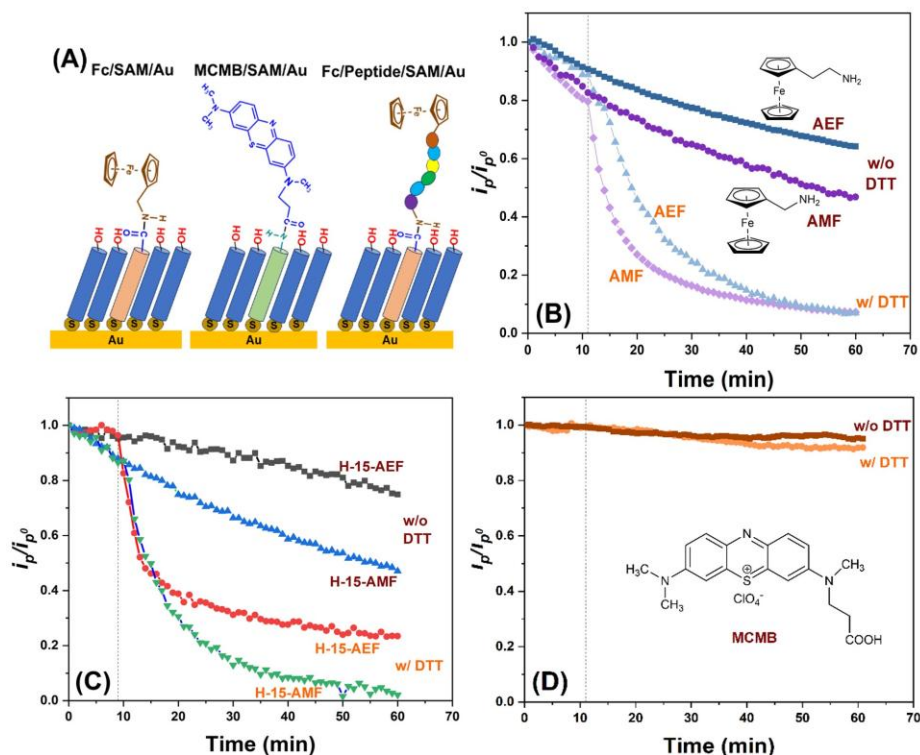


**Figure 2.2** (A) Schematic illustrations of the functionalization schemes of AMF (AEF), MCMB, and H-15-AEF peptide covalently attached to the Au electrodes via the thiol/Au-based SAM. The kinetic curve of the normalized peak current over time, during continuously repeated ACV cycling (B) for AEF and AMF, and (C) for AMF or AEF -tagged H-15 peptide. (D) The similar measurements with MCMB functionalized on SAM-covered Au disk electrodes. The measurements were done in the presence and absence of DTT (20  $\mu$ M) in 0.5X PB at 36  $^{\circ}$ C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

To check the first possibility, high-performance liquid chromatography (HPLC) was employed to assess potential disruptions to the peptide sequence or the AEF redox tag by DTT. Previous studies have reported that DTT can introduce nicks in the DNA backbone by breaking phosphodiester bonds in DNA-immobilized biosensors<sup>86</sup>. There was a concern that it could potentially lead to false signals in DNA sensors<sup>86</sup>. However, HPLC chromatograms in **Fig. A.3** in **Appendix A** show that incubating AEF with DTT for 1 hour at 36°C remained merely as a physically mixed solution. No chemical reactions were observed between AEF and DTT. HPLC chromatograms of H-15-AEF incubated with DTT (in **Fig. A.4**) showed similar results, confirming that both AEF and H-15-AEF were stable in the presence of DTT.

To investigate the second possibility, the SAM formation protocol was refined from **protocol-1** in water to a much more rigorous **protocol-2** in anhydrous ethanol, as detailed in the methods section. This ensures the formation of a more stable SAM on Au electrodes. As shown in **Fig. A.5A** in **Appendix A**, in the absence of DTT, AEF functionalized on the SAM/Au formed with **protocol-2** indeed demonstrates an improved stability (23%  $i_p$  drop) compared to that by **protocol-1** (36%  $i_p$  drop). However, DTT interference persisted, as indicated by the rapid peak current decay over time in **Fig. A.5B**. This excludes the second possibility.

### 2.3.4 Impact of redox potential window of different redox tags.

It is noteworthy that the potential window for ACV detection of the Fc signal (-0.45 V to 0.15 V vs. MSE) is substantially higher than that for detection of MCMB (-0.85 V to -0.40 V vs. MSE). We hypothesize that the high potential used for detecting the Fc signal may disrupt the SAM in the presence of DTT and cause the false signal decay. Although the stability of SAMs exposed to air, light, high temperatures, solvents, and reactive atmospheres has been extensively characterized<sup>87, 88</sup>, the effect of the potential window in combination with other strong adsorbates

on the Au surface (such as DTT) has not received adequate attention. Most electrochemical studies of SAMs focused on their reductive desorption from Au in basic electrolytes<sup>89, 90</sup>. Hydrophilic SAMs generally lower the electrochemical stability in both the oxidative and reductive directions, as they increase the ability for ions and water to reach the metal surface<sup>91</sup>. Variations in SAM hydrophilicity, orientation, adsorbate structures, coverage, and surface reconstruction can influence the electrochemical stability of thiols<sup>91</sup>. Applying a potential to gold electrodes can alter the work function, affecting its electronic properties and interactions with surrounding materials or molecules<sup>92</sup>. The stability of the Au-sulfur bond in SAMs can also be influenced by the electrochemical potential<sup>89</sup>. Small positive potentials applied to alkanethiols have been observed to enhance the surface packing of SAM<sup>93</sup>. Increasing the applied potential from -0.8 V to 0.4 V vs. saturated calomel electrode (SCE) first leads to the formation of SAMs with higher packing density, higher ordering, and fewer defects. However, going beyond 0.4 V vs. SCE, the packing density and the ordering of SAMs begin to deteriorate<sup>94</sup>. The reason that the redox tag MCMB shows a high stability in continuous ACV cycling in the presence of DTT (**Fig. 2.2D**) could be due to its low (relatively negative) redox potential window (-0.85 V to -0.4 V vs MSE), which is -0.45 to 0.00 V vs. SCE.

To confirm our hypothesis, MCMB was functionalized onto a SAM-covered Au disk electrode and subjected to ACV cycling in the higher potential region (-0.40 V to 0.15 V vs MSE) to simulate the condition for ferrocene detection, in a solution of 20  $\mu$ M DTT in 0.5X PB at 36°C, as shown in **Fig. 2.3A**. Before cycling in the high potential region, the initial ACV peak current was measured in the low potential window for normal MCMB detection (-0.85 V to -0.40 V vs MSE) as shown in **Fig. 2.3B**. Similar measurements were done periodically after every 11 continuous ACV cycles in the high potential region to monitor the change of the MCMB signal.

**Fig. A.6A** in **Appendix A** schematically illustrates how these experiments were done. The blank experiment was done following the same procedure but without DTT, as shown in **Fig. 2.3C** and **Fig. 2.3D**. **Fig. 2.3E** summarizes the change of the normalized peak current of Au/SAM/MCMB in these experiments. Notably, in the presence of 20  $\mu$ M DTT during continuous ACV cycling at normal lower potentials (-0.40 V to -0.85 V vs MSE, blue curve), the signal is highly stable with negligible decay (with only 4%  $i_p$  drop) over 23 cycles. In the absence of DTT, even though the main ACV cycles were carried out at the higher potentials (-0.40 V to 0.15 V vs MSE), the periodically checked signal remained stable. However, in the presence of DTT, the periodically checked ACV signal of MCMB showed a rapid decay. Clearly, it is the combination of ACV measurements at high potential and the presence of DTT that caused the rapid signal decay. It is likely that thiol molecules on gold could become more mobile and start to rearrange at higher potentials<sup>93</sup>. During this process, some of the redox-tagged thiol molecules could be replaced by DTT. Recent work has shown that the application of either a positive or negative potential over a certain threshold tends to lower the packing density of thiol molecules with a terminating Fc group

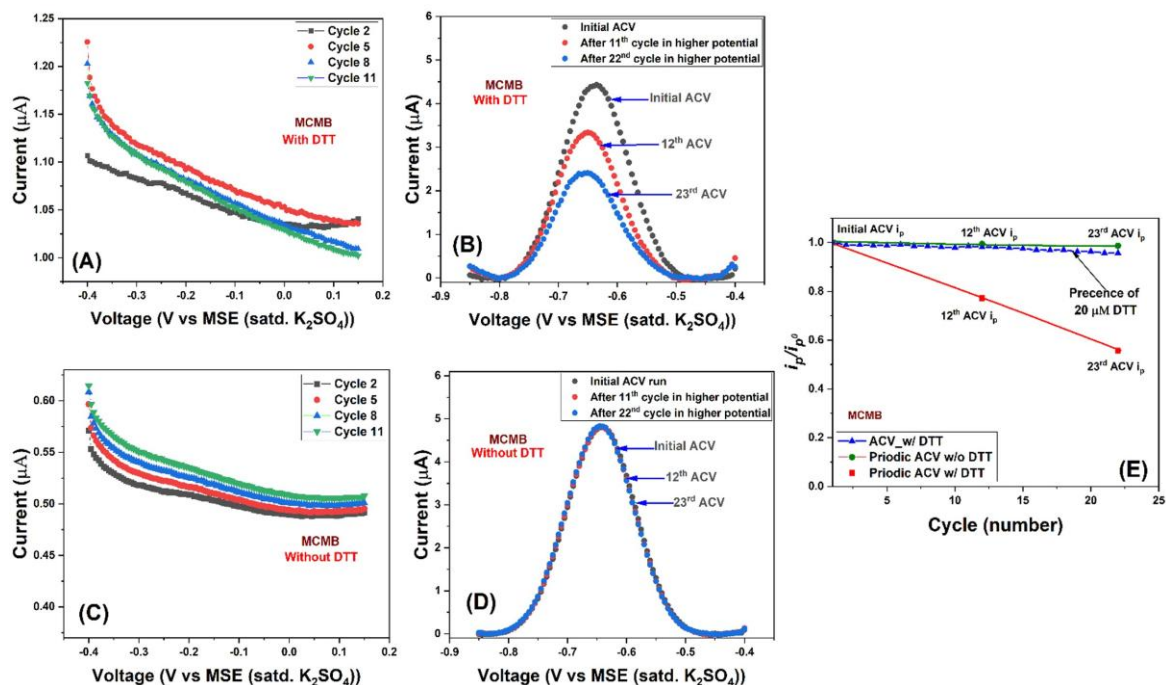


**Figure 2.3.** (A) Selected ACV curves showing no MCMB peak during continuous ACV cycling at the high Fc redox potential region (-0.40 to 0.15 V vs MSE) for MCMB/SAM/Au with DTT. (B) Periodic ACV probing of MCMB/SAM/Au at the low potential (-0.85 to -0.40 V vs MSE) during continuous ACV cycling simulating the conditions of AMF and AEF measurements at higher potentials (-0.40 to 0.15 V vs MSE) in the presence of 20  $\mu\text{M}$  DTT. (C) Similar experiment as panel (A) but without DTT. (D) in the high stability of the ACV peak current in similar experiments as panel (B) in the absence of DTT. (E) Comparison of the normalized peak current of MCMB/SAM/Au derived from panel B (with DTT, red curve) and panel D (without DTT, green curve). The latter shows a high stability similar to the control experiment of continuous ACV all at the low potentials (-0.40 to 0.15 V vs MSE) in the presence of 20  $\mu\text{M}$  DTT (blue curve). All measurements were done in 0.5X PB at 36  $^{\circ}\text{C}$  on Au disk electrodes. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

So far, Fc has been blamed for the low stability of electrochemical sensors. From the above experiments, it is clear that the problem is the relatively high potential needed for measuring the Fc signal rather than the chemical property of Fc itself. To illustrate this, AEF was functionalized onto a SAM/Au disk electrode and subjected to ACV cycling in the lower potential region (-0.35 V to -0.85 V vs MSE, simulating the redox potential window for MCMB) in a solution of 20  $\mu\text{M}$  DTT in 0.5X PB at 36 $^{\circ}\text{C}$ , as shown in **Fig. A.6B** and **Fig. A.7B** in **Appendix A**. Before cycling in

the negative potential region, the initial ACV peak current was measured at the high potential window (-0.45 V to 0.15 V vs MSE) to collect the Fc signal, as shown in **Fig. A.7A** in **Appendix A**. After 11 ACV cycles in the low potential region, the ACV peak current of AEF was remeasured at the high potential window to assess its change. The blank experiment without DTT, shown in **Fig. A.7C** and **Fig. A.7D**, indeed shows high stability comparable to MCMB. However, in the presence of DTT, even periodically checking the Fc signal with ACV at high potential causes a clear decrease in the peak current. Continuous ACV at the high potential window generates a large signal decay as discussed before.

### **2.3.5 Optimization of cathepsin B activity detection protocols with fluorogenic assays**

From the previous section, the interference of DTT could be mitigated by three approaches toward developing reliable electrochemical CB sensors, *i.e.*, using alternative activation agents (tris(2-carboxyethyl)phosphine (TCEP) or cysteine) to activate CB <sup>95</sup>, using MCMB tags, or removing DTT after CB activation. However, TCEP and Cysteine showed the same interference issue upon addition to the sensor, resulting in false signal decay in higher potentials (-0.40 to 0.15 V vs MSE) as shown in **Fig. A.8** in **Appendix A**. It was found that TCEP could displace thiolated DNA from Au nanoparticles, which may interfere with the stability of DNA-functionalized nanostructures. This potential issue suggests caution when considering TCEP as an alternative reducing agent in such systems <sup>96</sup>. In the second approach, it requires extensive efforts in re-synthesizing the peptide substrates by reversing the C- and N- terminus. In addition, MCMB is highly expensive. Therefore, we took the third approach in this study using the pre-synthesized and well-characterized AEF-tagged peptide substrates. To avoid the complication by electrode potentials, fluorogenic assays were used first to validate the protocol before applying it to electrochemical measurements.

### 2.3.5.1 Effect of the buffer composition and pH value on cathepsin B activity

FRET assays were conducted with the vendor-recommended FRET assay for CB activity detection using a 25 mM MES buffer at pH 5.0 with the Z-L-R-AMC substrate. Typically, 3.45 nM recombinant human CB (consisting of 60% inactive form) was activated in 25 mM MES (pH 5.0) with 5 mM DTT by incubating for 15 minutes at room temperature. However, in the activity (represented by the rate of fluorescence intensity increase) in 0.5X PB (pH = 7.4), the same buffer used in earlier electrochemical measurements, is much lower than that in the recommended 25 mM MES buffer (pH = 5.0), as shown by the blue and green curves in **Fig. 2.4A**. However, 10 nM AMC in these two buffers shows the same fluorescence intensity (**Fig. A.9 in Appendix A**). Thus, it is likely that the pH value, rather than buffer chemicals, affects the CB activity. Recent studies have indeed shown a strong pH dependence of CB activity in proteolyzing various peptide substrates <sup>97</sup>.

Additionally, the activity of non-activated CB was measured in both buffers, as seen in **Fig. 2.4A** (purple and gold curves). The activity was slightly higher in the 25 mM MES (pH 5.0) buffer compared to 0.5X PB (pH 7.4). Overall, CB activity was greater in the 25 mM MES buffer at pH 5.0. Therefore, the 25 mM MES (pH 5.0) buffer was used in further studies in this work.

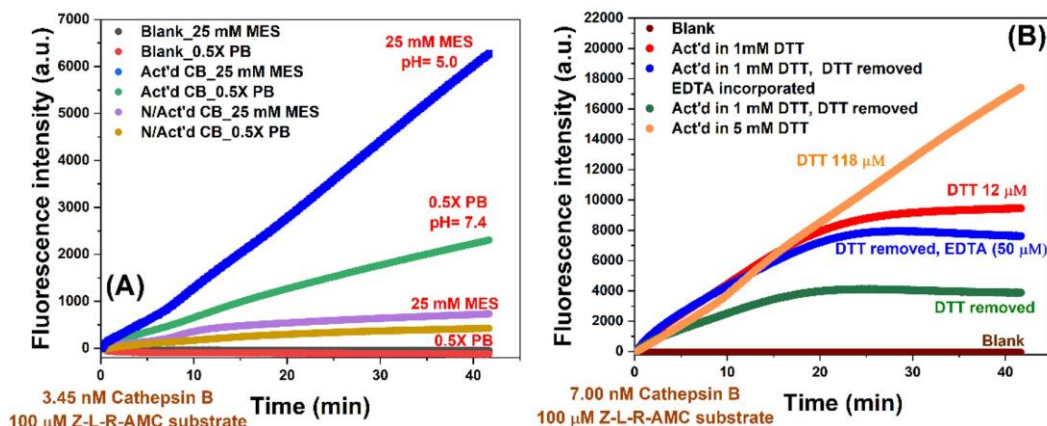


**Figure 2.4** (A) Validation of CB activity with FRET assay (100  $\mu$ M Z-L-R-AMC substrate at RT) using 3.45 nM of activated and non-activated CB in two buffers (25 mM MES at pH = 5.0 and 0.5X PB at pH = 7.4, respectively). (B) Comparison of kinetic proteolysis curves by 7.0 nM of CB using FRET assay (100  $\mu$ M Z-L-R-AMC substrate at RT) to investigate the effect of DTT and EDTA on the activity of CB. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

### 2.3.5.2 Effect of the DTT and EDTA on the activity of cathepsin B

Studies have shown that recombinant Pro CB can be activated in the presence of reagents such as DTT, cysteine, and EDTA<sup>95</sup>. However, the specific effects of DTT and EDTA on the long-term stability of CB activity have not been thoroughly investigated. To explore the impact of DTT concentration on CB activity, a fluorescence assay was performed using 7.00 nM CB activated in either 1 mM or 5 mM DTT in 25 mM MES buffer (pH 5.0). CB activated with 5 mM DTT introduced 118  $\mu$ M DTT into the final FRET assay solution, which helped to maintain consistent proteolytic activity over the entire 40-minute period, as shown in **Fig. 2.4B** (orange curve). In contrast, CB activated with 1 mM DTT only introduced 12  $\mu$ M DTT into the final FRET assay solution, which only exhibited consistent activity for the first 20 min, after which the activity began to decline (**Fig. 2.4B**, red curve).

To further investigate the effects of DTT, CB was activated in 1 mM DTT, and the DTT was removed using an Amicon Ultra 10k filter-based protocol (as described in **Section A.14** of

**Appendix A).** Assuming more than 98% recovery of the enzyme, 7.00 nM of activated DTT-free CB was used in the FRET assay (**Fig. 2.4B**, green curve). The enzyme showed a significantly reduced activity in the first 20 min, with a rapid decline in proteolytic function after that.

Next, CB was activated in 1 mM DTT, and the DTT was removed while 50  $\mu$ M EDTA was incorporated in the dilution buffer (25 mM MES, pH 5.0). Again, assuming more than 98% enzyme recovery, 7.00 nM of activated DTT-free CB with EDTA was used in the FRET assay (**Fig. 2.4B**, blue curve). Surprisingly, this condition resulted in nearly the same level of activity as the assay without removing DTT after activating CB in 1 mM DTT (red curve). This confirmed that CB can remain in an active state after DTT removal when EDTA is present.

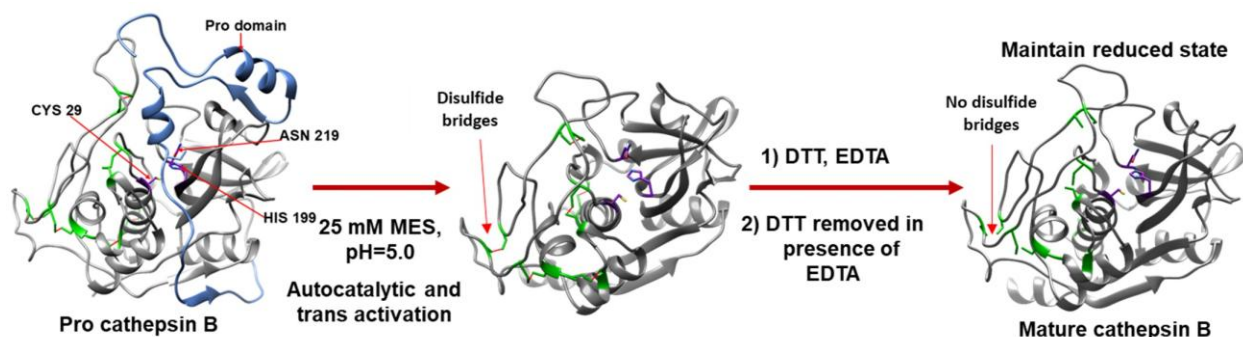


**Figure 2.5** Illustrative model for in vitro activation and processing of human pro-cathepsin B. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

CB activation occurs through both autocatalytic and trans-activation processes, converting the pro-enzyme into its mature form. This activation involves cleavage and dissociation of the 62-residue pro-region, as illustrated in **Fig. 2.5**<sup>98</sup>. At lower pH, protonation of carboxylate groups can disrupt salt bridges, potentially destabilizing the hydrophobic core of the pro-domain<sup>99</sup>. This leads to the dissociation of the pro-domain from the active site, triggering autocatalytic processing that cleaves the pro-domain and exposes the catalytic triad (CYS 29, HIS 199, and ASN 219) in the

enzyme<sup>100</sup>. This autocatalytic activation is particularly efficient in acidic environments<sup>99, 101</sup>, such as in a 25 mM MES buffer at pH 5.0.

The subsequent step involves bimolecular trans-activation, where the active CB molecule processes and activates additional pro-CB molecules in one or more steps<sup>99, 102</sup>. This results in a chain reaction, rapidly converting the remaining pro-CB into its mature form. Commercially obtained CB samples are dominated by the pro-form (~60%), with a small fraction of the mature enzyme capable of initiating this conversion. During activation, the 62-residue (7.5 kDa) pro-domain is cleaved from the N-terminus of pro-CB, generating a mature form consisting of 260 residues (29 kDa), as shown in **Fig. 2.5**<sup>99</sup>.

As mentioned earlier, DTT plays a key role in reducing the disulfide bridges in CB, increasing enzyme flexibility, and making the active site more accessible to substrates. Human CB contains six disulfide bridges, primarily located in the N-terminal half of the molecule, as shown in **Fig. 2.5**<sup>100</sup>. EDTA can further stabilize the reduced state of CB in the absence of DTT by chelating divalent cations in the medium, as these cations may promote disulfide bridge reformation. This observation was confirmed by FRET assays shown in **Fig. 2.4B**.

### 2.3.6 Substrate-dependent proteolysis by cathepsin B: Multiplex analysis on a single MEA chip

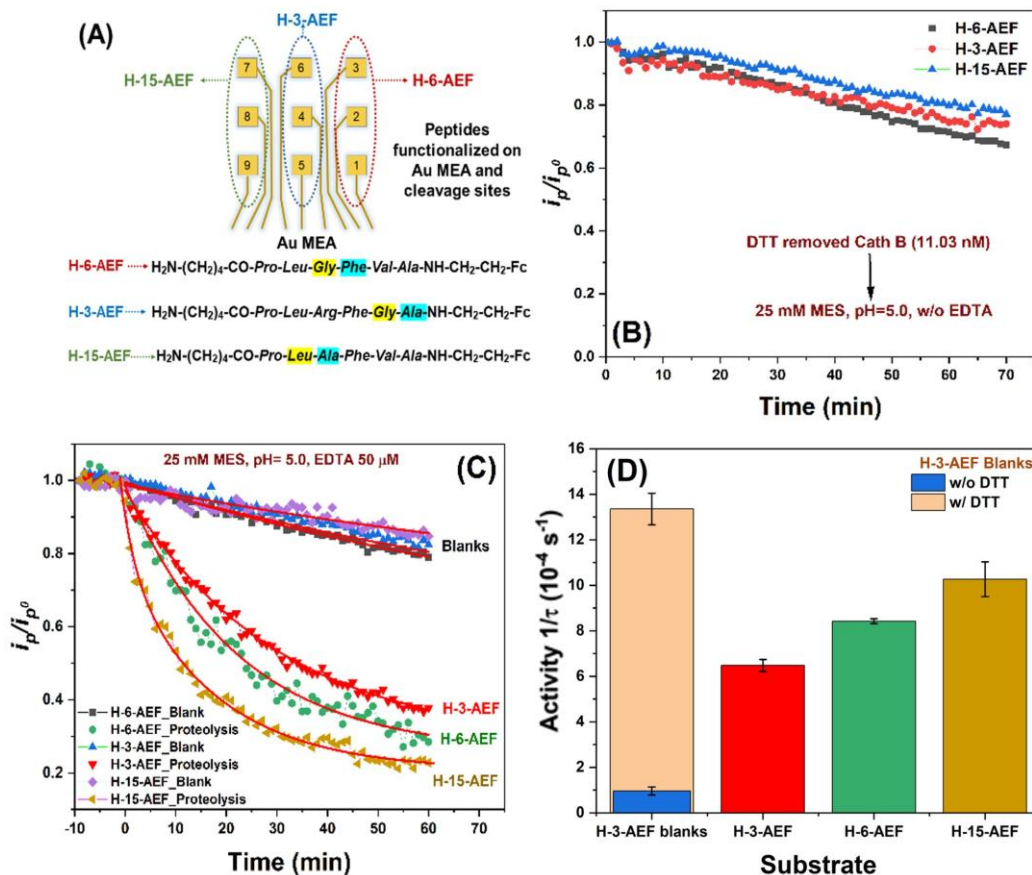


**Figure 2.6** (A) Schematic representation of how peptides were fictionalized on a single microelectrode array chip utilizing three microelectrodes for each peptide. (B) Kinetic curves of normalized peak current over time with DTT-removed (at 20 °C) CB on H-6-AEF, H-3-AEF, and H-15-AEF functionalized on SAM-covered Au MEA. EDTA was not incorporated during the DTT removal process and in the proteolysis buffer. (C) Representative normalized kinetic proteolysis curves for an Au MEA modified with three different peptide substrates, H-6-AEF, H-3-AEF, and H-15-AEF, which were proteolyzed by 11.03 nM activated CB (with DTT removed after activation) in 25 mM MES, pH = 5.0, with 50  $\mu\text{M}$  EDTA. The protease sample was added after the 10<sup>th</sup> ACV cycle to obtain proteolysis curves. Blanks were run without CB enzyme. The proteolysis curves are normalized to the peak current in the first ACV cycle after CB addition and fitted with Equation (2). (D) A bar graph comparing the  $1/\tau$  values for the faulty blank (without DTT removal) and the corrected blank (after employing the DTT removal protocol) for H-3-AEF. Additionally, the graph includes fitted and blank-subtracted  $1/\tau$  values for three peptide substrates, confirming substrate-dependent proteolysis after resolving the interference issue. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

Theoretically, a single highly specific peptide sequence is sufficient for detecting a single protease. However, designing a highly specific substrate is challenging due to the possibility of cross-reactivity with other proteases. By using multiple peptide substrates that are reasonably specific for a particular protease, an activity profile can be developed, providing more detailed information during the analysis of complex samples, such as human serum.

**Fig. 2.6** presents data obtained using multiple peptide substrates simultaneously on a single Au MEA chip. For these experiments, electrodes 1, 2, and 3 were functionalized with the H-6-AEF peptide, electrodes 4, 5, and 6 were functionalized with the H-3-AEF peptide, and electrodes 7, 8, and 9 were functionalized with the H-15-AEF peptide, as illustrated in **Fig. 2.6A**, which also indicates the cleavage sites, between the yellow and cyan highlighted amino acid moieties, identified in previous studies. Representative CV curves for the functionalized peptide substrates are shown in **Fig. A.11** in **Appendix A**. The average surface density ( $\Gamma_{ss}$ ) of peptides functionalized on Au MEA was found to be  $30.8 \pm 1.2$  pmol/cm<sup>2</sup>, corresponding to an average molecular spacing of  $2.49 \pm 0.05$  nm. This result was achieved using an improved functionalization protocol (**protocol-2**), where the resulting surface concentration was significantly lower than the maximum expected coverage for close-packed Fc-terminated SAM (763.8 pmol/cm<sup>2</sup>)<sup>103</sup>. **Fig. A.12** in **Appendix A** shows representative ACV curves for H-6-AEF functionalized on SAM-covered Au MEA, before and after the proteolysis, including the raw ACV curves and processed background-subtracted ACV curves in 25 mM MES, 50  $\mu$ M EDTA, pH = 5.0. **Fig. A.13** in **Appendix A** shows the comparison between blank runs for Au/SAM/H-3-AEF before and after incorporating the DTT removal protocol in the procedure. The DTT-removed blank showed higher stability with 20% of  $i_p$  drop, and the blank without DTT removal showed an 80% of  $i_p$  drop.

As shown in **Fig. 2.6B**, the CB enzyme could not be kept in its activated form without incorporating EDTA in the dilution buffer during DTT removal and in the proteolysis buffer in the electrochemical cell. Without EDTA, the DTT-removed CB resulted in no significant proteolytic decay. EDTA is critical in preserving CB activity after DTT is removed. **Fig. 2.6C** displays the normalized kinetic proteolysis curves after the addition of 11.03 nM DTT-removed CB to three peptide substrates in the presence of EDTA. The DTT-removed CB sample was added after the 10<sup>th</sup> ACV cycle and immediately produced the well-defined exponential decay curves, which can be fitted with Equation (2) to derive  $1/\tau$  values (i.e., proteolytic activity). The average values obtained were  $(6.48 \pm 0.26) \times 10^{-4} \text{ s}^{-1}$ ,  $(8.42 \pm 0.11) \times 10^{-4} \text{ s}^{-1}$ , and  $(10.27 \pm 0.77) \times 10^{-4} \text{ s}^{-1}$  for the H-3-AEF, H-6-AEF, and H-15-AEF peptides, respectively after subtracting the blank as shown in **Fig. 2.6D**. The  $1/\tau$  values for the faulty blank (without DTT removal) and the corrected blank (after employing the DTT removal protocol) for H-3-AEF are  $(13.35 \pm 0.69) \times 10^{-4} \text{ s}^{-1}$  and  $(0.96 \pm 0.18) \times 10^{-4} \text{ s}^{-1}$ , respectively, as shown in **Fig. 2.6D**. The variation of CB activity among different peptides shows the intrinsic substrate-dependent protease properties. This lays the foundation to use the Au MEA for rapid screening of more specific peptide substrates for detecting CB activity. It can be broadly applied for screening peptide substrates for other proteases as well.

## 2.4 Conclusion

In this study, we successfully demonstrated substrate-dependent proteolysis of CB using a 3×3 Au MEA, while overcoming previous interference issues caused by the activation agent DTT. By optimizing enzyme activation and the removal of DTT through the incorporation of EDTA, we were able to maintain CB in its active form and accurately assess its proteolytic activity across multiple peptide substrates. The critical roles of DTT and EDTA in cathepsin B activation were confirmed through both FRET assays and electrochemical experiments.

Our findings emphasize the importance of considering potential interference from activation agents like DTT when using Au-S-based electrochemical biosensors, particularly at higher electrical potential. It was observed that Fc-tagged peptide substrates immobilized on gold electrodes through a thiol monolayer are prone to interference from reagents such as DTT, which affects the Au-S interaction during the Fc redox reaction. This interference occurred regardless of the redox tag used but was only significant at higher potential ranges. At lower potential regions, no such interference was observed with MCMB, indicating that careful control of the operating potential can minimize the impact of activation agents like DTT.

Our results in this chapter lay the foundation for further exploration of complex matrices such as human serum to further evaluate the assay's robustness and specificity. The broader implications of this work extend far beyond the detection of CB by paving the way for developing biosensors for a variety of proteases such as MMP-9, MMP-7, and ADAM-17. These proteases could easily be adapted by customizing peptide substrates on the MEA capable of delivering precise enzyme profiling for early diagnosis and monitoring of diseases implicating proteases in their critical steps, like cancer, cardiovascular conditions, and neurodegenerative disorders. However, a potential challenge lies in designing highly specific substrates for individual proteases.

Conclusively, this work reveals one of the major technical barriers that exist with Au-S-based electrochemical biosensors and shows a path toward developing versatile, interference-resistant platforms for the detection of proteases, highlighting the need to account for activation agent interference at higher potentials for a wide array of applications with Au-S-based platforms.

# **Chapter 3 - Peptide Substrate Optimization for Sensitive Multiplex Electrochemical Detection of Cathepsin B under Neutral pH Conditions Using Au Microelectrode Arrays**

## **3.1 Introduction**

Proteases regulate a broad array of biological processes, from protein turnover to extracellular matrix remodeling, and their dysregulation is implicated in inflammation, neurodegeneration, cardiovascular disease, and cancer <sup>104-106</sup>. Proteases break peptide bonds at defined sequence positions within proteins or peptides <sup>107</sup>. Among the proteases, the cysteine cathepsin family (specifically cathepsin B (CB)) <sup>38, 108</sup>, matrix metalloproteinases (MMPs), and A Disintegrin And Metalloprotease (ADAM) family are recognized as key biomarkers <sup>40, 109</sup> in cancer and inflammatory diseases, which have obtained significant attention due to their overexpression and aberrant activities in tumorigenesis and metastatic progression <sup>43, 110</sup>. Developing novel methods for direct detection of extracellular proteases plays a vital role in improving disease diagnostics.

A variety of affinity-based or activity-based methods are available for quantifying proteases or evaluating their activities, respectively. Conventional enzyme-linked immunosorbent assays (ELISA) measure the proteases' abundance <sup>71, 111</sup> but fail to capture their functional activities. Fluorogenic techniques based on fluorescence-tagged peptide substrates <sup>112</sup> and activity-based probes (ABPs) <sup>113, 114</sup> are homogeneous assays that can provide activity readouts yet typically lack the multiplexing capability. In contrast, heterogeneous assays enable the simultaneous detection of multiple proteases through established microarray technologies <sup>52, 72, 73</sup>. Among these platforms, activity-based electrochemical biosensors <sup>74</sup> are recognized for their high

sensitivity, rapid response, and cost-effectiveness <sup>52</sup>. Our previous work demonstrated that nanoelectrode arrays (NEAs) constructed from vertically aligned carbon nanofibers (VACNFs) and functionalized with ferrocene-labeled peptides can effectively measure protease activity of CB <sup>25</sup>. However, the difficulties in fabrication posed challenges for its broader applications. To overcome this issue, a wafer-scale  $3 \times 3$  gold (Au) microelectrode array (MEA) was recently developed, evaluated, and validated for detecting CB activity using different peptide substrates <sup>27</sup>, <sup>28</sup>. This platform offers potential for rapid, multiplexed protease detection in early disease diagnosis, therapeutic monitoring, and inhibitor screening. In this system, short peptides consisting of 4 to 8 amino acids and a ferrocene (Fc) redox label, *i.e.*, (aminomethyl)ferrocene (AMF), at the distal terminus were immobilized on the Au MEA through a thiol-based self-assembled monolayer (SAM). The exponential decrease in peak current during continuous alternating current voltammetry (ACV) cycles was attributed to enzymatic cleavage of the immobilized peptide substrates <sup>27, 28</sup>. Though the thiol/Au linkage is susceptible to disruption by the enzyme activation reagent dithiothreitol (DTT), which can cause peptide desorption and lead to false signal decay during ACV cycling, it can be circumvented by removing DTT after CB activation using a centrifugal filtration procedure with the addition of EDTA to preserve the enzyme activity <sup>31</sup>. This enabled accurate measurement of CB proteolysis and revealed substrate-dependent cleavage for three peptide sequences on the MEA chip <sup>31</sup>, suggesting the specific peptide-CB interaction and the potential to optimize the peptide substrates for rapid, multiplex protease activity profiling.

To achieve clinically relevant multiplexed detection of proteases such as CB, MMP-9, and ADAM-17 in human serum samples, it is desired to carry out measurements in a unified, physiologically neutral pH environment. It is essential to develop peptide substrates that provide high specificity and activity in physiologically compatible conditions at neutral pH. However,

current activity-based assays cannot meet this requirement for multiplexing detection because each protease-substrate pair requires a different pH condition for optimal activity<sup>115-117</sup>. CB is optimally assayed in acidic MES buffer (25 mM MES, pH 5.0)<sup>28</sup>. In contrast, standard fluorogenic assays employed a Tris-based buffer (pH = 7.5) for MMP-9<sup>118</sup> and an alkaline buffer (pH = 9.0) for ADAM-17<sup>118</sup>, while an electrochemical assay employed a tricine buffer (pH = 7.4) for MMP-7<sup>16</sup>.

CB is a lysosomal cysteine protease that is optimally active at low pH (~4.6), yet it is also present in extra-lysosomal environments where it can function at neutral pH (~7.2) due to localized pH variations within cells<sup>119</sup>. Classic substrates like Abz-GIVRAK(Dnp)-OH show dominant dipeptidyl-carboxypeptidase (DPCP) activity, which comes under exopeptidase activity at acidic or low pH (requiring a free N-terminal amino group, C-terminal carboxyl group, or both<sup>120</sup>). In contrast, 7-Amino-4-methylcoumarin (AMC) labelled Z-Arg-Arg-AMC substrate exhibits a higher endopeptidase cleavage at neutral pH<sup>121</sup>. The peptidyl-AMC substrates like Z-Arg-Arg-AMC have blocked C- and N- termini and are the simplest peptide substrates for measuring the endopeptidase-type cleavage<sup>121</sup>. A recent multiplex substrate profiling by mass spectrometry (MSP-MS) revealed that the pH-dependent cleavage preferences of CB can be modulated by the substrate sequence rather than a simple transition between catalytic modes<sup>75</sup>. But these findings should be further evaluated with advanced substrate-enzyme binding structural analysis in different pH conditions. Peptide digestion studies indicate that cathepsin S exhibits reduced proteolytic specificity at extracellular pH, consistent with crystal structures showing a lysine residue entering the S3 pocket above pH 7.0 due to pH-dependent changes in protein surface charge<sup>122</sup>.

The rational design and optimization of peptide sequences is crucial for developing sensitive electrochemical sensors. Researchers have recently applied a variety of strategies, such as fluorescence resonance energy transfer (FRET) based assays <sup>75</sup>, MSP-MS <sup>123</sup>, phage-display biopanning <sup>124, 125</sup>, and computational prediction tools to enhance peptide specificity and activity <sup>126, 127</sup>. For example, MSP-MS has revealed unique P1-P3 substrate site preferences of CB at both acidic and neutral pH, which enabled the development of a highly specific fluorogenic tripeptide substrate (Z-Nle-Lys-Arg-AMC) with high activity in a broad pH range <sup>75</sup>.

In this study, three different peptide substrates immobilized independently on a single Au MEA platform were first used to examine the pH-dependent activity of CB. As expected, CB activity decreased when shifting from acidic to neutral pH, resulting in reduced detection sensitivity. The limit of detection (LOD) of CB activity with these three peptides was quantitatively derived at pH = 5.0 and 7.3. This validated our Au MEA protease sensor platform and showcased the strong pH-dependence of the CB activity. To further improve the CB activity at physiological pH, a series of fluorogenic Z-peptide–AMC substrates was evaluated across different pH conditions, revealing how modifying the specific amino acid at positions from P1 to P4 enhances CB activity near neutral pH.

Building on these findings, further optimization of the hexapeptide substrates for electrochemical detection of CB activity was carried out employing a minimal-change engineering strategy and subsequently tested on Au MEA chips under neutral pH conditions. A norleucine (Nle) was incorporated at the P3 position, which markedly improved the CB detection sensitivity at neutral pH. A low LOD of CB at  $0.43 \pm 0.08$  nM was obtained, sufficient for direct detection of

CB in serum samples. These results demonstrated the potential of the MEA platform for sensitive, multiplexed electrochemical detection of the activity of extracellular protease biomarkers in a single compartment at neutral pH.

### **3.2 Materials and Methods**

A detailed description of the materials and instruments used in this chapter is given in **Section B.1 in Appendix B**. The peptide substrates used in this chapter and the synthesis of peptide substrates for CB detection are described in **Sections B.2 and B.3 of Appendix B**. The methodology for structural modeling and molecular docking is described in **Section B.4 of Appendix B**. The method used to do the Au MEA functionalization is described in **Section 2.2.1 under Protocol-2**. Activation of the CB was done using the same methodology described in **Section 2.2.2 in Chapter 2**. Electrochemical proteolysis experiments were done in 25 mM MES buffer and 50  $\mu$ M EDTA at pH 5.0, 6.3, or 7.3 using the same methodology described in **Section 2.2.4**.

#### **Cathepsin B activity with fluorogenic assays in different pH conditions**

CB enzyme activity was measured using fluorogenic assays with a GloMax Multi+ Microplate Multimode reader (Promega, Madison, WI) in 96-well plates. The custom-synthesized and purchased CB substrates, Z-Lys-Arg-AMC, Z-Leu-Arg-AMC, Z-Nle-Arg-AMC, Z-Leu-Lys-Arg-AMC, Z-Nle-Lys-Arg-AMC (100  $\mu$ M each), were used to measure the activity of CB (3.45 nM) by monitoring the fluorescence increase over time (40 min) due to the lift of quenching of 7-amino-4-methylcoumarin (AMC) fluorophore upon proteolytic cleavage of it from the aforementioned CB substrates. The excitation and emission wavelengths were set to 365 nm and 460 nm, respectively. Activated CB (10  $\mu$ L) was added to the wells, and fluorescence measurements were initiated immediately after adding 90  $\mu$ L of substrate solution. The effects of

pH were examined by preparing the substrates in 25 mM MES buffer, tuning the pH (using 0.5 M NaOH) to pH = 5.0, 6.3, and 7.3, respectively. CB activities with different substrates in different pH conditions were compared by fluorescence intensity at a fixed time of 40 min ( $F_t = 40 \text{ min}$ ).

### 3.3 Results and Discussion

#### 3.3.1 Functionalization of Au Microelectrode Array with Multiple Peptides

**Fig. 3.1** shows an optical image of a single MEA chip and an illustrative zoomed-in representation of the 3×3 Au MEA. For the experiments, electrodes 1, 2, and 3 were functionalized with the H-6-Fc peptide ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Gly-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ ), electrodes 4, 5, and 6 were functionalized with the H-3-Fc peptide ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Arg-Phe-Gly-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ ), and electrodes 7, 8, and 9 were functionalized with the H-15-Fc peptide ( $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ ), as illustrated in **Fig. 3.1**. The cleavage sites are between the pink and cyan highlighted amino acid residues, identified in our previous study <sup>27</sup>.

As previously described in **Chapter 2**, upon applying ACV, the Fc tag undergoes reversible oxidation to  $\text{Fc}^+$ , transferring electrons to the electrode, as shown by the red arrows shown in **Fig. 2.1B**. Obtaining multiplex ACV signals from IVIUM-soft v2.561 as shown in **Fig. B.3** in **Appendix B**.

In this chapter, kinetics predicted by a heterogeneous Michaelis-Menten model described in the **Introduction section 1.8.3**.

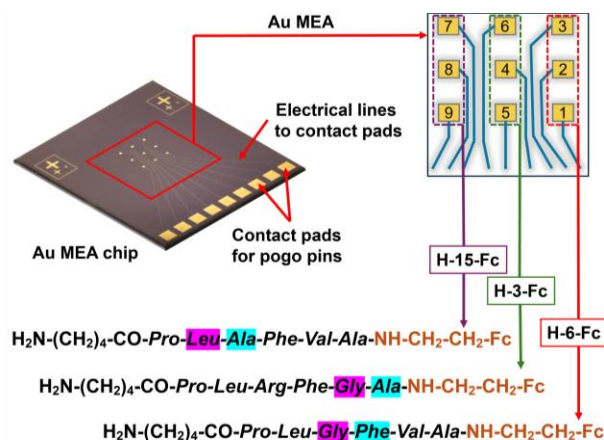


**Figure 3.1** An optical image of an Au MEA chip with 9 contact pads (1.0 mm × 1.0 mm) at the bottom and a schematic illustration of the 3 x 3 Au MEA with nine microelectrodes (200 μm × 200 μm), which were fictionalized with three types of hexapeptide substrates with three microelectrodes for each peptide.

### 3.3.2 pH-dependent proteolysis of CB

CB is a lysosomal cysteine protease whose catalytic performance is highly pH-dependent, exhibiting maximal activity at acidic pH of 4.5 – 5.5 and decreasing substantially as the matrix approaches neutrality<sup>128-130</sup>. This behavior arises from altered protonation of two catalytic amino acid residues (Cys29–His199) in its catalytic triad and conformational rearrangements in the occluding loop that affect substrate binding at a higher pH<sup>128</sup>. Nevertheless, CB is increasingly recognized as an extracellular protease that functions in near-neutral tumor microenvironments, inflamed tissues, and invasive cancer niches<sup>131-133</sup>.

To evaluate CB activity on the Au MEA platform under different pH conditions, three initially designed Fc-tagged hexapeptide substrates (H-6-Fc, H-3-Fc, H-15-Fc) were immobilized on Au MEA chips (**Fig. 3.1**), with each substrate assigned to three electrodes on the nine-electrode array. The activated CB sample was added after the 10<sup>th</sup> ACV cycle. The final CB concentration in the well was 11.03 nM. Continuous ACV measurements revealed clear pH-dependent proteolysis with well-defined exponential decay curves following **Equation 1.6** in **Section 1.8.3**,

with the  $1/\tau$  value representing the proteolytic activity. All substrates showed fast decay in the peak-current ( $i_p$ ) at pH 5.0, moderate decay at pH 6.3, and slow decay at pH 7.3, as shown in **Fig. 3.2A, 3.2B, and 3.2C** for H-6-Fc, H-3-Fc, and H-15-Fc, respectively. This decrease in proteolytic rate is consistent with the intrinsic pH dependence of CB shown in the literature with conventional fluorogenic peptide substrates<sup>97, 121</sup>.

To confirm these findings, a conventional fluorogenic substrate Z-Leu-Arg-AMC (100  $\mu$ M) was evaluated across the same pH conditions (5.0, 6.3, 7.3) as shown in **Fig. 3.2D** with 3.45 nM CB in 25 mM MES buffer. The fluorescence intensity increased over time (40 min) due to lift of quenching of the AMC fluorophore upon proteolytic cleavage. These reaction curves were fitted to a homogeneous Michaelis-Menten-derived function (**Equation B.8** in **Appendix B**) as explained in **Section B.6** in **Appendix B**, to obtain  $1/\tau$  values that were compared with the electrochemical proteolysis data<sup>134</sup>. The fluorogenic data also demonstrated markedly reduced activity as pH increased from 5.0 to 7.3. As shown in **Fig. 3.2E**, the dependence of  $1/\tau$  values on the pH value is highly consistent among all these measurements. The average  $1/\tau$  values obtained for H-3-Fc, H-6-Fc, and H-15-Fc are summarized in **Table 3.1**.

**Table 3.1** The average  $1/\tau$  values obtained for H-3-Fc, H-6-Fc, and H-15-Fc at pH 5.0, 6.3, and 7.3 with 11.03 nM CB.

| pH  | H-3-Fc<br>$1/\tau$ ( $s^{-1}$ )  | H-6-Fc<br>$1/\tau$ ( $s^{-1}$ )  | H-15-Fc<br>$1/\tau$ ( $s^{-1}$ )  |
|-----|----------------------------------|----------------------------------|-----------------------------------|
| 5.0 | $(6.91 \pm 0.18) \times 10^{-4}$ | $(8.61 \pm 0.04) \times 10^{-4}$ | $(12.27 \pm 1.26) \times 10^{-4}$ |
| 6.3 | $(5.57 \pm 0.58) \times 10^{-4}$ | $(6.60 \pm 0.06) \times 10^{-4}$ | $(4.18 \pm 0.59) \times 10^{-4}$  |
| 7.3 | $(1.51 \pm 0.33) \times 10^{-4}$ | $(4.42 \pm 0.50) \times 10^{-4}$ | $(1.69 \pm 0.02) \times 10^{-4}$  |

H-15-Fc substrate showed the highest proteolysis decay at pH 5.0, and H-6-Fc substrate showed the highest proteolysis decay at pH 6.3 and 7.3, showing delicate proteolysis properties due to different enzyme-substrate interactions at different pH conditions. The  $1/\tau$  values obtained for Z-Leu-Arg-AMC were  $(3.39 \pm 0.26) \times 10^{-4} \text{ s}^{-1}$ ,  $(2.17 \pm 0.06) \times 10^{-4} \text{ s}^{-1}$ , and  $(1.49 \pm 0.10) \times 10^{-4} \text{ s}^{-1}$  for pH 5.0, 6.3, and 7.3, respectively, showing the same trend over the pH as electrochemical proteolysis data. It is noteworthy that the  $1/\tau$  values for fluorogenic assay data were significantly lower than those by the electrochemical method. This may be due to the significantly lower concentration of CB used in the fluorogenic assays (3.45 nM) than in the electrochemical assays (11.03 nM). According to the literature, the number of amino acid residues in a peptide substrate likely plays a role in determining the rate of proteolysis. Substrate length influences enzyme accessibility, correct positioning of the scissile bond, and the affinity of substrate-enzyme interactions<sup>135</sup>. Generally, short peptides (e.g., di- or tripeptides) may be cleaved efficiently if they match the enzyme's preferred recognition motif, while longer substrates can provide additional residues that interact with secondary enzyme sub-sites, potentially increasing cleavage rate but sometimes causing steric hindrance or suboptimal binding<sup>136</sup>. Consistent with this rationale, the use of a hexapeptide substrate in our electrochemical assay is expected to yield larger decay values compared to conventional fluorogenic di- or tripeptide substrates, reflecting enhanced substrate-enzyme interactions and altered proteolytic kinetics.

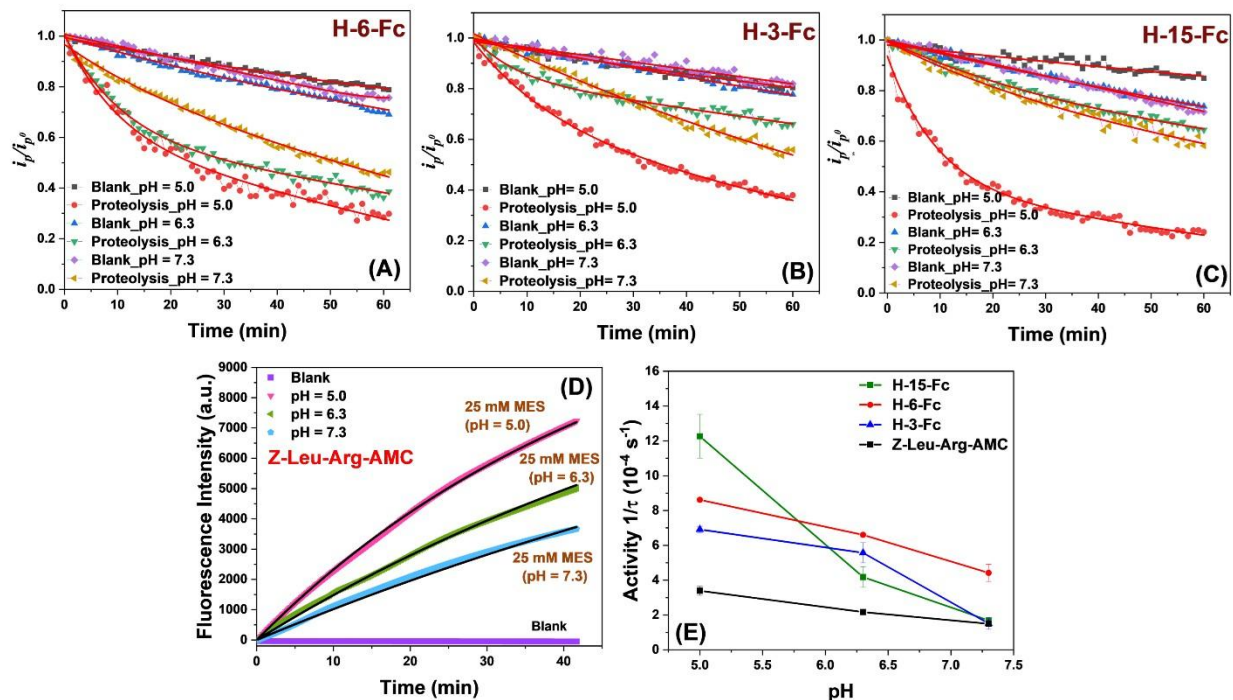


**Figure 3.2** (A-C) Representative normalized kinetic proteolysis curves for an Au MEA modified with three different hexapeptide substrates: A: H-6-Fc, B: H-3-Fc, and C: H-15-Fc, which were proteolyzed by 11.03 nM activated CB (with DTT removed after activation) in 25 mM MES, pH = 5.0/ 6.3/ 7.3 with 50  $\mu$ M EDTA. The protease sample was added after the 10<sup>th</sup> ACV cycle to obtain proteolysis curves. Blanks were run without CB enzyme. The proteolysis curves are normalized to the peak current in the first ACV cycle after CB addition and fitted with Equation 1.6 ( $y = A * \exp(-x / \tau) + b * x + c$ ). (D) The measurement of the activity of 3.45 nM activated CB by a fluorogenic assay with Z-Leu-Arg-AMC substrate (100  $\mu$ M) at pH = 7.3, pH = 6.3, and pH = 5.0, respectively. (E) Line plot showing the pH-dependent proteolytic activity of CB using three peptide substrates: H-15-Fc (green squares), H-6-Fc (red circles), H-3-Fc (blue triangles), and Z-Leu-Arg-AMC (black squares). Activity is expressed as  $1 / \tau \times 10^{-4}$  over a pH range of 5.0 to 7.3. Data are presented as mean  $\pm$  SD from independent replicates (n=3). Maximal cleavage observed at pH 5.0 for all substrates.

Since the proteolysis is much faster at pH 5.0 than at pH 7.3, as shown in **Fig. 3.2E**, a much higher LOD can be expected at pH 7.3. To assess the LODs, a series of proteolysis measurements was carried out at different activated CB concentrations using Au MEA chips functionalized with the three hexapeptide substrates at pH 5.0 and pH 7.3, respectively. As shown in **Fig. B.4A-C** in **Appendix B**, the proteolytic curves decay faster as the CB concentration is increased for all three

hexapeptide substrates at pH 5.0. **Fig. B.4D-F** shows the derived activity vs. CB concentration. The linear fitting parameters obtained over the CB concentration range of 0 to 15.04 nM for all three hexapeptide substrates are summarized in **Table 3.2**.

**Table 3.2** Fitting parameters and detection limits of electrochemical detection of cathepsin B using hexapeptide substrates.

| Peptide sequence                                                                                                                                                                                                                           | pH  | Fitted Calibration Curve<br>$1/\tau$ ( $s^{-1}$ ) vs. [CB] (nM)                      | LOD of activity<br>$1/\tau$ ( $s^{-1}$ ) | LOD of [CB] (nM) |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|--------------------------------------------------------------------------------------|------------------------------------------|------------------|
| (H-3-Fc)<br>A-Pro-Leu-Arg-Phe-<br>Gly-Ala-B <sup>a</sup>                                                                                                                                                                                   | 5.0 | $1/\tau = (5.22 \pm 0.46) \times 10^4 [\text{CB}] + (0.57 \pm 0.41) \times 10^{-4}$  | $1.09 \times 10^{-4}$                    | $1.39 \pm 0.95$  |
|                                                                                                                                                                                                                                            | 7.3 | $1/\tau = (2.19 \pm 0.21) \times 10^4 [\text{CB}] - (0.91 \pm 0.59) \times 10^{-4}$  | $7.49 \times 10^{-5}$                    | $2.09 \pm 1.12$  |
| (H-6-Fc)<br>A-Pro-Leu-Gly-Phe-<br>Val-Ala-B <sup>a</sup>                                                                                                                                                                                   | 5.0 | $1/\tau = (6.45 \pm 0.72) \times 10^4 [\text{CB}] + (0.28 \pm 0.64) \times 10^{-4}$  | $1.36 \times 10^{-4}$                    | $0.52 \pm 0.08$  |
|                                                                                                                                                                                                                                            | 7.3 | $1/\tau = (3.52 \pm 0.25) \times 10^4 [\text{CB}] + (0.78 \pm 0.67) \times 10^{-4}$  | $5.83 \times 10^{-5}$                    | $0.74 \pm 0.21$  |
| (H-15-Fc)<br>A-Pro-Leu-Ala-Phe-<br>Val-Ala-B <sup>a</sup>                                                                                                                                                                                  | 5.0 | $1/\tau = (13.94 \pm 0.35) \times 10^4 [\text{CB}] + (0.58 \pm 0.57) \times 10^{-4}$ | $1.92 \times 10^{-5}$                    | $0.09 \pm 0.07$  |
|                                                                                                                                                                                                                                            | 7.3 | $1/\tau = (2.05 \pm 0.14) \times 10^4 [\text{CB}] + (0.09 \pm 0.39) \times 10^{-4}$  | $3.96 \times 10^{-5}$                    | $0.58 \pm 0.09$  |
| (H-32-Fc)<br>A-Nle-Leu-Gly-Phe-<br>Val-Ala-B <sup>a</sup>                                                                                                                                                                                  | 7.3 | $1/\tau = (7.01 \pm 0.45) \times 10^4 [\text{CB}] + (1.17 \pm 0.37) \times 10^{-4}$  | $8.40 \times 10^{-5}$                    | $0.43 \pm 0.08$  |
| <sup>a</sup> A, linker $\text{H}_2\text{N}(\text{CH}_2)_4\text{CO}-$ at the N-terminus; B, redox tag $-\text{NH}(\text{CH}_2)_2\text{Fc}$ at the C terminus. Cleavage sites are between the pink and cyan highlighted amino acid residues. |     |                                                                                      |                                          |                  |

The LOD of activity can be determined as  $1.09 \times 10^{-4} s^{-1}$ ,  $1.36 \times 10^{-4} s^{-1}$ , and  $1.92 \times 10^{-5} s^{-1}$  for H-3-Fc, H-6-Fc, and H-15-Fc, respectively, as shown in **Table 3.2**, with the equation,

$$\text{LOD of activity} = 3\sigma_{\text{blank}} + (1/\tau)_{\text{blank}}, \quad (3.1)$$

Where  $\sigma_{\text{blank}}$  is the standard deviation and  $(1/\tau)_{\text{blank}}$  is the mean value of blank experiments.

The LOD of CB concentrations were  $1.39 \pm 0.95$  nM,  $0.52 \pm 0.08$  nM, and  $0.09 \pm 0.07$  nM for H-3-Fc, H-6-Fc, and H-15-Fc, respectively, calculated by the equation,

$$\text{LOD of [CB]} = 3\sigma_{\text{blank}} / m \quad (3.2)$$

Similarly, **Fig. 3.3A-C** shows that the proteolytic rate is faster as the CB concentration is increased for all three hexapeptide substrates at pH 7.3. **Fig. 3.3D-F** shows the derived activity  $1/\tau$  vs. CB concentrations. The linear fitting parameters obtained over the CB concentration range of 0 and 52.70 nM for all three hexapeptide substrates are summarized in **Table 3.2**. The LOD of activities can be determined as  $7.49 \times 10^{-5} s^{-1}$ ,  $5.83 \times 10^{-5} s^{-1}$ , and  $3.96 \times 10^{-5} s^{-1}$  for H-3-Fc, H-6-Fc

and H-15-Fc, respectively by **Equation 3.1**. The LOD of CB concentrations were  $2.09 \pm 1.12$  nM,  $0.74 \pm 0.21$  nM, and  $0.58 \pm 0.09$  nM for H-3-Fc, H-6-Fc, and H-15-Fc, respectively, by **Equation 3.2**.

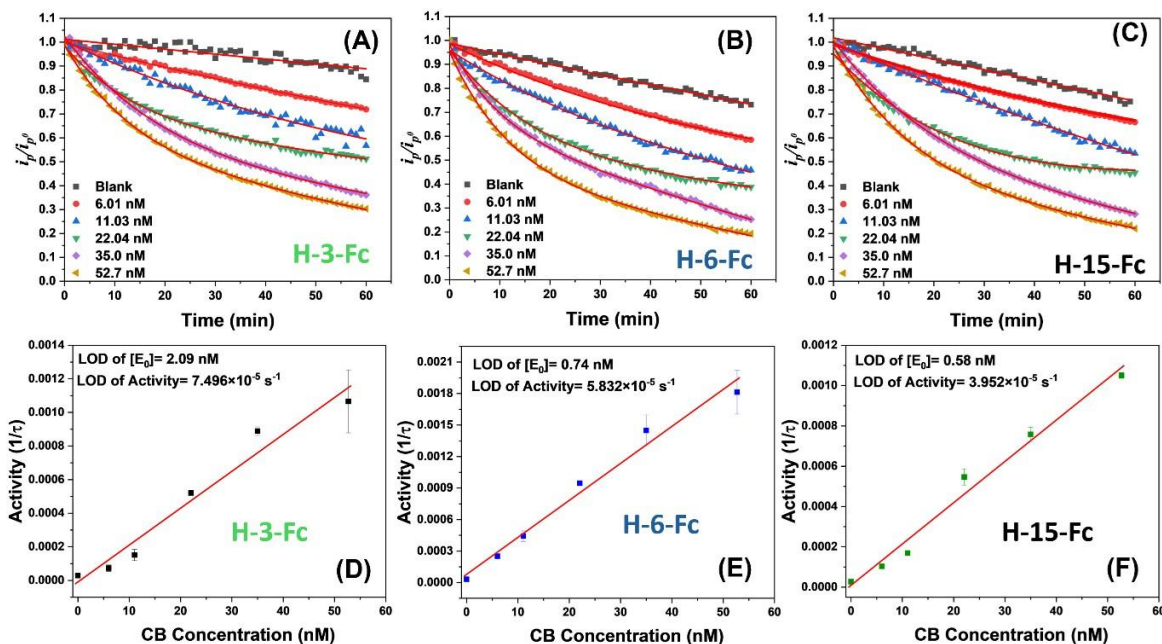


**Figure 3.3** (A-C) The representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the three hexapeptide-Fc substrates H-3-Fc, H-6-Fc, and H-15-Fc, respectively in activated CB (DTT removed after activation), concentrations of 0, 6.01, 11.03, 22.04, 35.00 and 52.7 nM in 25 mM MES, 50  $\mu$ M EDTA (pH = 7.3). (D-F) The linear calibration curves of CB protease activity in the activated CB with the hexapeptide-Fc substrates H-3-Fc, H-6-Fc, and H-15-Fc, respectively.

The activity dependency in pH 5.0 and 7.3 buffers further showed that the reduced catalytic efficiency at physiological pH lowers the electrochemical sensitivity. It is critical to engineer substrates to achieve high turnover rates at neutral pH conditions towards multiplex sensor platform development. As discussed in the introduction, CB, MMP-9, ADAM-17, and other related extracellular proteases may require different pH values for their optimal activity. This is a practical condition for directly measuring clinical samples such as serum, plasma, and tumor

interstitial fluid. An ideal multiplex assay would need to avoid acidifying or alkalifying the sample to prevent disrupting the activity of other proteases or compromising sample integrity.

CB has emerged as a clinically relevant biomarker whose circulating levels and activity levels are markedly altered across multiple pathological conditions. In healthy individuals, serum CB concentrations are typically around  $22.35 \pm 1.8$  ng/mL<sup>137</sup>, which can be translated to  $\sim 0.66$  nM with the assumption that the extracellular CB consists of  $\sim 60\%$  37 kDa proenzyme and  $\sim 40\%$  29 kDa active enzyme (with an average molecular weight of 33.8 kDa)<sup>28</sup> as in the human recombinant CB used in this study. Patients with malignancies, such as colorectal cancer, frequently show elevated levels more than 30 ng/mL ( $\sim 0.90$  nM)<sup>138,139</sup>. In esophageal carcinoma, mean serum CB (total) was  $\sim 38.35 \pm 4.3$  ng/mL ( $\sim 1.13$  nM) in patient<sup>137</sup>. Similar elevations have been reported in breast cancer, where serum CB levels are significantly higher<sup>138</sup>. Depending on the stage of colorectal cancer, plasma CB concentrations can span up to 63 ng/mL (1.87 nM)<sup>140</sup>. These increases correlate with tumor progression and invasion, reflecting CB's role in extracellular matrix degradation and metastatic dissemination.

Although the CB concentrations in patients' serum fall within the detectable range (LOD =  $0.58 \pm 0.09$  nM) of H-15-Fc under acidic conditions (pH = 5.0) in our sensor platform, the reduced sensitivity at neutral pH limits reliable detection in clinical samples. Enhancing CB sensitivity at physiological pH is therefore essential for achieving clinically meaningful detection. Here, we target improving the sensitivity of CB-specific peptide substrates at neutral pH through rational peptide engineering. This is a foundational requirement for real-world, multiplexed electrochemical protease profiling to support early cancer detection, tumor aggressiveness scoring, and point-of-care diagnostic applications, considering that most of the other cancer-related enzymes (MMP-9, MMP-7, ADAM-17) prefer neutral pH conditions<sup>115-117</sup>.

### 3.3.3 Fluorogenic assays for identifying amino acid sites that enhance CB cleavage at neutral pH

Optimal peptide substrates for proteases can be identified using biological or synthetic libraries. Phage display screens peptides on filamentous phage to isolate cleavable sequences <sup>141</sup>, while synthetic libraries rely on techniques like Edman degradation <sup>142</sup>, mass spectrometry, or chromatography to determine substrates and cleavage sites <sup>143</sup>. Support-bound FRET libraries, made by split-and-mix synthesis, allow selection of the most fluorescent beads after partial proteolysis, which are then sequenced to identify optimal substrates <sup>144</sup>. Positional-scanning combinatorial libraries perform site-specific screening to identify the optimal substrate sequences using FRET substrates <sup>145</sup>.

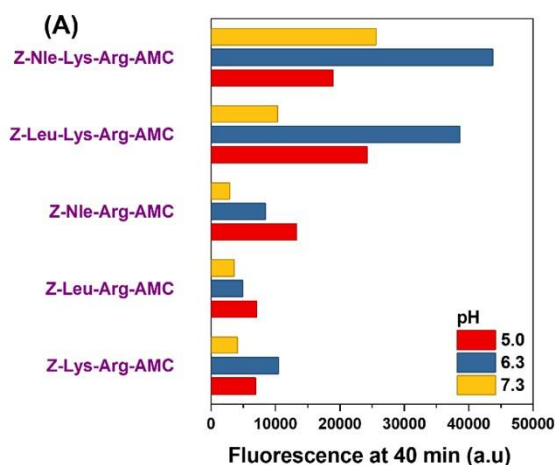
**Fig. B.5A** in **Appendix B** shows an illustrative representation of the catalytic triad (Cys29, His199, Asn219) of active human CB and possible salt bridge formation between His110 and Asp22. CB shows both endopeptidase and exopeptidase activity <sup>146</sup>. CB possesses a unique structural feature, the occluding loop, which contains His110 that interacts with Asp22 through ion pair stabilization, forming a salt bridge <sup>129</sup> as shown in **Fig. B.5A**. Below pH = 6.0 conditions, this salt bridge is formed, and the close-loop conformation is preferred, dominating the exopeptidase activity, *i.e.*, peptide substrates with a free C-terminal carboxyl group are cleaved by CB. This phenomenon can be explained by the pK<sub>a</sub> values of ionizable groups in folded proteins, with the aspartate residue (Asp) exhibiting a pK<sub>a</sub> value of approximately  $3.5 \pm 1.2$  and the histidine residue (His) approximately  $6.6 \pm 1.0$  <sup>147</sup>. Another key determinant of this exopeptidase activity is the occluding loop histidines, His110 and His111, which together form the S2' subsite and provide a positively charged environment that engages negatively charged or C-terminal carboxylate groups in the substrate at acidic pH <sup>148</sup>. The CB enzyme's S3, S2, S1, S1' and S2' subsites that

interact with respective substrate's P3, P2, P1, P1' and P2' residues are shown in **Fig. B.5B** in **Appendix B**.

In the neutral pH conditions, an open-loop conformation is formed, and larger endopeptidase substrates could be bound and cleaved <sup>149</sup>. Fluorogenic peptides having the C-terminal carboxyl group occupied by the fluorogenic AMC or ACC [7-amino-4-carbamoylmethyl)coumarin] groups were designed to investigate their endopeptidase specificities. In the literature, Arg was preferred at P1 while Leu, small residues like Ser and Ala, or even Gly (with no side chain), were also well accepted at P1 <sup>145</sup>. Most of the commercially available fluorogenic Z-peptide-AMC substrates for CB contain Arg as P1. CB favors bulky hydrophobic residues in P2 (*e.g.*, Phe, Leu, Ile, Val, Nle) when acting as an endopeptidase <sup>38, 146</sup>. The least favorable amino acids at the P3 position are Asn and the negatively charged residues Glu and Asp, indicating that the S3 subsite contains not only a hydrophobic pocket but also a positively charged environment <sup>145</sup>. Previous work identified Ile as a preferred residue at P3 <sup>145</sup>, and more recent studies showed that the non-proteinogenic norleucine (Nle), which also has a hydrophobic aliphatic side chain, provides the highest activity under neutral pH conditions <sup>75</sup>. To confirm and further extend these findings, we evaluated the impact of Nle at the P3 position using both commercially available and custom-synthesized fluorogenic peptide substrates.

Fluorogenic AMC-labeled peptide substrates used in this study enable real-time monitoring of proteolysis because the AMC moiety is non-fluorescent while conjugated to the peptide. Upon enzymatic cleavage, AMC is liberated, and its intrinsic fluorescence sharply increases, allowing the protease activity to be quantified by tracking the rise in fluorescence intensity <sup>150</sup>. In order to map CB preferences at the P1, P2, and P3 positions, we compared five fluorogenic peptide substrates (Z-Lys-Arg-AMC, Z-Leu-Arg-AMC, Z-Nle-Arg-AMC, Z-Leu-Lys-Arg-AMC, and Z-

Nle-Lys-Arg-AMC) by recording continuous fluorescence over 40 min. The final fluorescence intensity at 40 min was used as the metric to compare the CB activity at pH 5.0, 6.3, and 7.3, as shown in **Fig. B.6** in **Section B.9** of **Appendix B**. All assays were performed using 100  $\mu$ M fluorogenic peptide substrates and 3.45 nM activated CB in 25 mM MES buffer adjusted to pH 5.0, 6.3, or 7.3 at room temperature. The fluorescence measurements were initiated immediately after adding 10  $\mu$ L of CB solution to 90  $\mu$ L of substrate prepared in the corresponding buffer. Blanks were run without the CB enzyme. The data obtained for CB (3.45 nM) are plotted in a grouped bar chart in the **Fig. 3.4A**. Preferred amino acid residues at the P1, P2, and P3 positions adjacent to the cleavage site were determined for CB under each pH condition (7.3, 6.3, and 5.0) based on the comparative fluorescence data.



(B)

| P3  | P2  | P1  | AMC | Optimum pH |
|-----|-----|-----|-----|------------|
| Nle | Lys | Arg | AMC | 7.3, 6.3   |
| Leu | Lys | Arg | AMC | 6.3, 5.0   |
|     | Nle | Arg | AMC | 6.3, 5.0   |
|     | Leu | Arg | AMC | 6.3, 5.0   |
|     | Lys | Arg | AMC | 6.3, 5.0   |

**Figure 3.4** (A) The activity of 3.45 nM CB represented by the fluorescence intensity after mixing for 40 min ( $F_t = 40\text{min}$ ) with each of the Z-peptide-AMC substrates (100  $\mu\text{M}$  concentration) at pH 7.3 (gold bars), pH 6.3 (blue bars) and pH 5.0 (red bars) from Fig. B.6. (B) The table summary of the optimal pH conditions (pH = 7.3, 6.3 and 5.0) for each substrate assessed by the fluorescence data and preferred amino acid residues (P1, P2, P3) for cathepsin B adjacent to the peptide cleavage site (red arrow).

The data show a clear sequence- and pH-dependent pattern: substrates containing Lys at P2 (Z-Leu-Lys-Arg-AMC and Z-Nle-Lys-Arg-AMC) produced the highest signals at pH 6.3 (43,744 and 38,632, respectively), indicating that a basic side chain at P2 strongly enhances CB activity in this slightly acidic region <sup>145</sup>, a finding consistent with positional-scan and MSP-MS studies that emphasize substrate sequence (not a simple pH-mode switch) in determining CB cleavage preferences <sup>145</sup>. Notably, introducing Nle, a non-proteinogenic isomer of the more common amino acid leucine, at P3 markedly improved activity with Z-Nle-Lys-Arg-AMC at pH

7.3, giving 25,617 counts versus 10,316 with Z-Leu-Lys-Arg-AMC, representing  $\sim 2.5$ -fold increase. It is clear that a hydrophobic, linear-saturated hydrocarbon residue in P3 promotes CB recognition/cleavage under neutral conditions, a prediction previously made and validated by sequence-profiling work that identified Z-Nle-Lys-Arg-AMC as a preferred neutral-pH substrate<sup>75</sup>. Together, these results demonstrate that (i) pH-dependent activity of CB is strongly substrate-sequence dependent, (ii) Lys at P2 favors activity around pH 6.3, and (iii) Nle at P3 substantially rescues (and even enhances) cleavage at pH 7.3, supporting an initial rational design of peptide substrates for electrochemical detection of CB at neutral pH conditions. The table (**Fig. 3.4B**) summarizes the optimal pH conditions (pH = 7.3, 6.3, and 5.0) for each substrate assessed by the fluorescence data.

### 3.3.4 Impact of peptide substrate modification on electrochemical detection of CB

To improve the neutral-pH proteolysis efficiency of our redox-tagged peptide sensor, we pursued a minimal-change engineering strategy focused exclusively on the P3 position. CB exhibits a well-established preference for hydrophobic and aliphatic residues at the P3 site, whereas extensive mutations at multiple P-positions (P1, P2, P3) can significantly perturb the protease-substrate interaction interface and inadvertently shift the cleavage position<sup>75</sup>. Therefore, to avoid introducing unintended changes to the cleavage locus, we retained the native P2-P1-P1' motif (where Gly-Phe are P1-P1' sites at the scissile bond) and modified only the P3 residue while keeping the remaining five amino acid residues unchanged from the original H-6-Fc peptide substrate. Gly-containing P1 peptides are among the most frequently selected substrates for CB endopeptidase activity in the literature, supporting the choice of Gly at the P1 position in our original peptide substrates<sup>151</sup>. Leu is also commonly preferred, as studies have shown that the S2

site of the CB enzyme favors hydrophobic residues, particularly Phe and Leu <sup>38</sup>. **Fig. 3.5A** illustrates the structural modification to the peptide substrate.

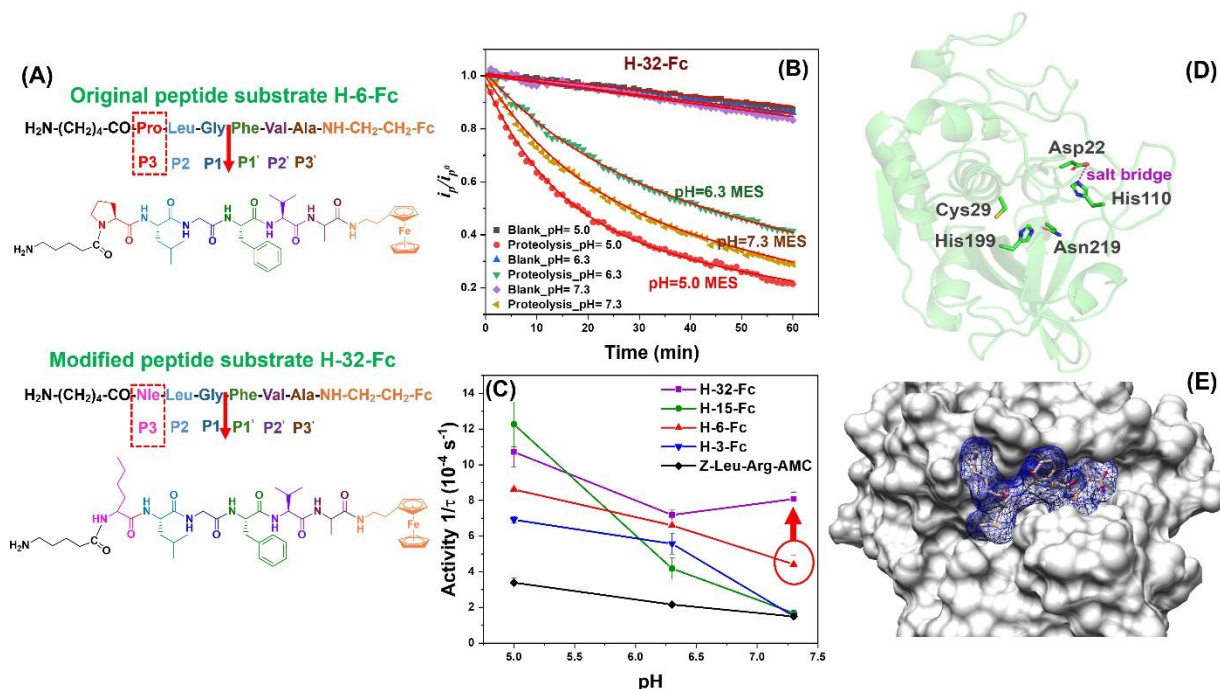


**Figure 3.5** (A) The original H-6-Fc hexapeptide substrate ( $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ ) and the modified H-32-Fc hexapeptide substrate ( $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Nle}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ ) with the Pro amino acid at P3 position replaced by a Nle. (B) The representative normalized kinetic proteolysis curves for an Au MEA modified with H-32-Fc, which was proteolyzed by 11.03 nM activated CB (with DTT removed after activation) in 25 mM MES, pH = 5.0, 6.3, and 7.3 with 50  $\mu\text{M}$  EDTA. The protease sample was added after 10<sup>th</sup> ACV cycle to obtain proteolysis curves. Blanks were run without CB enzyme. The proteolysis curves are normalized to the peak current in the first ACV cycle after CB addition and fitted with Equation 1.6 ( $y = A * \exp(-x / \tau) + b * x + c$ ). (C) Summary of the pH-dependent proteolytic activity of CB using four hexapeptide substrates on Au MEA in comparison with the Z-Leu-Arg-AMC fluorogenic peptide substrate. The activity is expressed as  $1/\tau \times 10^{-4}$  over a pH from 5.0 to 7.3. Data are presented as mean  $\pm$  SD from independent replicates ( $n=3$ ). Maximal cleavage observed at pH 5.0 for all substrates. (D) Illustrative representation of catalytic triad (Cys29, His199, Asn219) of active human cathepsin B and possible salt bridge formation between His110 and Asp22. (E) Docking pose of the peptide substrate (Nle-Leu-Gly-Phe-Val-Ala) within the active-site pocket of active cathepsin B (PDB ID: 1CSB), molecular surface (gray) while peptide is displayed as a stick model with a semi-transparent mesh representation (blue).

And **Fig. 3.5A** displays the amino acid sequences of the original (H-6-Fc) and the modified (H-32-Fc) substrates with their P-site assignments. The red-marked (red dashed-line square) region

highlights the single change at the P3 position: Pro→Nle. Importantly, the experimentally identified cleavage site remained between Gly and Phe in the modified substrate, with the minimal alteration to the interaction geometry of the scissile bond. The modified peptide, NH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-Nle-Leu-Gly-Phe-Val-Ala-(CH<sub>2</sub>)<sub>2</sub>-Fc sample (0.5 mM) was proteolyzed with CB (20 nM) and analyzed by analytical HPLC as shown in **Fig. B.7** in **Section B.10** of **Appendix B**. The proteolyzed peptide sample was injected after 1 hour, 2 hours, and 12 hours of incubation. The intact peptide appeared at 34.6 min retention time, and two new peaks appeared at the retention times of 19.5 min and 31.1 min, corresponding to the cleaved products. The intact peptide peak decreased over time, and the other two peaks' areas increased with the proteolytic time. The cleavage site of the modified peptide substrate was determined by high-resolution mass spectrometry (HRMS) analysis, as shown in **Fig. B.8** in **Section B.10** of **Appendix B** by analyzing 2 hours proteolyzed sample. As shown in HRMS spectrum, in **Fig. B.8** HRMS (ESI) calculated for Phe-Val-Ala-(CH<sub>2</sub>)<sub>2</sub>-Fc fragment C<sub>29</sub>H<sub>39</sub>FeN<sub>4</sub>O<sub>3</sub><sup>+</sup> [M + H]<sup>+</sup>: 547.2366, found 547.2424; and calculated for NH<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-Nle-Leu-Gly fragment C<sub>19</sub>H<sub>37</sub>N<sub>4</sub>O<sub>5</sub><sup>+</sup>[M + H]<sup>+</sup>: 401.2758, found 401.2809. This ability to retain the cleavage motif supports the idea of targeted single-site modifications when optimizing substrate performance in our sensor platform in the future.

CV characterization was carried out using Au/MEA (200 μm × 200 μm ) functionalized with H-32-Fc peptide substrate, as shown in **Fig. B.9** in **Section B.11** of **Appendix B**. The potentials of the redox pairs are consistent with the peak potential in ACV, and the peak areas reveal that the surface density ( $\Gamma_{ss}$ ) for H-32-Fc was  $40.59 \pm 4.65$  pmol/cm<sup>2</sup>. This corresponds to an average molecular spacing of  $2.18 \pm 0.12$  nm. After that, H-32-Fc on Au MEA was subjected to CB proteolysis analysis. 11.03 nM of activated CB was added after 10 ACV cycles in 25 mM MES, pH 5.0, 6.3, and 7.3. **Fig. B.10** in **Section B.12** of **Appendix B** shows the representative

ACV curves for H-32-Fc functionalized on SAM-covered Au MEA, before and after the proteolysis, including the raw ACV curves (**Fig. B.10A**) and background-subtracted ACV curves (**Fig. B.10B**) in 25 mM MES, 50  $\mu$ M EDTA, pH = 7.3. The normalized kinetic proteolysis curves in **Fig. 3.5B** demonstrate that the modified peptide (H-32-Fc) exhibited significantly enhanced proteolysis at pH 7.3, whereas the original substrate performed optimally under more acidic conditions (pH 5.0), which aligns with CB's canonical lysosomal activity profile. The increase in neutral-pH activity of H-32-Fc can be attributed to the ability of the Nle residue to support more favorable hydrophobic interactions with the CB S3 pocket under higher pH, where electrostatic contributions to binding become negligible and CB relies more heavily on hydrophobic contacts for substrate positioning. This behavior matches prior substrate engineering studies<sup>75</sup> and shows that the introduction of Nle at P3 indeed improves kinetic efficiency.

**Fig. 3.5C** quantitatively compares the pH-dependent activity ( $1/\tau$ ) across the Fc-labeled peptide substrates previously used in this study. The modified substrate H-32-Fc demonstrated the greatest enhancement at pH 7.3 (showed in purple line plot) showing  $(8.08 \pm 0.38) \times 10^{-4} \text{ s}^{-1}$  of  $1/\tau$  value, outperforming all the original substrates H-3-Fc, H-15-Fc and H-6-Fc whose  $1/\tau$  values were  $(1.51 \pm 0.33) \times 10^{-4} \text{ s}^{-1}$ ,  $(1.69 \pm 0.02) \times 10^{-4} \text{ s}^{-1}$ , and  $(4.42 \pm 0.50) \times 10^{-4} \text{ s}^{-1}$ , respectively, confirming the benefits of incorporating Nle at P3. While the original substrates showed the expected decline in activity when approaching neutral pH, H-32-Fc uniquely displayed an upward trajectory, indicating the successful optimization. Furthermore, H-32-Fc substrate showed higher  $1/\tau$  values of  $(10.72 \pm 0.86) \times 10^{-4} \text{ s}^{-1}$ , and  $(7.19 \pm 0.28) \times 10^{-4} \text{ s}^{-1}$  at pH 5.0 and 6.3, respectively. This improvement aligns with the fluorogenic peptide substrate screening results, where Nle-containing substrates also exhibited a superior performance at pH 7.3 compared to other substrates, reinforcing the validity of this design strategy.

**Fig. 3.5D** illustrates the active-site architecture of human CB, highlighting the catalytic triad (Cys29, His199, and Asn219) and a potential salt-bridge interaction between His110 and Asp22. No experimentally resolved structures of active human CB are available at near-neutral pH (pH 7.3); structures reported at this pH correspond only to the inactive proenzyme and are unsuitable for substrate docking. Therefore, the mature CB crystal structure (PDB ID: 1CSB)<sup>152</sup>, resolved under acidic conditions (pH  $\approx$  5.0), was used for docking after protonation and deprotonation, optimization of hydrogen positions at pH 7.3 as described in **Section B.5** of **Appendix B**. In parallel, a mature CB structure generated using AlphaFold2<sup>153</sup>, representing a more general enzyme conformation, was prepared at pH 7.3 and used for comparative docking. Three independent blind docking simulations were performed for both the crystal structure 1CSB and AlphaFold2 modeled CB using the CB-Dock2 server<sup>154</sup>, which identified four and five putative binding cavities, respectively. For both enzyme structures, the best-ranked pose of the peptide substrate *Nle-Leu-Gly-Phe-Val-Ala* was consistently located in cavity ID C1, yielding mean AutoDock Vina<sup>155</sup> binding energies of  $-7.5 \pm 0.3$  kcal/mol<sup>-1</sup> for the 1CSB and  $-7.5 \pm 0.1$  kcal/mol<sup>-1</sup> for AlphaFold2 modeled CB. Minor variations in Vina scores across repeated docking runs reflect the stochastic nature of the docking algorithm and the conformational flexibility of the peptide ligand. Nevertheless, the predicted binding poses were highly similar, with energy differences within  $\pm 0.3$  kcal/mol<sup>-1</sup>, indicating a consistent binding mode for both 1CSB and AlphaFold2 modeled CB structures.

The best-ranked docking pose for the 1CSB is shown in **Fig. 3.5E**, where the enzyme is rendered as a molecular surface (gray), illustrating the shape complementarity of the substrate-binding pocket. The peptide is displayed as a stick model with a semi-transparent mesh representation (blue). **Fig. B.11A** (**Section B.13 of Appendix B**) presents an additional surface

view of the 1CSB with the docked peptide shown as sticks, further demonstrating accommodation of the substrate within the catalytic groove. The peptide aligns along the S subsites of CB, positioning the putative scissile bond (Gly–Phe) proximal to the catalytic residue Cys29, while the Nle residue at the P3 position shows favorable alignment with the S3 subsite. Across all three docking runs, cavity C1 (**Fig. B.11A**) consistently produced the lowest Vina scores and exhibited a stable cavity volume of approximately 2963 Å<sup>3</sup> (1CSB), suggesting it represents the most probable substrate-binding region on the enzyme surface. Predicted interactions with catalytic residues Cys29 and His199 further support the potential proteolytic cleavage of the *Nle–Leu–Gly–Phe–Val–Ala* substrate.

For ease of comparison, the docking pose shown in **Fig. 3.5E** is reproduced in **Fig. B.11B**. Corresponding docking results for the AlphaFold2 modeled structure are shown in **Fig. B.11C** and **B.11D**, where the AlphaFold2 modeled CB is rendered as a surface representation (sky blue) with the peptide displayed as sticks, illustrating a similar mode of substrate accommodation within the binding groove. Overall, this preliminary docking analysis reveals the expected interaction between the P3 position of the modified peptide substrate and the S3 subsite of CB in both the modeled and experimentally obtained structures. These results highlight the utility of computer-aided substrate design as a complementary tool for developing protease-specific substrates. While the present study was limited to the docking of the peptide segment alone, future work will focus on incorporating redox-active tags to better represent the complete sensor architecture.

### 3.3.5 Assessment of the LOD in measuring activated CB with H-32-Fc in neutral conditions

After observing that the H-32-Fc substrate displayed higher proteolysis activity and sensitivity toward CB, particularly at pH 7.3 with 11.03 nM CB, we further investigated its analytical performance in terms of LOD. To accomplish this, we carried out kinetic measurements across a series of CB concentrations and monitored the time-dependent decrease in the peak current of continuous ACV. As shown in **Fig. 3.6A**, the proteolytic curves decay faster as the CB concentration increases from 1.5 nM to 20.0 nM. **Fig. 3.6B** shows the derived activity vs. the CB concentration. The linear fitting parameters obtained over the CB concentration range of 0-14.50 nM for all three hexapeptide substrates are summarized in **Table 3.2**. The LOD of activity and CB concentration can be determined as  $8.40 \times 10^{-5} \text{ s}^{-1}$  for H-32-Fc by **Equation 3.1** and  $0.43 \pm 0.08 \text{ nM}$  by **Equation 3.2**, respectively.

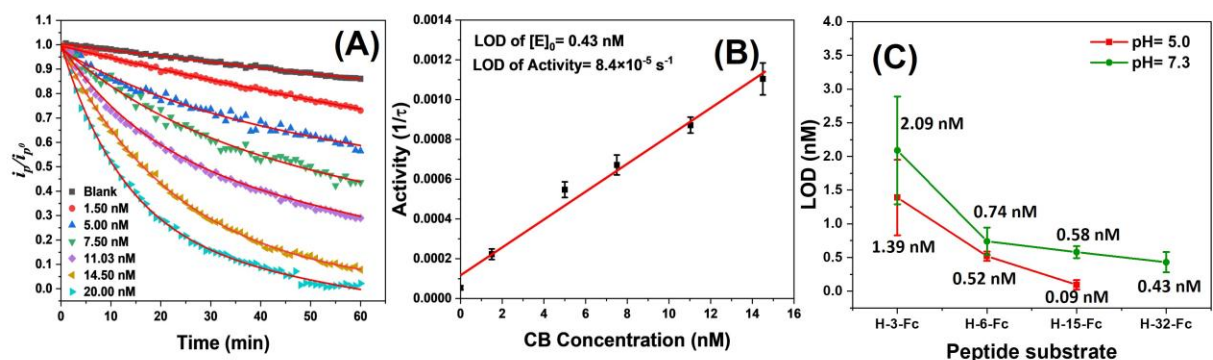


**Figure 3.6** (A) Representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the peptide-Fc substrate H-32-Fc at activated CB ( DTT removed after activation) concentrations of 0, 1.5, 5.0, 7.5, 11.03, 14.5, and 20.0 nM in 25 mM MES, 50  $\mu\text{M}$  EDTA (pH = 7.3). (B) Linear calibration curve of CB protease activity in the activated CB with the peptide-Fc substrate H-32-Fc. The error bars represent the standard deviation of three replicates for each measurement. (C) A line plot containing the summary of LODs of CB using different peptide substrates at pH 5.0 and 7.3.

As shown in **Fig. 3.6C**, H-15-Fc shows the lowest LOD at pH 5.0, indicating it is highly efficiently cleaved under acidic (lysosomal-like) conditions. However, its LOD is worsened at neutral pH to  $0.58 \pm 0.09$  nM, showing that its catalytic advantage is pH dependent. In contrast, H-32-Fc demonstrates the lowest LOD of  $0.43 \pm 0.08$  nM at pH = 7.3, making it a better candidate for neutral-pH multiplexed sensing. Although these LOD values were not significantly different by performing the two sample T-test, there was an improvement when considering their individual standard deviations. For a single-compartment multiplex electrochemical sensor designed to operate at neutral pH (to accommodate other proteases such as MMPs and ADAMs), H-32-Fc or other substrate exhibiting similar behavior is preferred because it retains an acceptable sensitivity for CB detection at pH 7.3. This likely enables reliable discrimination between healthy individuals and diseased patients in clinical samples, given that CB levels in healthy individuals are typically around  $22.35 \pm 1.8$  ng/mL ( $\sim 0.66$  nM) <sup>137</sup>. In addition, plasma CB concentrations in cancer patients, which span a broader range of approximately 63 ng/mL (1.87 nM) <sup>139</sup>, can be readily detected using this platform with H-32-Fc at neutral pH conditions.

A potential challenge lies in designing highly specific substrates for individual proteases, given the overlapping cleavage preferences among cysteine cathepsins and matrix metalloproteinases. For papain-like cysteine proteases, numerous structural and mutational studies have established that the S2 subsite is the primary determinant of substrate specificity <sup>156, 157</sup>, highlighting it as a key target for future substrate engineering to further enhance specificity. Nevertheless, the platform presented here offers immense potential for rapid, cost-effective, and reliable multiplex assays. By integrating optimized peptide chemistry with electrochemical readouts, this approach supports advanced diagnostic and therapeutic monitoring through multivariate data analysis and pattern recognition. Ultimately, the strategy outlined in this work

establishes a scalable foundation for next-generation biosensors that can operate under unified neutral pH conditions, enabling clinically meaningful detection of protease activity in complex biological systems.

### 3.4 Conclusion

This chapter demonstrates an optimized peptide substrate,  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Nle-Leu-Gly-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ , for sensitive electrochemical detection of CB under neutral pH conditions using Au MEA. By systematically evaluating fluorogenic substrates and confirming the sequence-dependent cleavage, we established that incorporation of the non-proteinogenic Nle amino acid at the P3 position markedly enhances CB activity at physiological pH and obtained a very low LOD of CB at  $0.43 \pm 0.09$  nM. These results validate prior reports of the pH-dependence of CB activity, using three hexapeptide substrates and extend them by providing direct electrochemical evidence for rational peptide substrate design. Importantly, the optimized substrates improved detection sensitivity at neutral pH. Moreover, a minimal-change engineering strategy focused exclusively on the P3 position proved successful, encouraging further site-directed modifications of the substrate sequence to enhance both the sensitivity and specificity of peptide substrates employed on the Au MEA platform. The specificity of H-32-Fc is discussed in the next chapter (**Chapter 4**).

## **Chapter 4 - Electrochemical Response from Human Serum on Au-MEA and evaluating the cross reactivity from MMP-9 and ADAM-17 like proteases on cathepsin B detection**

### **4.1 Introduction**

Translating electrochemical protease biosensors from controlled buffer systems to analyzing complex biological fluids remains one of the central challenges in diagnostic sensor development. Human serum is a chemically and biologically complex matrix containing a high abundance of proteins ( $\sim 60\text{--}80\text{ mg mL}^{-1}$ ), electrolytes, lipids, metabolites, and enzymes, all of which can interfere with surface-confined sensing strategies<sup>158</sup>. These components can contribute to nonspecific adsorption, electrode fouling, background currents, and unintended enzymatic degradation of peptide substrates, ultimately complicating signal interpretation. Therefore, evaluating biosensor performance directly in serum is essential for assessing analytical robustness and clinical relevance<sup>159</sup>.

Au-MEAs offer distinct advantages for serum-based electrochemical sensing due to their high surface-to-volume ratio, enhanced mass transport, and the ability to spatially confine sensing events<sup>160</sup>. Microelectrode architectures reduce ohmic drop and capacitive charging currents, enabling improved signal-to-noise ratios even in highly conductive biological media<sup>161</sup>. Furthermore, the use of self-assembled monolayers and peptide-based recognition elements on gold surfaces has been widely adopted for probing proteolytic activity through changes in electron transfer kinetics or signal decay of redox reporters tethered to the electrode surface<sup>162</sup>.

In earlier chapters, this platform was demonstrated for the sensitive detection of CB, a lysosomal cysteine protease overexpressed in multiple cancer types and active predominantly under mildly acidic conditions (pH = 5.0). While CB-specific peptide substrates enable selective detection under optimized buffer conditions, real biological samples introduce additional complexity. Human serum contains a broad spectrum of proteases, including MMPs) and ADAM family members<sup>40, 43</sup>, which can contribute to nonspecific or partially overlapping cleavage events. Among these, MMP-9 and ADAM-17 are of particular interest due to their well-documented roles in extracellular matrix remodeling, inflammation, and cancer progression<sup>40, 43, 66</sup>.

MMP-9 is a zinc-dependent endopeptidase<sup>40</sup> that retains activity near physiological pH, while ADAM-17 is a membrane-associated metalloprotease responsible for ectodomain shedding of multiple signaling proteins<sup>43, 118</sup>. Both enzymes have been reported in circulating blood and tumor microenvironments, and their proteolytic activity can overlap with peptide motifs designed for cysteine proteases. As a result, the electrochemical signal decay observed in serum cannot be trivially attributed to a single protease without carefully evaluating cross-reactivity and matrix effects.

Another major challenge in multiplex or multi-protease sensing within a single compartment arises from incompatible optimal operating conditions. CB exhibits maximal activity under acidic pH, whereas MMP-9 and ADAM-17 require neutral pH and divalent metal ions such as  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$  for catalytic function<sup>42</sup>. Buffer composition, ionic strength, chelating agents, and redox mediator stability must therefore be carefully balanced to maintain functionalized electrode performance while preserving enzymatic activity. In addition, serum proteins such as albumin and globulins can sterically hinder substrate accessibility or alter local microenvironments at the electrode surface, further influencing observed kinetics<sup>159</sup>.

Nonspecific signal decay is also induced by surface fouling and partial sensing element desorption in serum, particularly during the initial exposure period, as people have seen in DNA-based sensors <sup>163</sup>. Rapid decay observed at early time points has been frequently reported in surface-confined protease assays and is often attributed to fast cleavage of highly accessible peptide populations <sup>52</sup>, loosely bound substrate fractions, or initial adsorption-driven rearrangement of the sensing layer<sup>164</sup>. Distinguishing these effects from true enzymatic activity is critical for quantitative interpretation.

In this chapter, the electrochemical response of peptide-modified Au MEAs is systematically evaluated in real human serum to assess sensor robustness and specificity. The contribution of MMP-9 and ADAM-17-like proteolytic activity to the overall electrochemical signal decay is examined, highlighting the challenges of discriminating against protease activity in a complex enzymatic environment. Despite these challenges, the platform demonstrates a clear and reproducible linear relationship between human serum concentration and electrochemical response on Au MEA. Additionally, a faster initial decay is observed for certain peptide substrates, followed by a more stable, concentration-dependent response regime, underscoring the importance of kinetic analysis in real-sample measurements.

Overall, this chapter provides critical insight into the behavior of multiplex protease-responsive electrochemical sensors in human serum and establishes key considerations for advancing Au MEA-based platforms toward clinically relevant protease diagnostics.

## 4.2 Materials and Methods

A detailed description of the materials and instruments used in this chapter is given in **Section C.1** in **Appendix C**. Au MEA functionalization and methodology of electrochemical experiments are described in the materials and methods sections of **Chapter 2** under **2.2.1** and **2.2.4** subsections, respectively.

### 4.2.1 Activating MMP-9

Recombinant human MMP-9 (rhMMP-9) was activated using *p*-aminophenylmercuric acetate (APMA). The assay buffer consisted of 50 mM Tris, 10 mM CaCl<sub>2</sub>, 150 mM NaCl, and 0.05% (w/v) Brij-35, adjusted to pH 7.5 (TCNB buffer). Inactive MMP-9 was initially diluted to a concentration of 100 µg/mL (1298.7 nM) in the assay buffer.

APMA (Sigma-Aldrich) was prepared as a 100 mM stock solution in dimethyl sulfoxide (DMSO) and added to the rhMMP-9 solution to achieve a final concentration of 1 mM. The mixture was incubated at 37 °C for 24 h (4.18 µL final volume) to allow proteolytic activation of the zymogen. Following incubation, the activated MMP-9 sample was then mixed with 495.82 µL of TCNB dilution buffer in an Amicon ultra 10K 0.5 mL filter (total volume = 500 µL). The device spun down at 14,000 g for 5 min in a 40° fixed-angle rotor at 20°C with  $\sim 50 \pm 7$  µL remaining in the vial at the end. After that, another  $450 \pm 7$  µL of dilution buffer was added and spun down to  $\sim 50 \pm 7$  µL using the same procedure. The remaining residue was taken as the final activated APMA removed MMP-9 sample for proteolysis experiments.

In parallel experiments, a separate batch of activated rhMMP-9 with APMA removed was purchased from Sigma-Aldrich. This enzyme was employed to validate the proteolytic activity of in-house APMA-activated and APMA-removed MMP-9 samples.

For electrochemical proteolysis experiments, 25 mM MES, pH = 7.3, 50  $\mu$ M EDTA buffer was used after validating and comparing the activity in its original buffer using FRET assays.

#### **4.2.2 Preparation of active ADAM-17 samples**

10  $\mu$ g of lyophilized from a 0.2  $\mu$ m filtered solution in Tris, NaCl, and Brij-35 was received from R&D Systems, and the sample was reconstituted in deionized water at 100  $\mu$ g/mL stock concentration (1923.08 nM). Recombinant human ADAM-17 (rhTACE) original assay buffer consists of 25 mM Tris, 2.5  $\mu$ M ZnCl<sub>2</sub>, and 0.005% (w/v) Brij-35, adjusted to pH 9.0. The enzyme was diluted to a final desired concentration using stock ADAM-17 (1923.08 nM) in the assay buffer and used directly for subsequent experiments.

For electrochemical proteolysis experiments, 25 mM MES, pH = 7.3, 50  $\mu$ M EDTA buffer was used after validating and comparing the activity in its original buffer using FRET assays.

#### **4.2.3 Validation of enzyme activity with fluorogenic assays**

MMP-9 and ADAM-17 enzyme activities were validated using a fluorogenic assay based on fluorescence resonance energy transfer (FRET) with a BLACK-Comet StellaNet UV-VIS Spectrometer (Tampa, FL). The recommended MMP-9 and ADAM-17 substrate, Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub> (20  $\mu$ M), was used with the desired enzyme concentration to measure fluorescence increase over time due to proteolytic cleavage by active MMP-9/ ADAM-17 at the Gly–Leu bond and by separating the fluorophore (Mca) and quencher (Dpa), resulting in a fluorescence increase proportional to enzymatic activity. The excitation and emission wavelengths were set to 295 nm and 405 nm, respectively. Activated MMP-9 or ADAM-17 (75  $\mu$ L) was added to a quartz fluorescence cuvette, and fluorescence measurements were initiated after 1 min of adding 75  $\mu$ L of substrate solution. The effect of different proteolysis buffers was examined by preparing the substrate in various buffers, including TCNB buffer (pH = 7.5) for MMP-9, Tris

buffer (pH = 9.0), and 25 mM MES with 50  $\mu$ M EDTA (pH = 7.3) or without EDTA. Additionally, the activity of APMA removed and not removed MMP-9 samples compared for persistent enzyme activity.

Furthermore, the GloMax Multi+ Microplate Multimode reader (Promega, Madison, WI) in a 96-well plate system was optimized to use and validate both MMP-9 (10 nM) and ADAM-17 (5 nM) protease with Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub> (100  $\mu$ M) substrate to see continuous proteolysis activity through 40 min time period.

## 4.3 Results and Discussion

### 4.3.1 Sensor platform stability in the presence of Human Serum

To evaluate the intrinsic stability of the Au/SAM/peptide-Fc sensing interface under biologically relevant conditions, the platform was tested in 3% pooled healthy human serum (HS). In this experiment, the AEF reporter was immobilized on the self-assembled monolayer (SAM) without incorporating a protease-cleavable peptide sequence (Au/SAM/AEF). This design eliminates the possibility of proteolytic signal loss and enables isolation of nonspecific signal decay arising from matrix effects such as protein adsorption, ionic interactions, or disruption of the SAM layer.

This strategy was previously used in **Chapter 2** to identify nonspecific current decay caused by DTT. Here, a similar approach was applied to evaluate the stability of the sensing interface in complex biological media.

As shown in **Figure 4.1A**, the Au/SAM/AEF platform exhibited excellent stability in 25 mM MES pH = 7.3, 50  $\mu$ M EDTA over the monitored period, maintaining nearly constant normalized peak current ( $i_p/i_{p0}$ ) as shown in the brown stability curve. Upon addition of 3% (v/v) human serum (indicated at ~11 min), a modest but gradual decrease in current was observed, as

shown in the orange stability curve. No abrupt current drop occurred immediately after HS addition, and the total signal reduction was approximately 13% from the initial current over the measurement period. Because no peptide substrate was present, this decrease cannot originate from proteolysis. Instead, it likely arises from partial SAM desorption and nonspecific adsorption of serum proteins on the sensor platform. This indicates that the Au-thiol linkage remains largely intact even in complex biological matrices.

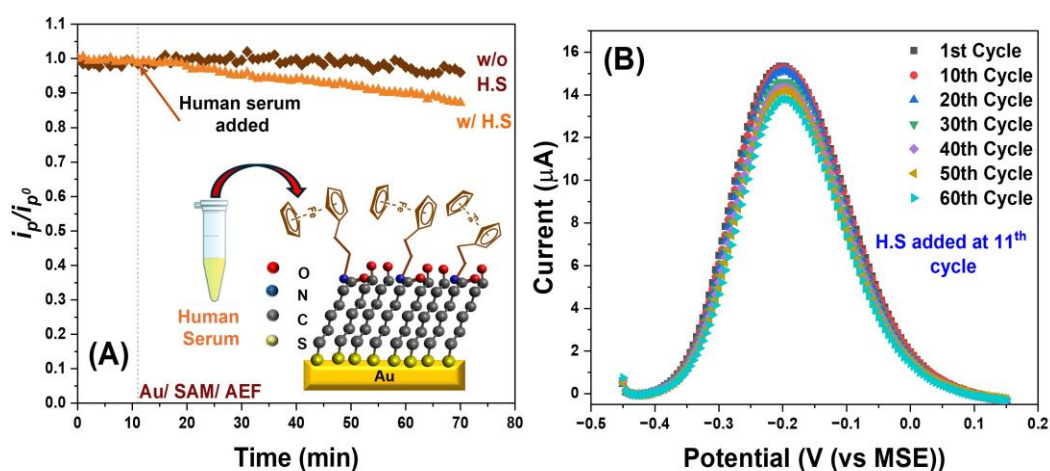


**Figure 4.1.** Stability of the Au/SAM/AEF sensor platform in the presence of human serum. (A) Normalized peak current as a function of time for the Au/SAM/AEF interface in 25 mM MES pH = 7.3, 50 μM EDTA at 36 °C (brown) and after addition of human serum (orange). Human serum was introduced at ~11 min (dashed line). The AEF layer was immobilized without a protease-cleavable peptide to isolate nonspecific interfacial effects. (B) Background-subtracted ACV curves recorded at different cycles following serum addition (HS added at the 11th cycle). The peak shape and potential remain largely unchanged, with only a modest reduction in peak current over 60 cycles, confirming the electrochemical stability of the interface in complex biological media.

Background-subtracted ACV curves (**Figure 4.1B**) provide further insight into the stability of the interface. The peak potential remains essentially unchanged, and the peak shape is preserved across cycles, with only a gradual decrease (approximately 13% current reduction after 60 cycles) in peak current is observed.

#### 4.3.2 Electrochemical assessment of effective cathepsin B activity spiked in Human serum and modelling kinetic response.

To evaluate effective proteolytic activity in biologically relevant conditions, Au MEA electrodes functionalized with H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc were exposed to 3% human serum (HS), and the normalized electrochemical current decay ( $i_p/i_{p0}$ ) was monitored over time in 25 mM MES, pH=7.2, 50  $\mu$ M EDTA at 36 °C.

For H-3-Fc, H-6-Fc, and H-15-Fc, an immediate and pronounced drop in current was observed upon serum addition, as shown in **Fig.4.2A, B, and C**, followed by a slower, gradual decay phase. This biphasic behavior suggests the presence of at least two kinetically distinct processes contributing to signal loss.

In contrast, H-32-Fc, previously optimized for selective CB detection, did not exhibit the sharp initial decay (**Fig. 4.2D**) observed for the other substrates. Instead, it showed a more gradual and continuous decrease in signal. This indicates improved resistance to nonspecific or rapid surface-associated cleavage and supports the enhanced selectivity of H-32-Fc developed in **Chapter 3**.

HS contains very complex components, which may affect the assay sensitivity and reproducibility<sup>165</sup>. To assess the matrix effects, a study was carried out by spiking active CB (11.03 nM) into the 3% HS in 25 mM MES, pH=7.3, 50  $\mu$ M EDTA at 36 °C as shown in **Fig. 4.2A, B, C, and D**. The same rapid initial decay was observed with H-3-Fc, H-6-Fc, and H-15-Fc peptide substrates. The overall magnitude of decay increased compared to serum alone.

Previously, the current decay was fitted using a linear decay term combined with a single exponential function in **Chapter 3** and explained in **Section 1.8.3**. However, this model failed to adequately describe the present data due to the abrupt signal drop immediately after serum addition

and could not fit the data. This deviation suggests that decay is not governed by a single homogeneous kinetic process. To better describe the observed behavior, both single- and double-exponential decay models were evaluated. The double exponential model (**Equation 4.1**) provided significantly improved fitting across all peptides by providing fraction of unexplained error ranging around 0.18% to 0.6% after residual analysis ((residual/ corrected total)× 100%), supporting the presence of two kinetically distinct decay components.

$$(y)_{(t)} = A_1 e^{\left(-\frac{t}{\tau_1}\right)} + A_2 e^{\left(-\frac{t}{\tau_2}\right)} + (y)_{(0)} \quad (4.1)$$

In this equation fast component is given by  $\tau_1$ , and the slow component is given by  $\tau_2$ . The fitted curves are shown in black in **Fig. 4.2** proteolysis curve panels. However, due to overlapping time constants and signal complexity, complete separation of these processes was not possible.

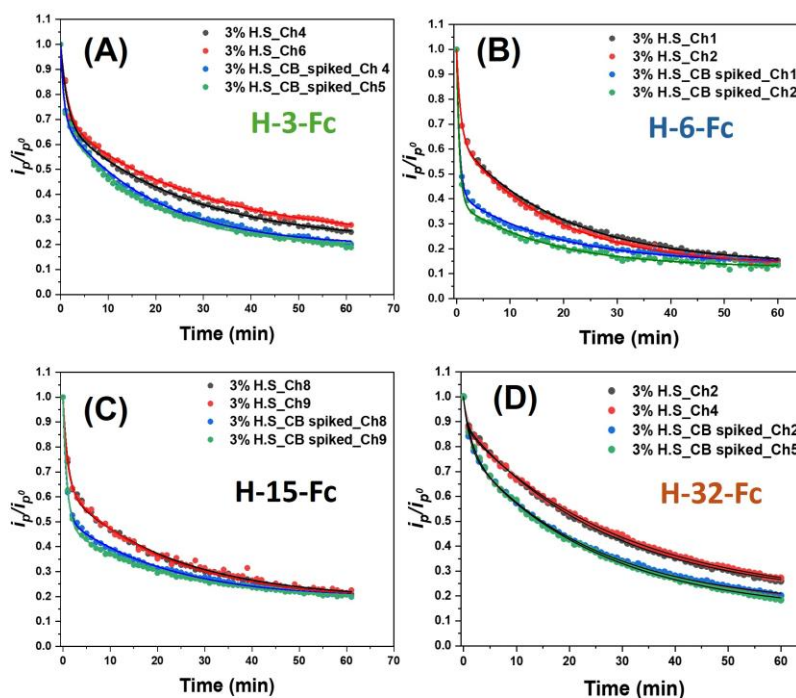


**Figure 4.2** Electrochemical monitoring of peptide cleavage in 3% human serum and CB-spiked serum. Normalized current decay as a function of time for Au-MEA electrodes functionalized with (A) H-3-Fc, (B) H-6-Fc, (C) H-15-Fc, and (D) H-32-Fc upon exposure to 3% human serum (HS)

and 3% HS spiked with 11.03 nM cathepsin B (CB). H-3-Fc, H-6-Fc, and H-15-Fc exhibit a pronounced initial rapid decay followed by a slower phase, whereas H-32-Fc shows a more gradual decay profile. Enhanced signal decay in CB-spiked samples confirms effective CB activity in serum. Solid lines represent double-exponential fits used for kinetic modeling.

Depending on the fitting results,  $1/\tau_1$  and  $1/\tau_2$  values were calculated as shown in **Table 4.1**, and corresponding values are shown in a grouped column graph (**Fig. 4.3A** and **Fig. 4.3B**) showcasing apparent proteolytic activity in HS, CB-spiked HS, and the corresponding activity difference.

**Table 4.1** Calculated  $1/\tau_1$  and  $1/\tau_2$  values for 3% HS added and CB spiked 3% HS added proteolysis decay curves.

| Peptide                   | Fast decay component<br>$1/\tau_1$ ( $s^{-1}$ ) | Slow decay component<br>$1/\tau_2$ ( $s^{-1}$ ) |
|---------------------------|-------------------------------------------------|-------------------------------------------------|
| H-3-Fc (3% HS)            | $(1.21 \pm 0.06) \times 10^{-2}$                | $(6.15 \pm 0.04) \times 10^{-4}$                |
| H-3-Fc (3% HS+CB spiked)  | $(2.37 \pm 0.05) \times 10^{-2}$                | $(7.08 \pm 0.07) \times 10^{-4}$                |
| H-6-Fc (3% HS)            | $(2.18 \pm 0.08) \times 10^{-2}$                | $(8.91 \pm 0.06) \times 10^{-4}$                |
| H-6-Fc (3% HS+CB spiked)  | $(2.97 \pm 0.14) \times 10^{-2}$                | $(9.49 \pm 0.04) \times 10^{-4}$                |
| H-15-Fc (3% HS)           | $(1.71 \pm 0.15) \times 10^{-2}$                | $(7.07 \pm 0.03) \times 10^{-4}$                |
| H-15-Fc (3% HS+CB spiked) | $(2.09 \pm 0.25) \times 10^{-2}$                | $(7.82 \pm 0.02) \times 10^{-4}$                |
| H-32-Fc (3% HS)           | $(1.03 \pm 0.04) \times 10^{-2}$                | $(5.62 \pm 0.36) \times 10^{-4}$                |
| H-32-Fc (3% HS+CB spiked) | $(1.24 \pm 0.12) \times 10^{-2}$                | $(6.59 \pm 0.39) \times 10^{-4}$                |

The rapid initial decay observed in H-3-Fc, H-6-Fc, and H-15-Fc likely arises from nonspecific proteolysis by multiple serum proteases, cleavage of highly accessible peptide substrates due to heterogeneity in surface coverage and packing density. Surface heterogeneity in thiolated monolayers is well documented, particularly for mixed or peptide-terminated SAMs<sup>30</sup>. Variations in molecular packing create domains with different steric accessibility and enzyme susceptibility. The slower decay component likely corresponds to proteolysis of peptides embedded within well-packed monolayer regions. Surface-confined enzymatic reactions frequently exhibit multi-phase kinetics due to restricted enzyme diffusion and substrate

heterogeneity<sup>166</sup>. For H-32-Fc, the absence of a strong fast component at the beginning upon addition of H.S suggests enhanced specificity toward CB and improved packing.

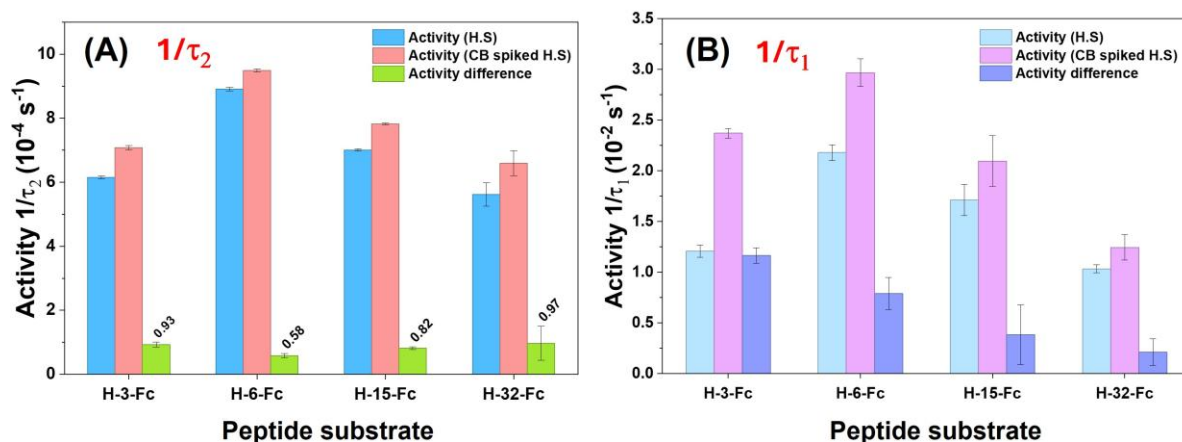


**Figure 4.3** Extracted kinetic parameters from double-exponential fitting of electrochemical decay profiles. (A) Slow decay component ( $1/\tau_2$ ) and (B) fast decay component ( $1/\tau_1$ ) obtained from double-exponential modeling of current decay curves for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc in 3% human serum (HS) and 3% HS spiked with 11.03 nM CB. Bars represent apparent proteolytic activity in HS, CB-spiked HS, and the corresponding activity difference.

According to **Fig. 4.3**, the slow decay component and the fast decay component show the same activity trend for HS and CB spiked HS added experiments with each peptide substrate. But the activity differences do not show a clear similar trend for slow and fast decay components. This confirms the complexity of the kinetics with complex biological samples or the difficulty of separating multi-phase kinetics due to the overlapping of time constants during the fitting process. These results highlight the importance of improving kinetic fitting strategies and developing more selective peptide substrates.

### 4.3.3 Linear response of the sensor platform to increasing Human serum concentration.

To evaluate the concentration-dependent behavior of the electrochemical protease sensor, Au-MEA electrodes functionalized with H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc were exposed to increasing concentrations of HS (0.5%, 1%, 3%, 5%, and 6%). For all peptide substrates, the magnitude and rate of current decay increased systematically with increasing serum concentration (**Fig. 4.4A, D, G, and J**), indicating enhanced overall proteolytic activity and/or possible matrix-driven interfacial effects at higher serum levels.

Consistent with previous observations, the decay profiles exhibited biphasic behavior and were best described using a double-exponential model as shown in **Equation 4.1**. The extracted kinetic constants  $1/\tau_1$  and  $1/\tau_2$  were then plotted as a function of serum concentration to assess linearity, as shown in **Fig. 4.4B, E, H, K**, and **Fig. 4.4C, F, I, L** respectively for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc. The slow decay component  $1/\tau_2$  exhibited a strong linear correlation with serum concentration for all peptide substrates, showing H-6-Fc:  $R^2 = 0.965$ , H-3-Fc:  $R^2 = 0.881$ , H-15-Fc:  $R^2 = 0.990$ , and H-32-Fc:  $R^2 = 0.92$ . This linear dependence strongly suggests that the slow decay component reflects concentration-dependent enzymatic turnover under stable interfacial conditions. The near-linear relationship indicates that, within the tested range (0.5-6%), the system operates below saturation and that effective protease activity scales proportionally with serum concentration. Importantly, the high  $R^2$  values for H-15-Fc and H-6-Fc ( $\geq 0.96$ ) indicate robust quantitative performance, while H-32-Fc maintains good linearity despite its more controlled decay behavior. Collectively, these results demonstrate that the slow decay constant is the most reliable kinetic descriptor for quantitative serum activity assessment if the double exponential model is used.

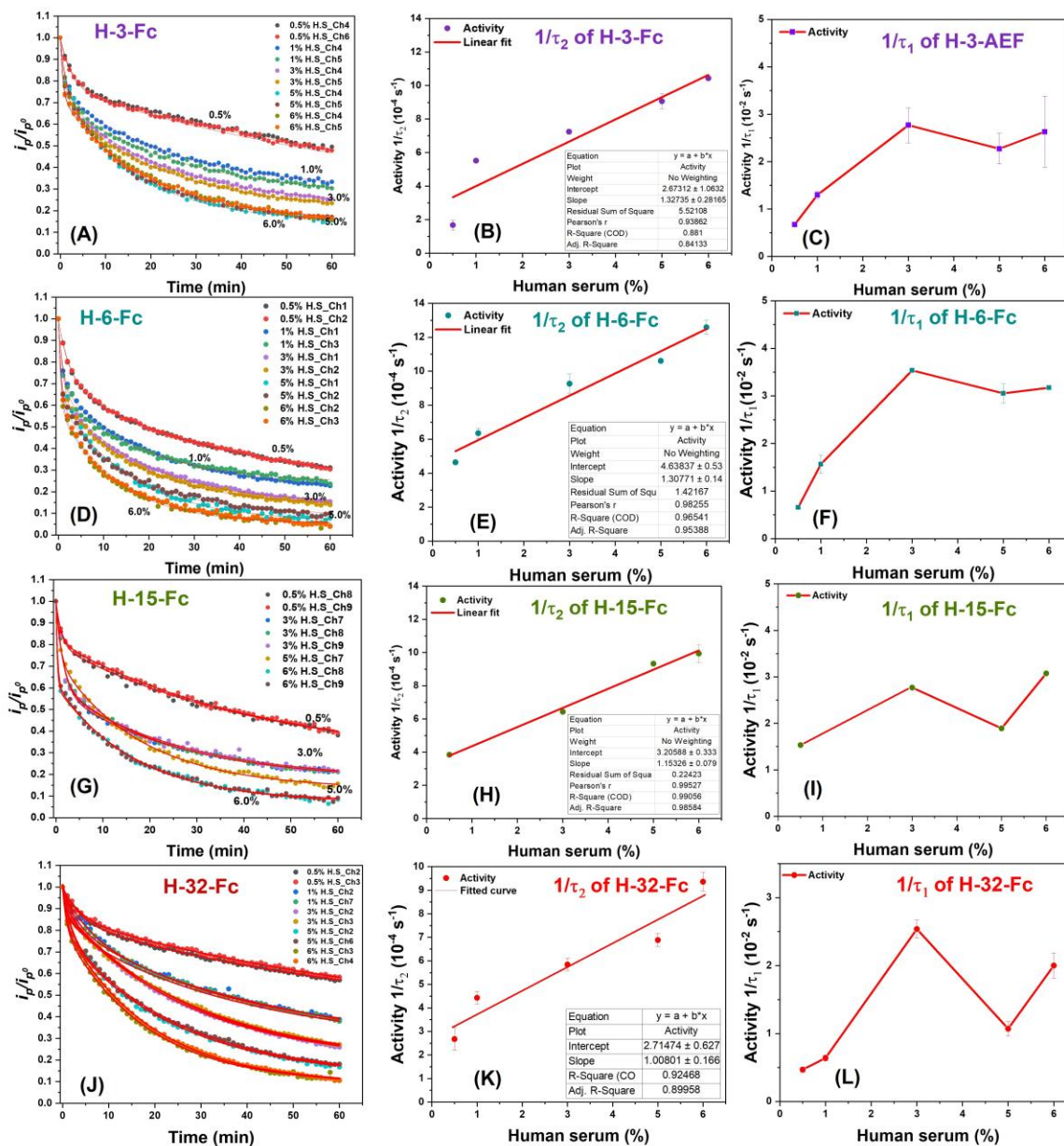


Figure 4.4 Linear response of peptide-functionalized Au-MEA sensors to increasing human serum concentration. (A, D, G, J) Normalized current decay profiles  $i_p/i_{p0}$  for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc, respectively, upon exposure to increasing human serum (HS) concentrations (0.5-6%). (B, E, H, K) Slow decay rate constants ( $1/\tau_2$ ) extracted from double-exponential fitting plotted against H.S concentration. (C, F, I, L) Fast decay rate constants ( $1/\tau_1$ ) as a function of H.S concentration.

In contrast, the fast decay component  $1/\tau_1$  did not exhibit a clear linear relationship with serum concentration. Instead, rapid increase at low concentrations (0.5-3%), partial saturation or plateau behavior at higher concentrations (5-6%) were observed. The fast component likely reflects cleavage of highly accessible or loosely packed peptide domains. These sites are rapidly depleted at higher enzyme concentrations, leading to apparent saturation behavior. As serum concentration increases,  $\tau_1$  and  $\tau_2$  may approach similar magnitudes, making mathematical separation less reliable. This overlap introduces fitting uncertainty, particularly in early time points, where small experimental variations significantly affect  $\tau_1$  extraction. At higher serum concentrations, multiple proteases and matrix components may contribute simultaneously, producing a fast decay response that does not scale linearly. Overall, the strong linearity confirms that the slow component  $1/\tau_2$  can serve as a quantitative metric for protease activity in complex biological matrices.

#### **4.3.4 Nonspecific decay due to other proteases like MMP-9 and ADAM-17 in Human serum**

To evaluate potential sources of nonspecific signal decay, proteolysis experiments were performed using two clinically relevant proteases: MMP-9 and ADAM-17. These studies were critical to distinguish and understand any nonspecific proteolysis that potentially contributes from H.S samples and causes initial rapid current decay.

##### **4.3.4.1 Possible interference to the Au/SAM platform from other activation agents**

Before investigating proteolytic decay, careful attention was given to enzyme activation strategies. In previous chapters (**Chapter 2**), the reducing agent DTT, used for CB activation, was found to interfere with the Au/SAM interface, particularly at elevated redox potentials. This observation raised serious concerns regarding the influence of activation reagents on monolayer stability and electrochemical readout integrity. Given that Au/thiol self-assembled monolayers are

highly sensitive to chemical perturbation, any reagent capable of altering surface chemistry could compromise kinetic interpretation.

ADAM-17 was obtained directly in its active form, eliminating the need for chemical activation. In contrast, MMP-9 is commonly supplied as a zymogen and requires activation. This can be achieved either by treatment with APMA (4-aminophenylmercuric acetate) or by purchasing pre-activated enzyme preparations in which APMA has been removed through purification (e.g., Bio-Gel P-6 column chromatography)<sup>41</sup>. In nature, these proteases are activated by cathepsins and furin-like proprotein convertases<sup>167</sup>. However, given our prior experience with DTT-induced surface disruption, it was essential to determine whether APMA could similarly affect the Au/SAM interface. APMA is known to contain a mercurial moiety capable of interacting with thiol groups, raising the possibility of direct interference with the gold-thiolate bond or alteration of monolayer packing. APMA is widely described as a mercurial thiol-reactive compound<sup>168</sup> used to activate pro-MMPs by attacking a cysteine thiol in the “cysteine switch”<sup>169</sup>. APMA, as an organomercurial compound, reacts with the sulfhydryl group of this cysteine residue. This interaction disrupts the cysteine-zinc coordination, leading to a conformational change in the enzyme. This change exposes the catalytic site, resulting in autolytic cleavage of the pro-domain and full activation of the MMP<sup>169</sup>. Such interactions could induce apparent current decay independent of enzymatic cleavage, thereby leading to misinterpretation of kinetic data. To rigorously assess this possibility, a carefully controlled experimental design was implemented. These experiments address a critical and often overlooked aspect of electrochemical biosensing: chemical compatibility between enzyme preparation protocols and surface-confined sensing interfaces.

#### 4.3.4.2 APMA interference to the Au/SAM platform

To systematically evaluate possible interference from APMA on the Au/SAM interface, a dedicated experiment was designed using a self-assembled monolayer system without peptide functionalization. A 2 mm diameter gold disk electrode was chemically and electrochemically cleaned as described in the methodology sections in previous chapters (**Chapter 3**). A mixed SAM was then formed by immersing the electrode for 12 h in an ethanolic solution containing 6-mercapto-1-hexanoic acid and 6-mercapto-1-hexanol in a 1:10 molar ratio. After thorough rinsing with ethanol and deionized water, the SAM-modified electrode was used for electrochemical characterization. To verify complete SAM coverage and the absence of pinholes, cyclic voltammetry (CV) was performed in a 1:1 mixture of  $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$  (1.0 mM total) prepared in 0.1 M  $\text{KNO}_3$  at 36 °C. The electrode was cycled between -0.45 V and 0.15 V vs. MSE at a scan rate of 100  $\text{mV s}^{-1}$ .

For a bare gold electrode, oxidation of  $\text{Fe}(\text{CN})_6^{4-}$  to  $\text{Fe}(\text{CN})_6^{3-}$  would typically produce an anodic peak near - 0.11 V vs. MSE, followed by a cathodic peak near - 0.21 V vs. MSE on the reverse scan. However, in the presence of an intact and densely packed SAM, electron transfer to the redox probe is suppressed, no well-defined redox peaks should be observed, and peak separation can be more pronounced.

As shown in **Fig. 4.5A** (black trace), no Ferri/ferrocyanide peaks were detected, confirming effective surface passivation and good monolayer integrity before APMA exposure.

The SAM-modified electrode was then immersed in 0.5X phosphate buffer containing 20  $\mu\text{M}$  APMA. Alternating current voltammetry (ACV) was performed between -0.4 V and 0.15 V vs. MSE to mimic the potential window typically used for ferrocene-based peptide measurements.

The AC amplitude was 50 mV at a frequency of 300 Hz. During the first two ACV scans, no distinct redox feature was observed. However, after cycling continued, a redox peak began to emerge near -0.3 V vs. MSE. Over the next ~12 ACV cycles, the peak current progressively increased (Fig. 4.5B), indicating the development of an electroactive process at the interface.

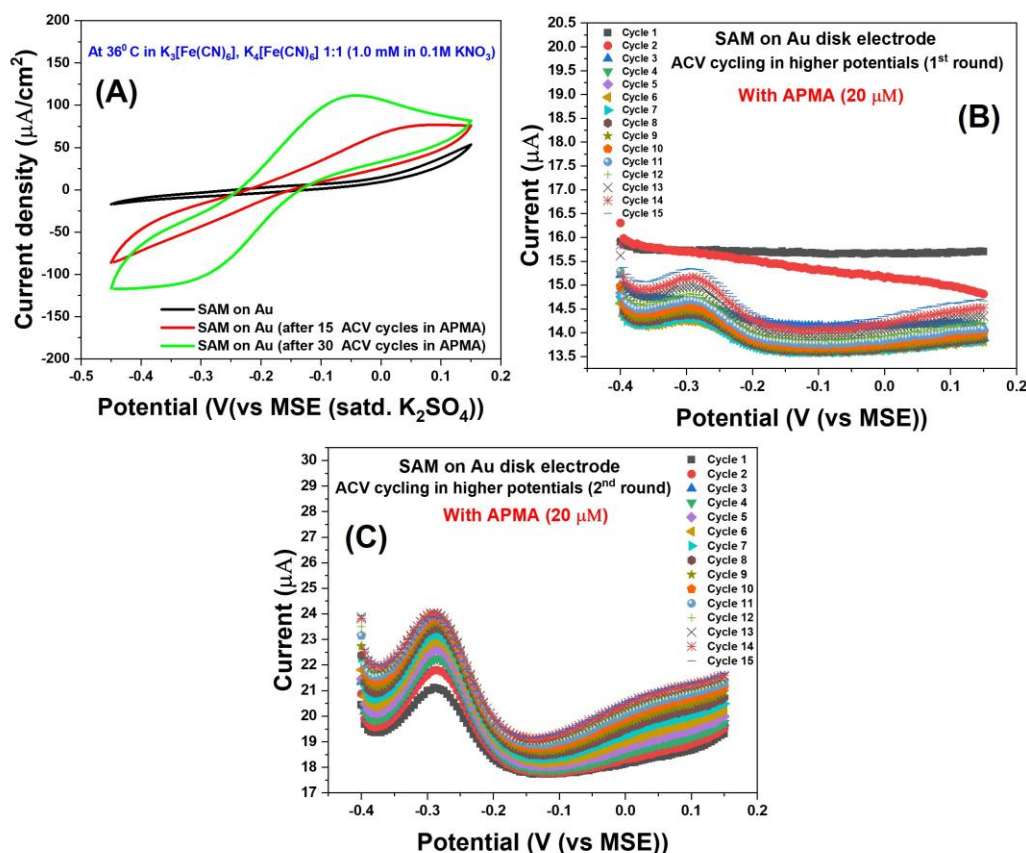


**Figure 4.5** Electrochemical evaluation of APMA-induced interference and SAM degradation on Au disk electrodes. (A) Cyclic voltammograms recorded in 1:1  $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$  (1.0 mM in 0.1 M  $KNO_3$ ) at 36 °C for a freshly prepared mixed SAM on a Au disk electrode (black trace), after 15 ACV cycles in 20  $\mu M$  APMA (red trace), and after 30 ACV cycles in APMA (green trace). The intact SAM suppresses Ferri/ferrocyanide redox peaks, confirming effective surface blocking. After exposure to APMA, progressively pronounced redox peaks appear, indicating loss of monolayer integrity and formation of pinholes. (B) Alternating current voltammograms (ACV) recorded during the first round of potential cycling (-0.4 to 0.15 V vs. MSE, 50 mV amplitude, 300 Hz) in 0.5X phosphate buffer containing 20  $\mu M$  APMA. An emerging redox feature near -0.3 V vs. MSE develops and increases over successive cycles, suggesting formation of electroactive sites upon APMA exposure. (C) ACV response during a second round of cycling in APMA following initial surface damage. A pronounced and continuously increasing redox peak at ~-0.3

V vs. MSE is observed over 15 cycles, consistent with progressive SAM degradation and enhanced exposure of the underlying gold surface.

Following APMA exposure, the electrode was thoroughly rinsed with deionized water and re-evaluated in the Ferri/ferrocyanide solution to assess monolayer integrity. In contrast to the initial measurement, clear redox peaks corresponding to  $\text{Fe}(\text{CN})_6^{3-/4-}$  were now observed (**Fig. 4.5A, red trace**), demonstrating partial loss of SAM blocking behavior and the formation of pinholes or defects.

To further evaluate this effect, the electrode was reintroduced into 0.5X phosphate buffer containing 20  $\mu\text{M}$  APMA and subjected to an additional 15 ACV cycles. A pronounced redox peak at approximately -0.3 V vs. MSE was again observed, with increasing peak current over successive scans (**Fig. 4.5C**).

After a final washing step, CV in Ferri/ferrocyanide solution revealed (**Fig. 4.5A, green trace**) an even more pronounced redox response, indicating further deterioration of the monolayer structure. To further confirm that the redox feature observed at approximately -0.3 V vs. MSE originated from APMA itself, a control experiment was performed using a clean (SAM-free) Au disk electrode.

Cyclic voltammetry was first carried out in 0.5X phosphate buffer (PB) by scanning the potential from -0.6 V to 0.15 V vs. MSE at a scan rate of 100  $\text{mV s}^{-1}$  (**Fig. 4.6, black trace**). As expected, no distinct faradaic peaks were observed apart from the water hydrolysis at lower potentials, confirming the absence of electroactive species in the supporting electrolyte within this potential window. The same experiment was then repeated in 0.5X PB containing 1 mM APMA (**Fig. 4.6, red trace**). In contrast to the blank measurement, a well-defined redox couple appeared with a formal potential ( $E_{1/2}$ ) centered at approximately -0.3 V vs. MSE. Notably, this potential

coincides with the peak observed during ACV measurements on SAM-modified electrodes exposed to APMA, indicating that the electrochemical signal arises directly from APMA.

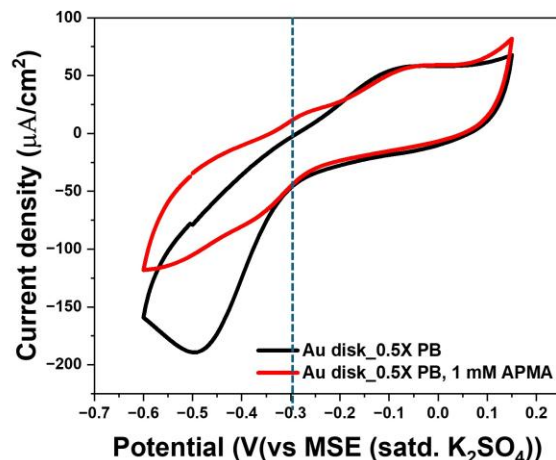


**Figure 4.6** Electrochemical response of APMA (1 mM) at a clean Au disk electrode. Cyclic voltammograms recorded at a clean Au disk electrode in 0.5X phosphate buffer (PB) (black trace) and in 0.5X PB containing 1 mM APMA (red trace), scanned from -0.6 V to 0.15 V vs. MSE at 100 mV s<sup>-1</sup>.

The redox potential observed here is also consistent with previously reported electrochemical responses of Hg-containing species on gold surfaces. For example, differential pulse voltammetry (DPV) studies of Hg<sup>2+</sup> on Au nanorod-modified electrodes show well-defined stripping peaks around 0.077 V vs. SCE<sup>170</sup>, which corresponds to approximately -0.33 V vs. MSE after reference conversion. This value closely matches the redox potential detected in our system.

Given that APMA (4-aminophenylmercuric acetate) contains a mercurial (Hg-aryl) moiety, it is highly plausible that under the applied potentials, APMA undergoes electrochemical transformation at the gold surface. The mercurial group may interact strongly with gold, leading to reduction processes and possible formation or deposition of metallic mercury (Hg<sup>0</sup>) or Au-Hg surface species.

Therefore, the data strongly suggest that APMA is electroactive on gold electrodes and can participate in surface reactions that modify or damage the Au/SAM interface. This finding is fundamentally important, as it demonstrates that the activation reagent commonly used for MMP-9 is not chemically inert toward gold electrodes. Any proteolysis experiments performed in the presence of residual APMA may therefore produce misleading electrochemical signals arising from mercury-gold interactions rather than true enzymatic cleavage. Therefore, it was decided to use purified activated MMP-9 samples to perform electrochemical proteolysis experiments.

#### **4.3.5 Validation of the activity of MMP-9 and ADAM-17 proteases**

To validate the enzymatic activity of MMP-9 and ADAM-17 before electrochemical measurements, fluorescence resonance energy transfer (FRET) based assays were performed using a commercially available peptide substrate Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub> (R&D Systems). This substrate is commonly used for activity screening of both MMP-9 and ADAM-17. It incorporates a fluorophore-quencher pair within the peptide sequence. The N-terminal 7-methoxycoumarin-4-acetic acid (Mca) serves as the fluorescent donor, while the 2,4-dinitrophenyl (Dpa) group acts as an efficient resonance energy transfer quencher<sup>171</sup>.

In the intact peptide, fluorescence from Mca is strongly suppressed due to intramolecular FRET to the Dpa group. Upon enzymatic cleavage of the peptide bond between Gly and Leu, the fluorophore and quencher are spatially separated, disrupting energy transfer and resulting in a measurable increase in fluorescence intensity. Protease activity was quantified by monitoring the increase in fluorescence emission at 405 nm following excitation at 295 nm. The rate of fluorescence increase directly correlates with enzymatic cleavage efficiency. All fluorescence measurements were performed using a StellarNet fluorescence spectrophotometer (Tampa, FL). This assay provided independent confirmation of protease activity before surface-based

electrochemical experiments, ensuring that any absence of electrochemical response could not be attributed to inactive enzyme preparations.

#### 4.3.5.1 MMP-9 activity

Inactive MMP-9 purchased from R&D Systems was activated according to the procedure described in the **Materials and Methods section 4.2.1**. Following activation, it was necessary to validate the activity of both APMA-removed and APMA-containing enzyme preparations to determine whether removal of the activation reagent affected enzymatic performance. Residual APMA was removed using 10k Amicon Ultra centrifugal filters using the method explained. Fluorescence measurements were conducted using a StellarNet fluorescence spectrophotometer.

To confirm the effective removal of APMA using the 10k Amicon Ultra centrifugal filter, analytical HPLC measurements were performed. First, a 1.29 mM APMA solution was injected (50  $\mu$ L) into the analytical HPLC system using a gradient elution method (0-95% acetonitrile over 60 min, flow rate 1.0 mL min<sup>-1</sup>). A well-defined peak was observed at a retention time of 19.6 min, corresponding to APMA (**Appendix Fig. C.1A**), establishing its chromatographic signature under the applied conditions.

To simulate the concentration typically present after MMP-9 activation, a 0.07 mM APMA solution (55  $\mu$ L) was prepared, and 50  $\mu$ L was injected into the HPLC system. As shown in **Appendix Fig. C.1B**, a clear peak at 19.6 min was again observed, with an intensity of approximately 700 mV, confirming detectability at this lower concentration. The same sample was then diluted to a total volume of 500  $\mu$ L and subjected to centrifugal filtration using a 10k Amicon Ultra filter (14,000 g, 5 min), reducing the volume to approximately 57  $\mu$ L. This filtrate was subsequently injected (50  $\mu$ L) into the HPLC under identical conditions. In this case, the APMA peak at 19.6 min was nearly eliminated, showing only a trace signal of approximately 35 mV as

shown in **Appendix Fig. C.1C**. The substantial reduction in peak intensity demonstrates efficient removal of APMA by centrifugal filtration, confirming that the 10k Amicon Ultra method effectively minimizes residual activation reagent in the enzyme preparation after two spin rounds.

FRET assays were performed to compare the activity of (i) activated MMP-9 (35.4 nM) with APMA present and (ii) activated MMP-9 (35.4 nM) after APMA removal. Measurements were carried out in both the original TCNB buffer (pH 7.5) and in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA, as shown in **Fig. 4.7**, which is the buffer used for electrochemical proteolysis experiments. According to the FRET results, there was no activity change in APMA removed and not removed samples in TCNB buffer, according to blue and green curves in **Fig. 4.7**. As shown in the teal color curve, the APMA not removed active MMP-9 sample showed 35% loss of its original activity in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA.

In addition to these preparations, a commercially available MMP-9 sample from MilliporeSigma was also evaluated. This enzyme was supplied in an already activated form, with APMA removed and purified using a Bio-Gel P-6 column. This sample was included to further compare enzymatic activity across different preparation methods and to use these samples in future experiments. The MMP-9 sample that was purified by the vendor showed comparable activity with in-house activated MMP-9, as shown by the black color curve in TCNB at pH 7.5, as shown in **Fig. 4.7**. When the same sample was evaluated in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA, the activity loss was around 35% approximately. Overall, there was no impact from using an APMA-containing or removed sample for the activity.

Since the FRET assay revealed approximately 35% loss of activity in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA compared to TCNB buffer (pH 7.5), it was necessary to determine whether EDTA was responsible for this reduction in enzymatic performance. To

investigate this, a 25 mM MES buffer (pH 7.3) was prepared without EDTA, and the FRET assay was repeated using the vendor-purified MMP-9 sample (35.4 nM), which had been supplied in activated form with APMA removed. Under these conditions, the enzyme exhibited activity, comparable to that observed in TCNB buffer at pH 7.5, as shown in the purple color curve in **Fig. 4.7**.

This result confirms that the reduced activity observed in the MES buffer containing EDTA was due to the presence of EDTA rather than pH or buffer composition. As MMP-9 is a matrix metalloproteinase, its catalytic activity depends on the presence of a catalytic  $\text{Zn}^{2+}$  ion. EDTA, being a strong metal chelating agent, can block essential metal ions and thereby inhibit enzyme activity. This may influence the intrinsic MMP-9 activity in electrochemical proteolysis data when it is performed in 25 mM MES buffer (pH 7.3) containing 50  $\mu\text{M}$  EDTA.

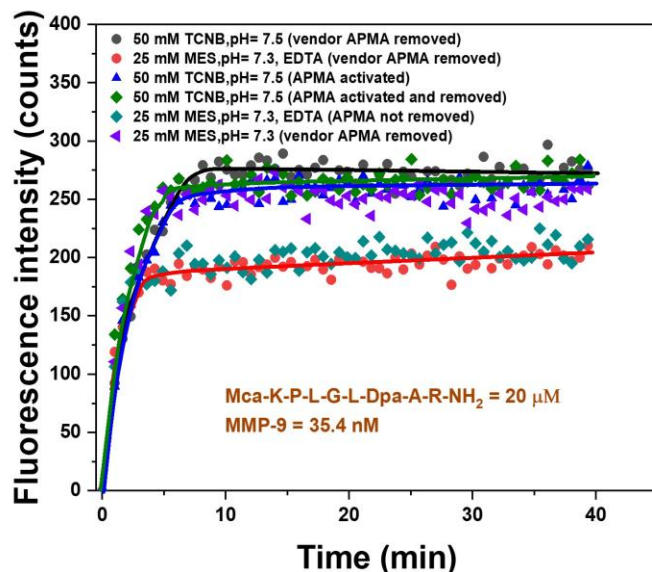


**Figure 4.7** FRET-based validation of MMP-9 activity under different activation and buffer conditions. Fluorescence intensity as a function of time for cleavage of the FRET peptide substrate (Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub>, 20  $\mu\text{M}$ ) by MMP-9 (35.4 nM) under various conditions. Measurements were performed in 50 mM TCNB buffer (pH 7.5) and 25 mM MES buffer (pH 7.3) with or without 50  $\mu\text{M}$  EDTA, using vendor-purified (APMA removed) MMP-9, APMA-activated MMP-9, and APMA-activated followed by removal in-house using a 10k Amicon Ultra filter.

Although the FRET assays performed using the StellarNet fluorescence spectrophotometer provided clear validation of MMP-9 activity under the standard conditions (20  $\mu$ M substrate and 35.4 nM enzyme), the fluorescence signal exhibited a saturation-like behavior after approximately 5 minutes of proteolysis. This plateau suggested that the reaction conditions may not have been optimized with respect to the substrate-to-enzyme ratio. To determine whether this apparent saturation resulted from rapid substrate depletion rather than loss of enzymatic activity, additional experiments were conducted using a higher substrate concentration (40  $\mu$ M) and varying concentrations of MMP-9 (2.0 nM, 4.0 nM, and 35.4 nM), as shown in **Appendix Fig. C.2**.

At the highest enzyme concentration (35.4 nM), the fluorescence signal increased rapidly and reached saturation within less than 10 minutes, consistent with fast substrate turnover. In contrast, experiments performed at lower enzyme concentrations (2.0 and 4.0 nM), corresponding to larger substrate to protease ratios, displayed a continuous and steady increase in fluorescence over the full 40 minutes measurement period without early plateau formation as shown in **Appendix Fig. C.2**. These results confirm that the initial saturation behavior was due to substrate limitation rather than enzyme instability or deactivation. Under conditions with an appropriate substrate to enzyme ratio, MMP-9 maintained sustained catalytic activity over extended time periods, demonstrating its long-term activity and suitability for subsequent electrochemical proteolysis experiments.

#### **4.3.5.2 ADAM-17 activity**

Active ADAM-17 purchased from R&D Systems was used after reconstitution to become the final concentration of 1923.08 nM as described in the **Materials and Methods section 4.2**. FRET assays were performed to compare the activity of active ADAM-17 protease. Measurements were carried out in both the original Tris buffer (pH 9.0) and in 25 mM MES buffer (pH 7.3)

containing 50  $\mu\text{M}$  EDTA, as shown in **Fig. 4.8**, which is the buffer used for electrochemical proteolysis experiments. FRET substrate Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub> (20  $\mu\text{M}$ ) and ADAM-17 (35.4 nM) were used. According to the FRET results, ADAM-17 in Tris buffer (pH = 9.0) showed higher activity according to the purple curve in **Fig. 4.8**. As shown in the teal color curve, ADAM-17 showed 36% loss of its original activity in 25 mM MES buffer (pH 7.3) containing 50  $\mu\text{M}$  EDTA.

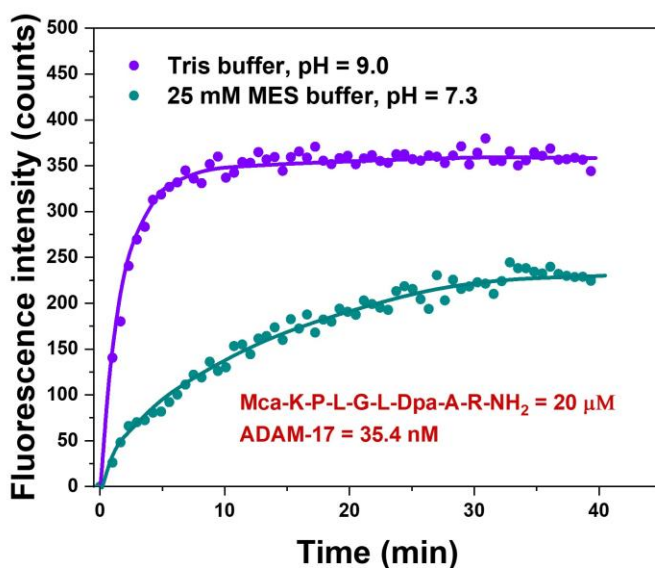


**Figure 4.8** Time-dependent fluorescence response of the peptide substrate (Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub>, 20  $\mu\text{M}$ ) upon cleavage by ADAM-17 (35.4 nM) under different buffer conditions. Reactions were carried out in Tris buffer (pH 9.0) and 25 mM MES buffer (pH 7.3). A significantly higher fluorescence intensity and faster signal saturation were observed in Tris buffer compared to MES buffer, indicating enhanced enzymatic activity and/or substrate turnover at alkaline pH.

#### **4.3.5.3 Optimization of FRET Assay Conditions for Long-Term Evaluation of MMP-9 and ADAM-17 Activity**

Initial measurements of MMP-9 and ADAM-17 activity using the StellarNet fluorescence system exhibited rapid signal saturation under the tested conditions, limiting the ability to evaluate long-term enzymatic activity. To overcome this limitation, the FRET-based assay was optimized using the ProMega fluorescence system, enabling continuous monitoring of protease activity over 40 minutes.

For the optimized ProMega assay conditions, MMP-9 activity was measured using 10 nM enzyme and 100  $\mu$ M substrate in a total reaction volume of 100  $\mu$ L (90  $\mu$ L substrate solution and 10  $\mu$ L enzyme solution). Similarly, ADAM-17 activity was evaluated using 5 nM enzyme and 100  $\mu$ M substrate under the same total reaction volume. Increasing the substrate to enzyme ratio allowed sustained fluorescence growth without early saturation, thereby enabling reliable assessment of long-term enzymatic performance. Fluorescence measurements were recorded at an excitation wavelength of 365 nm and an emission range of 410 - 460 nm.

As shown in **Fig. 4.9A**, MMP-9 exhibited continued enzymatic activity for over 40 minutes under the optimized conditions. However, when comparing fluorescence intensity at 40 minutes, a 45% reduction in activity was observed in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA relative to the original TCNB buffer (pH 7.5).

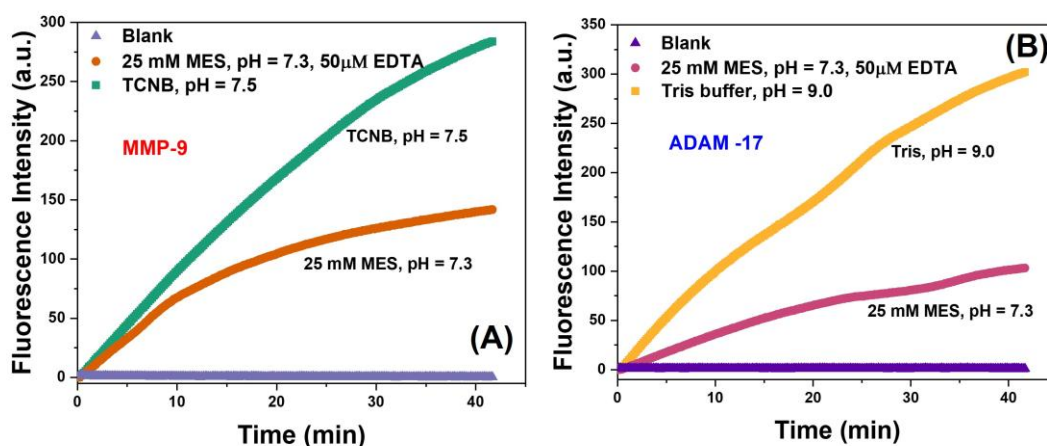


**Figure 4.9** (A) FRET-based long-term activity measurement of MMP-9 (10 nM) with 100  $\mu$ M substrate in a total volume of 100  $\mu$ L (90  $\mu$ L substrate + 10  $\mu$ L enzyme) using the ProMega system (Ex = 365 nm, Em = 410-460 nm). (B) FRET-based long-term activity measurement of ADAM-17 (5 nM) with 100  $\mu$ M substrate in a total volume of 100  $\mu$ L (90  $\mu$ L substrate + 10  $\mu$ L enzyme) using the ProMega system (Ex = 365 nm, Em = 410-460 nm).

Similarly, as shown in **Fig. 4.9B**, ADAM-17 demonstrated sustained activity under optimized conditions. Nevertheless, a 57% decrease in activity at 40 min was observed in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA compared to its original Tris buffer (pH 9.0).

These results indicate that buffer composition and the presence of EDTA significantly influence protease activity, even under conditions optimized for long-term monitoring. These findings highlight the importance of carefully selecting assay buffers during enzyme validation and biosensor development to avoid underestimation of protease activity.

#### 4.3.6 Electrochemical proteolysis by MMP-9 and ADAM-17 using Au MEA

To further evaluate potential cross-reactivity on the Au MEA platform, electrochemical proteolysis experiments were performed using four peptide substrates, H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc, functionalized on the Au MEA surface. Measurements were conducted in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA at 36 °C. After confirming that APMA interferes with the Au/SAM interface, MMP-9 was activated separately, followed by the removal of APMA before electrochemical measurements. Active 30.2 nM MMP-9 and 98 nM ADAM-17 were introduced to the system.

Higher concentrations of these proteases were intentionally employed to assess the possibility of detectable proteolysis under conditions where EDTA was present, as EDTA was previously shown to potentially suppress metalloprotease activity through metal chelation. Despite this inhibitory environment, measurable electrochemical decay was observed for H-3-Fc, H-6-Fc, and H-15-Fc upon addition of both MMP-9 and ADAM-17 (**Fig. 4.10A** and **4.10B**). This indicates that both proteases retain partial catalytic activity even in the presence of EDTA.

In contrast, the Nle-containing H-32-Fc peptide exhibited negligible decay with either MMP-9 or ADAM-17 under identical conditions. **Fig. 4.10C** shows the improved cleavage of H-32-Fc by CB, compared to other peptide substrates at pH = 7.3. The absence of electrochemical signal loss suggests that this substrate is not susceptible to cleavage by these metalloproteases, reinforcing its enhanced specificity toward CB. This observation is particularly significant, as it demonstrates that rational peptide modification can effectively suppress nonspecific proteolysis in complex multiplex detection settings.

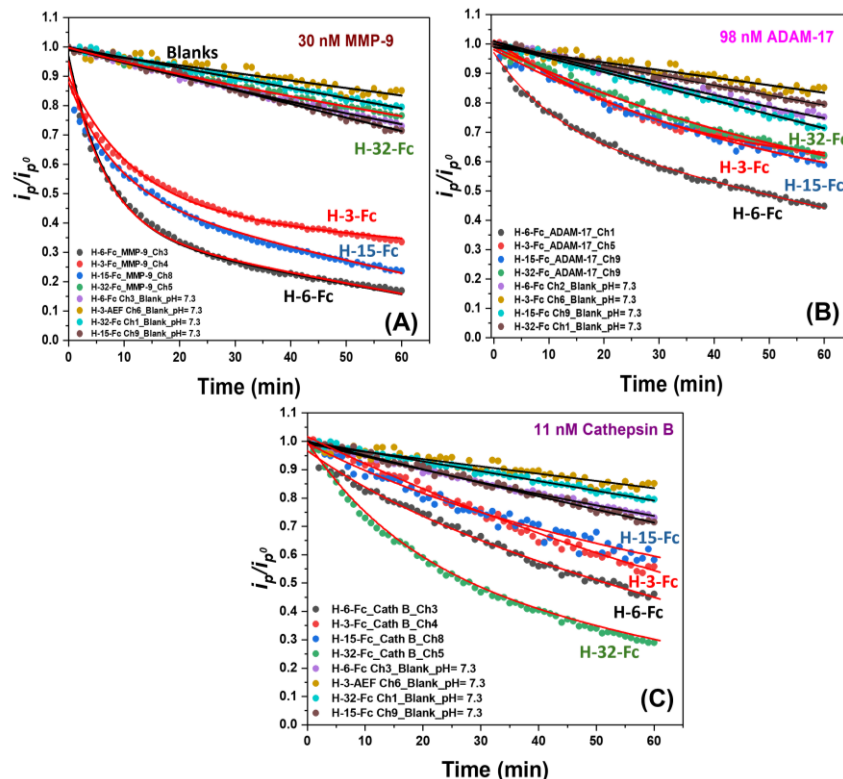


**Figure 4.10** Electrochemical proteolysis of surface-immobilized peptide substrates by MMP-9, ADAM-17 and cathepsin B on the Au MEA platform. Normalized peak current ( $i_p/i_{p0}$ ) versus time obtained by ACV measurements for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc functionalized on Au MEAs in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA at 36  $^{\circ}$ C. (A) Response after addition of 30 nM active MMP-9 (APMA removed before measurement). (B) Response after addition of 98 nM active ADAM-17. (C) Response after addition of 11 nM active cathepsin B.

The decay profiles observed for H-3-Fc, H-6-Fc, and H-15-Fc followed time-dependent signal reduction consistent with proteolytic cleavage of the surface immobilized substrates. Compared to blank measurements, the protease-treated electrodes showed substantially greater current loss, confirming that the decay originated from enzymatic activity rather than surface instability. Notably, MMP-9 produced pronounced decay at 30.2 nM despite the presence of EDTA, whereas ADAM-17 required a higher concentration (98 nM) to achieve comparable signal reduction, indicating differences in catalytic efficiency and substrate preference under these assay conditions. The calculated  $1/\tau$  values for MMP-9 and ADAM-17 proteolysis are presented as

grouped column charts for the peptide substrates H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc in **Fig. 4.11A** and **Fig. 4.11B**, respectively.

As shown in **Fig. 4.11A**, the H-6-Fc peptide substrate exhibited the highest proteolysis rate toward MMP-9, followed by H-15-Fc and H-3-Fc in decreasing order of activity. In contrast, H-32-Fc showed negligible decay, indicating minimal or no significant proteolysis by MMP-9 under the tested conditions.

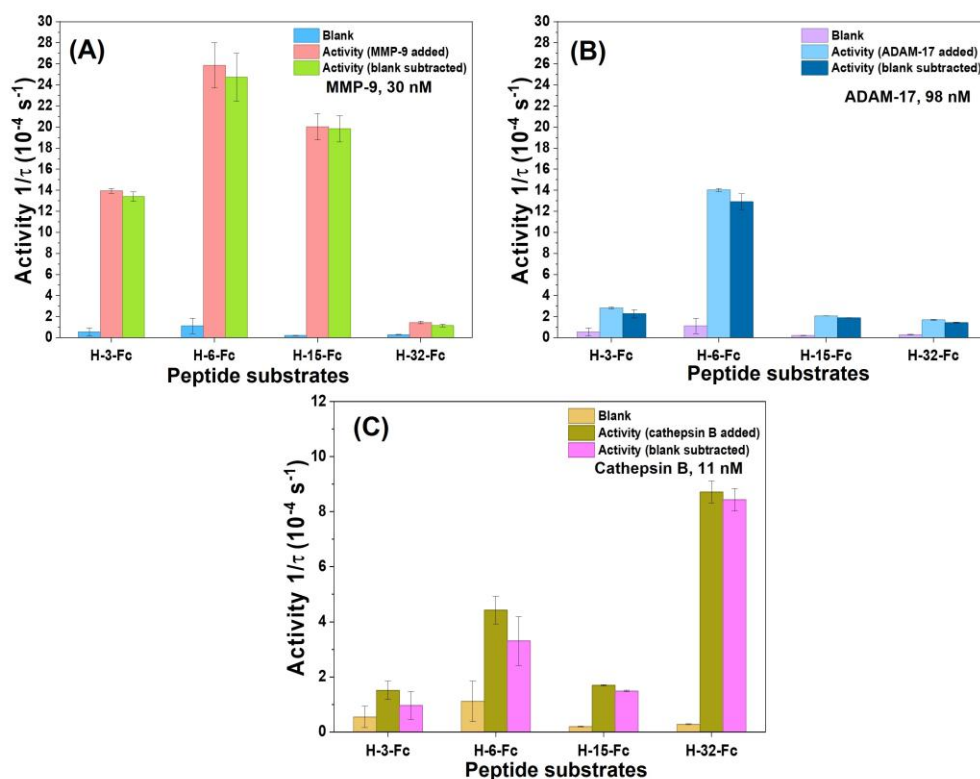


**Figure 4.11** Proteolytic activity of MMP-9 (A), ADAM-17 (B), and cathepsin B (C) toward peptide substrates H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc, expressed as the calculated  $1/\tau$  values ( $10^{-4} \text{ s}^{-1}$ ) obtained from electrochemical decay measurements. Bars represent the blank response, activity in the presence of the protease, and blank-subtracted activity for each peptide substrate.

As shown in **Fig. 4.11B**, H-6-Fc also displayed a measurable decay rate in the presence of ADAM-17 (98 nM). However, H-3-Fc, H-15-Fc, and H-32-Fc exhibited very low and nearly identical decay rates, suggesting that the observed signal changes are unlikely to arise from true

proteolytic activity. Instead, these small responses may be attributed to matrix effects associated with the relatively high concentration and volume of rhADAM-17 introduced into the electrochemical cell. From **Fig. 4.11C**, it can be observed that H-32-Fc showed considerably enhanced activity with cathepsin B at pH = 7.3 compared to other peptide substrates.

To identify the cleavage sites responsible for the observed electrochemical decay, solution-phase proteolysis experiments were performed. Each peptide (1 mM) was incubated with 15 nM MMP-9 in TCNB buffer (pH 7.5) and with 30 nM ADAM-17 in Tris buffer (pH 9.0). Aliquots collected at initial and 2-hour time points (and additionally at 20 hours for ADAM-17) were analyzed by analytical HPLC using a 0-95% acetonitrile gradient over 60 minutes at a 1 mL/min flow rate. The resulting chromatograms are listed in **Appendix C.4** for MMP-9 and ADAM-17, respectively. Newly emerged peaks are marked and described in respective chromatograms starting from H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc peptide substrates for each protease. Newly emerged peaks corresponding to cleavage fragments were collected and subjected to high-resolution mass spectrometry (HRMS) for structural confirmation. The HRMS data are provided in **Appendix C.5** for MMP-9 and **Appendix C.6** for ADAM-17 proteolysis data, and the summarized cleavage sites are presented in **Table 4.2** along with cathepsin B cleavage data for direct comparison. The molecular weight confirmations for respective cleavage sites are described on relevant HRMS spectra in **Appendix C.5** and **C.6**.

**Table 4.2** Different peptide substrates used in this study and their cleavage sites with different proteases.

| Peptide | Protease    | Sequence and cleavage site (between highlighted residues)                                                                                   |
|---------|-------------|---------------------------------------------------------------------------------------------------------------------------------------------|
| H-3-Fc  | MMP-9       | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Arg-Phe-Gly-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-6-Fc  | MMP-9       | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Gly-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-15-Fc | MMP-9       | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Ala-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-32-Fc | MMP-9       | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO-Nle- <i>Leu-Gly-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc (no cleavage) |
| H-3-Fc  | ADAM-17     | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Arg-Phe-Gly-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-6-Fc  | ADAM-17     | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Gly-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-15-Fc | ADAM-17     | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Ala-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc (no cleavage) |
| H-32-Fc | ADAM-17     | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO-Nle- <i>Leu-Gly-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc (no cleavage) |
| H-3-Fc  | Cathepsin B | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Arg-Phe-Gly-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-6-Fc  | Cathepsin B | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Gly-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-15-Fc | Cathepsin B | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO- <i>Pro-Leu-Ala-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |
| H-32-Fc | Cathepsin B | H <sub>2</sub> N-(CH <sub>2</sub> ) <sub>4</sub> -CO-Nle- <i>Leu-Gly-Phe-Val-Ala</i> -NH-CH <sub>2</sub> -CH <sub>2</sub> -Fc               |

The combined electrochemical and solution phase analyses confirm that while H-3-Fc and H-6-Fc contain sequences susceptible to cleavage by MMP-9 and ADAM-17 and H-15-Fc is not cleaved by ADAM-17 protease. H-32-Fc substrate resists hydrolysis by these metalloproteases. This selective resistance supports its suitability for CB-specific detection in multiplex platforms. Overall, these findings demonstrate that metalloprotease cross-reactivity can be systematically evaluated and minimized through rational substrate engineering, strengthening the reliability of the Au MEA system for selective protease sensing in complex biological environments. This nonspecific decay confirms the larger initial decay observed upon addition of HS samples.

## 4.4 Conclusion

Electrochemical monitoring of peptide-functionalized Au-MEA electrodes revealed biphasic decay behavior in human serum, which was best described using a double-exponential kinetic model. The fast decay component likely originates from cleavage of highly accessible peptide sites or nonspecific serum proteases, while the slow component reflects enzymatic activity under more stable surface conditions. Among the tested substrates, H-32-Fc exhibited improved resistance to rapid nonspecific decay, indicating enhanced specificity.

Further investigation into possible interference from metalloproteases demonstrated that MMP-9 and ADAM-17 can cleave several peptide substrates, particularly H-3-Fc, H-6-Fc, and H-15-Fc, contributing to nonspecific signal decay in complex samples. Importantly, H-32-Fc showed negligible cleavage by both MMP-9 and ADAM-17, while maintaining strong responsiveness toward cathepsin B (CB). Complementary solution-phase proteolysis experiments and HRMS analysis confirmed the absence of metalloprotease cleavage sites in H-32-Fc.

Overall, these results demonstrate that rational peptide design can significantly improve protease selectivity and identify H-32-Fc as a highly specific substrate for CB detection. This work highlights the capability of the Au-MEA electrochemical platform for selective protease sensing in complex biological matrices and provides important insights into minimizing nonspecific decay and cross-reactivity in multiplex biosensing applications.

## **Chapter 5 - Conclusion and Future Perspective**

In this dissertation, a gold microelectrode array was employed as a platform for the development of an electrochemical biosensor targeting cancer-associated proteases. This work is motivated by the growing need for reliable point-of-care diagnostic tools capable of detecting cancer at early stages, thereby improving treatment outcomes and reducing overall healthcare costs. The findings presented here not only contribute to the advancement of protease-based biosensing strategies but also provide a foundation for future device miniaturization and multiplexed detection using microelectrode array architectures.

A central outcome of this research is the identification of critical interference mechanisms that can arise during sensor validation, particularly in the detection of cathepsin B and MMP-9. These interferences originated from factors such as enzyme activation reagents (e.g., DTT and APMA), buffer components (e.g., EDTA), and unintended electrochemical reactions at the Au-thiol interface. Such effects can generate misleading electrochemical signals, induce monolayer degradation, or suppress enzymatic activity, ultimately compromising data interpretation. These observations highlight an important but often underappreciated challenge in practical biosensor development: ensuring chemical compatibility between biological reagents, activation protocols, buffer systems, and the electrode interface.

### **5.1 Study I: Overcoming Activation-Agent Interference in Cathepsin B**

#### **Detection**

In the first study, substrate-dependent proteolysis of CB was successfully demonstrated using a 3×3 Au MEA platform while resolving persistent interference issues caused by the activation agent DTT. By optimizing enzyme activation protocols (DTT removal after activation) and incorporating EDTA to maintain its active form, the proteolytic activity of CB was accurately

evaluated across multiple peptide substrates. The essential roles of DTT and EDTA in CB activation were validated through both fluorogenic assays and electrochemical measurements.

A key finding of this study was the identification of DTT-induced interference with Au-S monolayers, particularly at higher operating potential. Fc-tagged peptide substrates immobilized via thiol chemistry were susceptible to disruption during Fc redox cycling when residual DTT was present. At lower potentials, such as those employed with Methylene blue-based tags, the interference was minimized. These results demonstrate that careful control of enzyme activation chemistry and electrochemical operating potential is essential for reliable Au-S-based biosensor performance. More broadly, this study reveals a major technical barrier in thiol-based electrochemical biosensing and provides practical strategies for developing interference resistant platforms.

## **5.2 Study II: Rational Peptide Engineering for Enhanced Cathepsin B**

### **Detection at Physiological pH**

The second study focused on improving CB detection sensitivity under physiologically relevant conditions. An optimized peptide substrate,  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Nle-Leu-Gly-Phe-Val-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ , was developed for electrochemical detection using the Au MEA platform. Through systematic evaluation of fluorogenic substrates and confirmation of sequence-dependent cleavage, it was established that substitution of norleucine (Nle) at the P3 position significantly enhanced CB activity at neutral pH.

This minimal site-directed modification strategy resulted in a low limit of detection of  $0.43 \pm 0.09$  nM for CB. These findings not only validate the well-established pH dependence of CB activity but also extend prior work by providing direct electrochemical evidence supporting rational substrate design. Importantly, the selective enhancement achieved through a single residue

substitution demonstrates the power of targeted sequence engineering to improve both sensitivity and specificity. The optimized substrates therefore provide a strong foundation for future multiplexed biosensing applications targeting extracellular proteases in clinical matrices.

### **5.3 Study III: Human Serum Matrix Effects and Protease Cross-Reactivity on the Au-MEA Platform**

The third study examined the impact of HS as a complex biological matrix on the Au MEA platform and evaluated potential cross-reactivity from MMP-9 and ADAM-17 during CB detection. Introduction of 3% HS, with and without spiked CB, resulted in a pronounced initial signal decay across most peptide-modified electrodes. This rapid decay was attributed to surface heterogeneity and nonspecific proteolysis arising from limited substrate specificity within the complex matrix.

Kinetic analysis revealed that a single exponential model was insufficient to describe the decay behavior, whereas a double exponential function more accurately captured the two distinct processes. The slower decay component exhibited a linear dependence on serum concentration, suggesting a protease-related contribution, while the faster decay component saturated at higher concentrations, indicating a matrix or surface-driven heterogeneity effect. Among the tested substrates, the Nle-containing H-32-Fc peptide consistently demonstrated the lowest initial decay, highlighting its improved resistance to nonspecific cleavage and enhanced selectivity.

Further investigation into protease cross-reactivity revealed that the activation agent APMA, used for MMP-9 activation, disrupted the Au-thiol self-assembled monolayer and introduced electrochemical interference. Importantly, removal of APMA following activation did not compromise enzymatic activity, allowing the use of purified MMP-9 in subsequent studies. Additionally, EDTA was found to significantly suppress metalloprotease activity. While this

inhibitory effect is beneficial for selective CB detection, it presents challenges for multiplexed assays requiring a unified buffer system.

Electrochemical proteolysis experiments demonstrated that MMP-9 retained measurable activity even in EDTA-containing buffer, whereas ADAM-17 exhibited minimal activity under similar conditions. Notably, the Nle modified peptide showed no detectable cleavage by either MMP-9 or ADAM-17, confirming its high specificity toward CB. Collectively, these findings emphasize that matrix effects, activation reagents, and buffer composition critically influence electrochemical protease sensing. More importantly, they demonstrate that rational peptide engineering, particularly targeted residue modification, can significantly enhance specificity and minimize cross-reactivity. This study establishes a framework for developing selective, multiplex-capable electrochemical protease sensors suitable for complex biological environments.

#### **5.4 Design Principles Need to be Considered in the Future**

- 1) Activation reagents such as DTT and APMA can introduce significant interference in Au/ thiol-based systems (DTT in relatively higher potentials, APMA- regardless of the potential window).
- 2) Chelating agents like EDTA may suppress metalloprotease activity.
- 3) Operating potential selection plays an important role in minimizing surface disruption.
- 4) Rational peptide engineering can significantly enhance both sensitivity and specificity.

#### **5.5 Future Perspectives**

Although complex biological samples were not comprehensively analyzed in this study, the results provide a strong foundation for future validation in matrices such as human serum.

Further investigation of matrix effects, nonspecific decay, and long-term monolayer stability will be essential for clinical translation.

Future work will focus on several key directions:

- 1) **Redox Tag Optimization:** Preliminary synthesis and characterization of methylene blue-derived redox tag (with an -NH<sub>2</sub> terminal) was conducted to shift the operating potential to lower, more stable regions. Although the initial compound exhibited limited stability due to still overlapping with a higher potential region, ongoing efforts aim to develop more robust derivatives with improved electrochemical durability and minimized monolayer perturbation. By applying this, it should be able to completely get rid of DTT-like interference and minimize the interference from APMA-like activation agents. This may also help to remove EDTA-like metal chelating agents during sensor validation studies with CB enzyme and help to detect intrinsic metalloprotease activity.
- 2) **Enhanced Substrate Design Using Computational Tools:** Integration of MEROPS database analysis (protease-substrate database) with AI-assisted peptide design will facilitate systematic identification of protease-specific cleavage motifs and final binding analysis with molecular docking studies. Such approaches will enable the rational construction of highly selective substrates for multiplex profiling.
- 3) **Multiplex and Miniaturized Platforms:** The Au MEA provides inherent capability for parallel functionalization with distinct peptide substrates with an

increased number of microelectrodes, enabling simultaneous detection of multiple proteases such as CB, MMP-9, and ADAM-17. Coupled with multivariate data analysis and pattern recognition, this strategy could support advanced diagnostic profiling, even though it has considerable cross-reactivity in peptide substrates.

- 4) **Electrochemical Proteolysis Kinetics Extraction:** Further electrochemical studies will quantify decay kinetics and differentiate between enzymatic cleavage, monolayer degradation, and matrix-induced signal suppression using improved and advanced fitting functions. A well-trained AI platform can be integrated to automate data analysis and predictions about the state of the cancer.

## 5.6 Final Remarks

In conclusion, this dissertation not only advances the development of Au MEA-based electrochemical protease biosensors but also reveals one of the fundamental technical barriers associated with Au-S chemistry in enzyme-based sensing. By systematically identifying and mitigating activation-agent interference, optimizing peptide substrate design, and establishing robust validation protocols, this work provides a clear pathway toward interference-resistant, multiplex-capable biosensing platforms. The principles established here contribute to the rational design of next-generation electrochemical diagnostics for early disease detection and therapeutic monitoring across a wide range of protease-mediated pathologies.

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## Appendix A - Supplementary Information for Chapter 2

### A.1 Materials and Instruments

The peptide sequences (H-3:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Arg-Phe-Gly-Ala-COOH}$ , H-6:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Gly-Phe-Val-Ala-COOH}$ , and H-15:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Ala-Phe-Val-Ala-COOH}$ ) were purchased from Biomatik USA LLC (Wilmington, DE). Aminomethylferrocene (AMF) and (2-aminoethyl)ferrocene redox tags were synthesized in D. H. Hua's laboratory at Kansas State University. The synthetic procedure for attaching the amine-terminated ferrocene redox tag to the substrate peptides and the characterization of the peptide sequence are described in later sections. Monocarboxymethylene was purchased from emp BIOTECH LLC (Manalapan, NJ), and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) was bought from Chem-Impex International, Inc. (Wood Dale, IL). *N*-Hydroxysulfosuccinimide (Sulfo-NHS), 6-mercapto-1-hexanol, 6-mercapto-1-hexanoic acid, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate dibasic heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ), sodium phosphate monobasic monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ), dithiothreitol, and 2-(4-morpholino)ethanesulfonic acid (MES) were purchased from Fisher Scientific (Hampton, NH). The 1X phosphate buffer (PB) was prepared as 7.17 mM potassium phosphate dibasic and 4.63 mM potassium phosphate monobasic (pH = 7.4, adjusted using potassium hydroxide) purchased from Fisher Scientific (Fair Lawn, NJ) in deionized water. This was modified from the standard phosphate buffer saline (PBS) by removing other salts to lower the ionic strength. The 0.5X PB was prepared by a 2-fold dilution of 1X PB with deionized water to further lower the ionic strength. Carrier-free recombinant human cathepsin B (rhCB) (~60% 37 kDa proenzyme, ~40% 29 kDa mature enzyme), cathepsin B specific fluorogenic peptide substrate (Z-Leu-Arg-

Mca), and human total cathepsin B were acquired from R&D Systems Inc. (Minneapolis, MN). Cathepsin B solutions were activated in 25 mM MES buffer containing 1 mM or 5 mM DTT for 15 min to activate the proenzyme.

Cathepsin B enzyme activity was validated using a fluorogenic assay with a GloMax Multi+ Microplate Multimode reader (Promega, Madison, WI) in 96-well plates. Electrochemical measurements were carried out using an IVIUM n-Stat potentiostat (Eindhoven, The Netherlands), which allowed for simultaneous measurement of up to 10 independent working electrodes with a shared mercury mercurous sulfate, saturated K<sub>2</sub>SO<sub>4</sub> (MSE) reference electrode, and a common platinum coil counter electrode.

## **A.2 Design and selection of peptide-Fc substrates for cathepsin B**

We have designed, synthesized, and tested a small library of peptide-Fc substrates, each consisting of 6-8 amino acids, in previous studies, to be cleaved by cathepsin B based on analyses of specific recognition sequences reported in the literature. The activity of cathepsin B on these peptide-Fc substrates was evaluated using a combination of electrochemical measurements (employing Au disk electrodes and VACNF nanoelectrode arrays), fluorogenic assays, and HPLC-MS analysis. From studies of a library of substrate peptides, three cathepsin B-selective peptides H-3, H-6, and H-15 were chosen from the library for further study due to their higher proteolysis rates by cathepsin B compared to other candidates after tagging them with (2-aminoethyl)ferrocene (AEF) redox reporter which found to be more stable than previously used (aminomethyl)ferrocene (AMF). The cleavage sites, identified in our previous reported studies, are highlighted in yellow and cyan for reference.

**Appendix Table A.1** Peptide sequences screened for cathepsin B in this study and their cleavage sites highlighted.

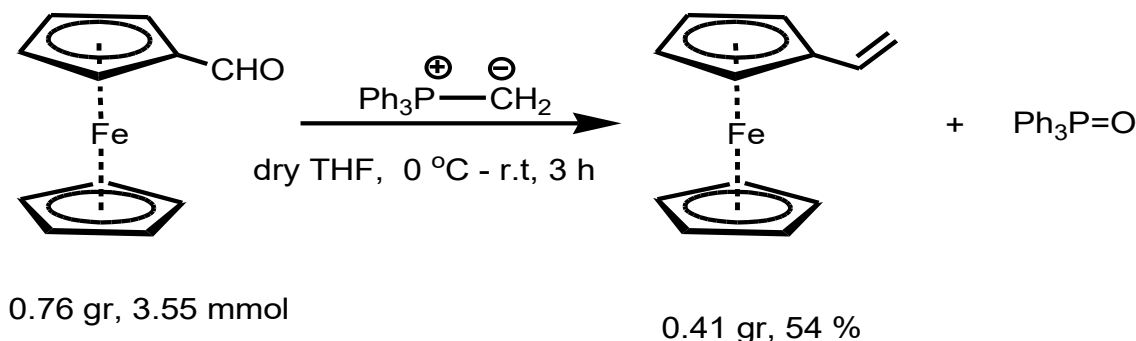
| Substrate | Peptide substrate sequence                                                                                                                                   |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| H-3-AEF   | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Arg}-\text{Phe}-\text{Gly}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |
| H-6-AEF   | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |
| H-15-AEF  | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Ala}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |

### A.3 Peptide Synthesis and Characterization

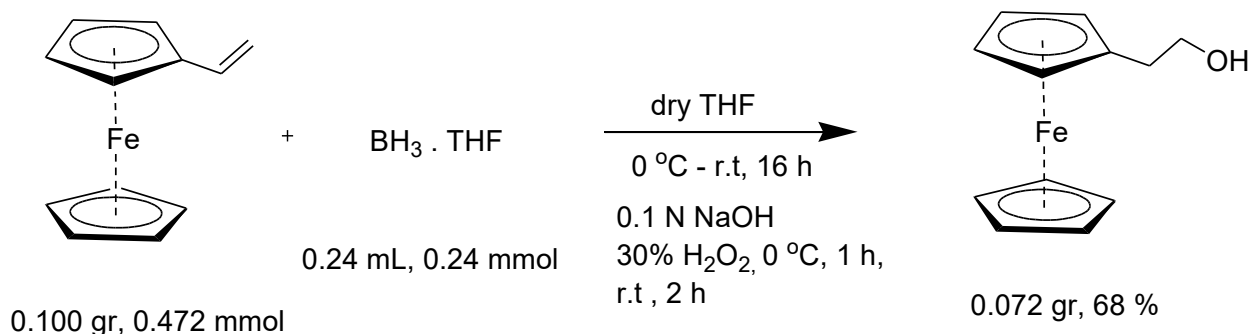
- Synthesis protocol for (2-aminoethyl) ferrocene (AEF), a previously unreported molecule. The synthesis was carried out in a five-step sequence starting from commercially available ferrocenecarboxaldehyde.

**a. Ethenylferrocene.** A 250-mL dried flask containing 1.79 g (5.01 mmol) of methyltriphenylphosphonium bromide was dried under vacuum and maintained under argon. To it, 20 mL of dried THF was added, and the reaction mixture was cooled to  $-20^\circ\text{C}$  over a dry ice/acetone bath. Then, 5.0 mL (6.0 mmol, 1.2 eq) of 1.2 M *n*-BuLi in hexane was added. The mixture was stirred at  $-20^\circ\text{C}$  for 2 h, and the resulting solution was allowed to warm to  $0^\circ\text{C}$ . To it, a solution of 0.76 g (3.55 mmol) of ferrocenecarboxaldehyde in 10 mL of dry THF was injected into the flask via a cannula, then the reaction solution was stirred at  $25^\circ\text{C}$  for 3 h. A small sample from the reaction mixture was taken and analyzed by  $^1\text{H}$ -NMR spectroscopy, which indicated that the formation of the desired product with no starting material presence. To the mixture, an aqueous  $\text{NH}_4\text{Cl}$  solution was added and extracted with ethyl acetate (five times, with 40 mL each time). The organic extracts were combined, washed with  $\text{H}_2\text{O}$ , and brine, followed by drying over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure to yield 1.5 g of a reddish color liquid. The crude product was purified by silica-gel column chromatography using hexanes as eluent to yield a 0.41 g (54 % yield) of ethenylferrocene as an orange-color solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.44 (dd,  $J = 17.6, 10.8$  Hz, 1H, =CH), 5.31 (dd,  $J = 17.4, 4$  Hz, 1H, =CH<sub>2</sub>), 5.01 (dd,  $J = 10.8, 4$  Hz,

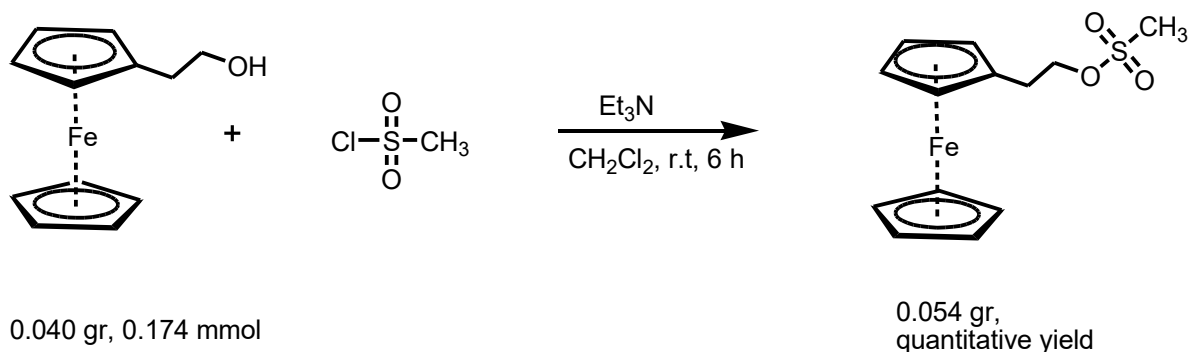
$^1\text{H}$ ,  $=\text{CH}_2$ ), 4.35 (t,  $J = 2$  Hz, 2H, Cp), 4.20 (t,  $J = 2$  Hz, 2H, Cp), 4.10 (s, 5H, Cp);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  134.7 ( $=\text{CH}$ ), 111.1 ( $=\text{CH}_2$ ), 69.2 (5C, Cp), 68.7 (2C, Cp), 66.7 (2C, Cp).



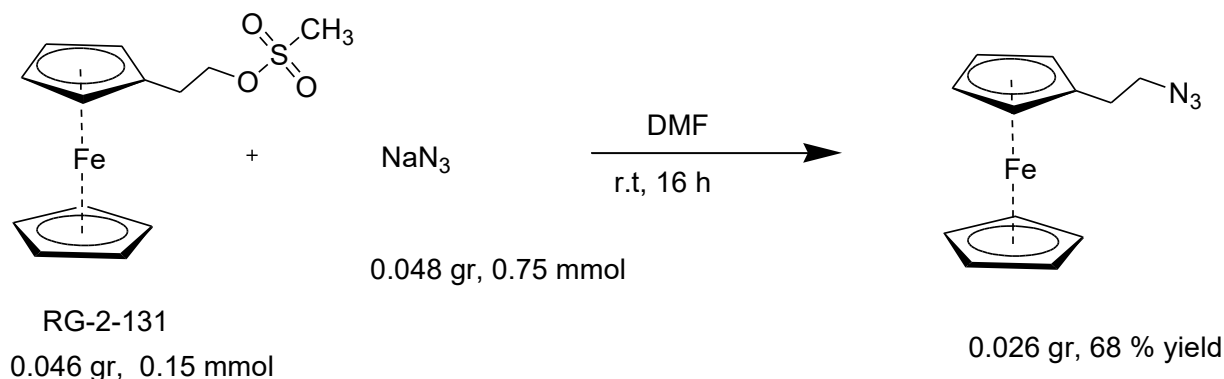
**b. (2-Hydroxyethyl)ferrocene.** A 50-mL dried flask containing 0.10 g (0.472 mmol) of ethenylferrocene was dried under vacuum and maintained under argon. To it, was added 5 mL of dried THF, cooled to  $-20^\circ\text{C}$ , and added 0.24 mL (0.24 mmol, 0.5 eq.) of 1.0 M of  $\text{BH}_3\cdot\text{THF}$  dropwise. The solution was stirred and allowed to warm to  $0^\circ\text{C}$  for 2 h and then to  $25^\circ\text{C}$  for 16 h. After stirring this time, the reaction mixture was cooled to  $0^\circ\text{C}$  and added 4.72 mL (4.72 mmol, 10.0 eq.) of 1 N NaOH followed by 0.48 mL (4.72 mmol, 10.0 eq.) of 30% aqueous  $\text{H}_2\text{O}_2$ , and stirred at  $0^\circ\text{C}$  for 1 h followed by stirring at  $25^\circ\text{C}$  for 2 h. The reaction mixture was then diluted with 10 mL of aqueous  $\text{NH}_4\text{Cl}$ , and extracted with ethyl acetate (five times with 40 mL each time). The organic extracts were combined, washed with  $\text{H}_2\text{O}$ , and brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure to yield 0.118 g of a crude product as a yellow-colored liquid. The crude product was purified by silica-gel column chromatography using hexane and ethyl acetate as eluents starting from a ratio of 10:1 to 3:1 and yield 0.072 g (68% yield) of (2-hydroxyethyl)ferrocene as a yellow-colored oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.11 (m, 9H, Cp), 3.73 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 2.60 (t,  $J = 6.8$  Hz, 2H,  $\text{CH}_2\text{Cp}$ ), 1.64 (bs, 1H, OH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  84.8 (C-O), 68.5 (5C, Cp), 67.7 (2C, Cp), 63.5 (2C, Cp), 32.8 ( $\text{CH}_2\text{Cp}$ )<sup>172</sup>.



**c. 2-(Methanesulfonyloxy)ethylferrocene.** A 50-mL dried flask containing 0.040 g (0.17 mmol) of (2-hydroxyethyl)ferrocene was dried under vacuum and maintained under argon. To it, 2 mL of dry  $\text{CH}_2\text{Cl}_2$  was added followed by 74  $\mu\text{L}$  (0.52 mmol, 3.0 eq.) of trimethylamine and 21  $\mu\text{L}$  (0.26 mmol, 1.5 eq.) of freshly distilled methanesulfonyl chloride. After stirring at  $25^\circ\text{C}$  for 6 h, the reaction mixture was diluted with 5 mL of aqueous  $\text{NH}_4\text{Cl}$ , and extracted with ethyl acetate (5 times with 40 mL each time). The organic extracts were combined, washed with  $\text{H}_2\text{O}$ , and brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure to yield 0.054 g (quantitative yield) of [2-(methanesulfonyloxy)ethyl]ferrocene as a yellow-colored oil, which was used in the subsequent synthesis without purification. This molecule is unstable and decomposed gradually upon standing at room temperature and on silica gel column.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.31 (t,  $J = 7.2$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.13 (m, 9H, Cp), 2.92 (s, 3H,  $\text{CH}_3$ ), 2.78 (t,  $J = 7.2$  Hz, 2H,  $\text{CH}_2\text{Cp}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  82.8 (C-O), 70.3 (2C, Cp), 68.5 (5C, Cp), 67.9 (2C, Cp), 37.5 ( $\text{CH}_2\text{Cp}$ ), 29.7 (S- $\text{CH}_3$ ).

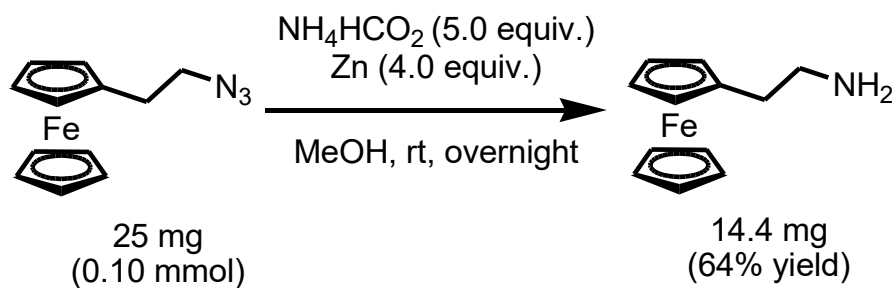


**d. (2-Azidoethyl)ferrocene.** A 50-mL dried flask containing 0.046 g (0.15 mmol) of [2-(methanesulfonyloxy)ethyl]ferrocene under argon was added 2 mL of dried DMF (distilled over  $\text{CaH}_2$ ). To it, 0.048 g (0.75 mmol, 5.0 eq.) of  $\text{NaN}_3$  was added and stirred at 25°C for 16 h, and the mixture was diluted with 5 mL of aqueous  $\text{NH}_4\text{Cl}$  and extracted with diethyl ether (5 times, 40 mL each time). The organic extracts were combined, washed with  $\text{H}_2\text{O}$ , and brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure to yield 0.035 g of crude product as a yellow colored oil. This crude mixture was subjected to silica-gel column chromatography using hexane and diethyl ether as eluents starting from a ratio of 20:1 to 5:1, yielding 0.026 g (68% yield) of (2-azidoethyl)ferrocene as a yellowish oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.11 (m, 9H, Cp), 3.38 (t,  $J$  = 7.2 Hz, 2H,  $\text{CH}_2\text{N}$ ), 2.64 (t,  $J$  = 7.2 Hz, 2H,  $\text{CH}_2\text{Cp}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  68.6 (5C, Cp), 68.4 (2C, Cp), 67.7 (2C, Cp), 52.3 ( $\text{CH}_2\text{-N}_3$ ), 29.3 ( $\text{CH}_2\text{Cp}$ ).

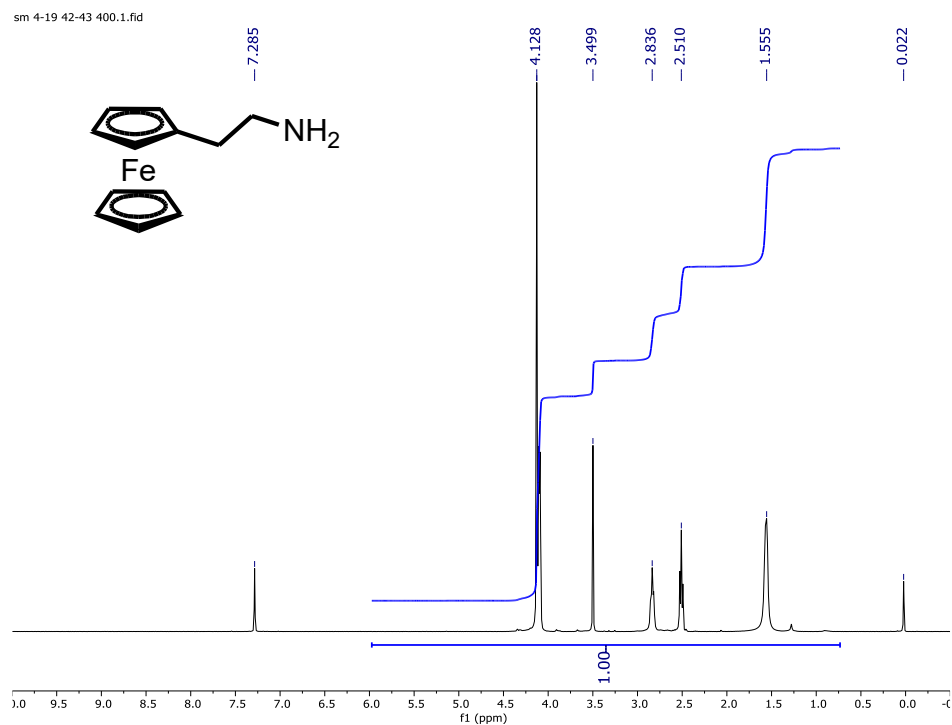


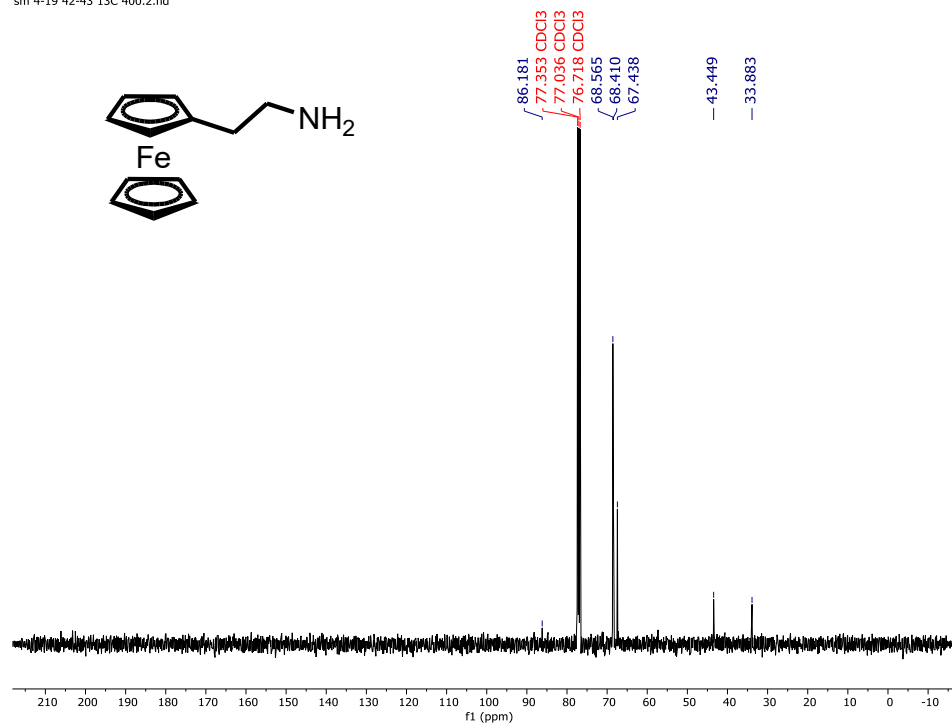
**e. (2-Aminoethyl)ferrocene.** A flask containing 25 mg (0.10 mmol) of (2-azidoethyl)ferrocene and 31 mg (0.49 mmol) of ammonium formate was vacuum dried and then maintained under argon. To it, 0.6 mL of methanol (pre-dried over 3 Å molecule sieves) and 26 mg (0.40 mmol) of activated zinc dust were added, and the mixture was stirred overnight at 25°C then filtered through a pad of Celite. The solid was washed carefully with ethyl acetate, and the filtrates were combined and was washed with 2.0 mL of 0.1 N  $\text{NaOH}$ , followed by 2.0 mL of  $\text{H}_2\text{O}$  and 3.0 mL of brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure. The

crude produce was column chromatographed over silica gel using hexane and diethyl ether as eluents from a ratio of 10:1 to 0:1, and followed by dichloromethane and methanol and aqueous ammonium hydroxide in a ratio of 5:1:0.01, yield 14.4 mg (64% yield) of (2-aminoethyl)ferrocene.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.13 (m, 9H, Cp), 3.50 (s, 2H,  $\text{NH}_2$ ), 2.84 (t,  $J = 7$  Hz, 2H,  $\text{CH}_2\text{N}$ ), 2.51 (t,  $J = 7$  Hz, 2H,  $\text{CH}_2\text{Cp}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  68.6 (5C, Cp), 68.4 (2C, Cp), 67.4 (2C, Cp), 43.4 ( $\text{CH}_2\text{-NH}_2$ ), 33.9 ( $\text{CH}_2\text{Cp}$ ). HRMS (ESI) calcd for  $\text{C}_{12}\text{H}_{16}\text{FeN}^+ [\text{M} + \text{H}]^+$ : 230.0627, found 230.0620.



**f.  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  (100 MHz) NMR Spectra of to (2-Aminoethyl)ferrocene.**





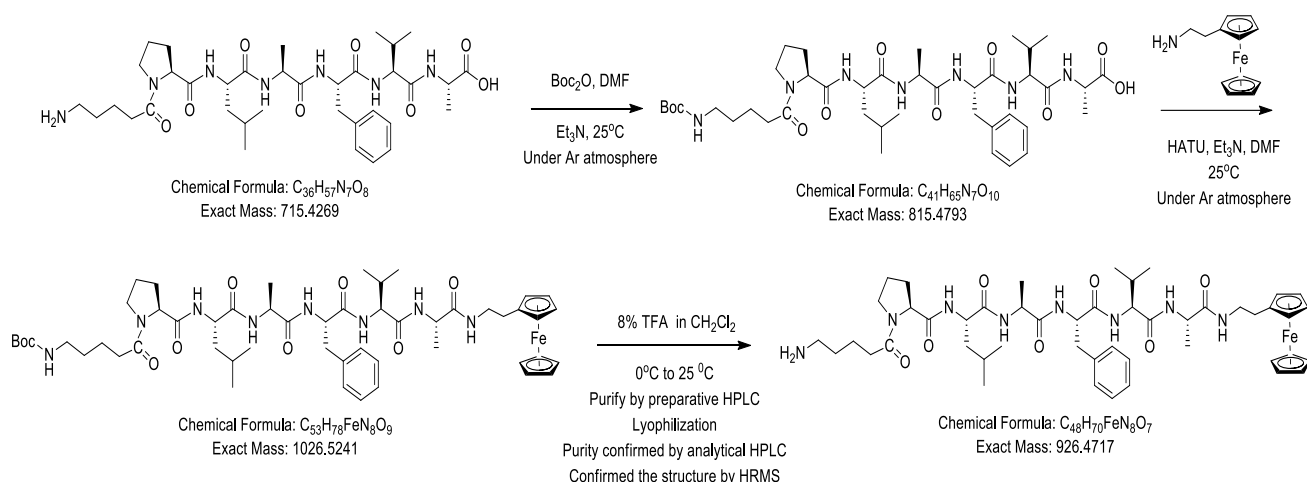
- Synthesis protocol for attaching AEF to purchased H-15 peptide substrate as a representative coupling methodology. H-3-AEF and H-6-AEF were similarly synthesized.



Firstly, a flask containing 0.030 g (0.042 mmol) of  $\text{H}_2\text{N}(\text{CH}_2)_4\text{CO}-\text{Pro}-\text{Leu}-\text{Ala}-\text{Phe}-\text{Val}-\text{Ala}-\text{OH}$  (MW = 716 g/mol) was dried under vacuum and maintained under argon. After that, 0.5 mL of DMF and 20  $\mu\text{L}$  (0.16 mmol) of trimethylamine were added, and the solution was stirred for 5 min., followed by the addition of a solution of 0.018 g (0.063 mmol) of di-*tert*-butyl dicarbonate ( $\text{Boc}_2\text{O}$ ) in 0.5 mL of DMF using a syringe under argon. The solution was stirred at room temperature for 1 h, and HPLC analysis of an aliquot of the solution showed the absence of starting material peptide and only the presence of the desired Boc-protected peptide. The solution was concentrated under reduced pressure for 3 h to give the Boc-protected crude product, which was used without purification in the following reaction. To the aforementioned *N*-Boc- $\text{HN}(\text{CH}_2)_4\text{CO}-\text{Pro}-\text{Leu}-\text{Ala}-\text{Phe}-\text{Val}-\text{Ala}-\text{OH}$  under argon, a solution of 0.019 g (0.0504 mmol) of

1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium-3-oxide

hexafluorophosphate (HATU) in 1 mL of DMF was added, and the mixture was stirred for 1.5 h at 40°C. A solution of 13.4 mg (0.0504 mmol) of (2-aminoethyl)ferrocene (AEF) HCl salt (neutralized with trimethylamine) in 1 mL of DMF was added. The solution was stirred at 40 °C for 12 h and an aliquot of the reaction mixture was removed and analyzed by HPLC, showing the absence of starting peptide. The solution was concentrated under reduced pressure for 3 h to give the Boc-protected and AEF functionalized crude product. The reaction mixture was filtered through a Chromafil Xtra PTFE-20/25 syringe filter (0.20 µm) and the filtrate was purified by preparative HPLC using deionized water and acetonitrile (0% to 95% acetonitrile gradient in 60 min; 8 ml/min; the deionized water contains 0.1% of TFA) as the running solvent to give *N*-Boc-HN(CH<sub>2</sub>)<sub>4</sub>CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH<sub>2</sub>-CH<sub>2</sub>-Fc (Fc = ferrocenyl). After removal of majority of the eluents (acetonitrile and water), the collected sample from HPLC was freeze-dried to obtain the solid product. Freeze dried powder, *N*-Boc-HN(CH<sub>2</sub>)<sub>4</sub>CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH<sub>2</sub>-CH<sub>2</sub>-Fc, was diluted with 8% TFA in dichloromethane (DCM) at 0°C under argon and gradually warmed to room temperature for 1 hour, and DCM and most of the TFA were removed on a rotary evaporator. The final Boc sample was dissolved in water and freeze dried to give a yellow/green solid. The HPLC graphs and HRMS charts are shown in the following section.



**Scheme A.1.** Synthesis of H-15-AEF substrate peptide.

- Characterization of H-3-AEF, H-6-AEF, and H-15-AEF:

**$H_2N-(CH_2)_4-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH_2-CH_2-Fc$  (H-15-AEF)**

HRMS (ESI) calcd for  $C_{48}H_{71}FeN_8O_7^+ [M + H]^+$ : 927.4790, found 927.4785; and calcd for  $C_{48}H_{72}FeN_8O_7^{2+} [M + 2H]^{2+}$ : 464.2431, found 464.2436.

**$H_2N-(CH_2)_4-CO-Pro-Leu-Arg-Phe-Gly-Ala-NH-CH_2-CH_2-Fc$  (H-3-AEF):**

$^1H$  NMR and HRMS spectra and HPLC graph are depicted in Section 15. HRMS (ESI) calcd for  $C_{48}H_{72}FeN_{11}O_7^+ [M + H]^+$ : 970.4960, found 970.4998; and calcd for  $C_{48}H_{73}FeN_{11}O_7^{2+} [M + 2H]^{2+}$ : 485.7517, found 485.7805.

**$H_2N-(CH_2)_4-CO-Pro-Leu-Gly-Phe-Val-Ala-NH-CH_2-CH_2-Fc$  (H-6-AEF):**

HRMS spectra and HPLC graphs are depicted in Section 15. HRMS (ESI) calcd for  $C_{47}H_{69}FeN_8O_7^+ [M + H]^+$ : 913.4633, found 913.4739; and calcd for  $C_{47}H_{71}FeN_8O_7^{2+} [M + 2H]^{2+}$ : 457.2317, found 457.2444.

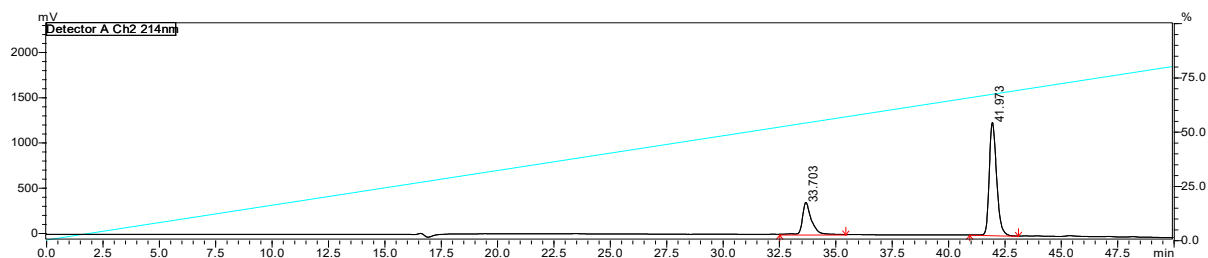
### HRMS, HPLC, and NMR spectra of H-3-AEF, H-6-AEF, and H-15-AEF:

HPLC column and solvent program used in the analysis and purification of H-3-, H-6-, and H-15-AEF peptides:

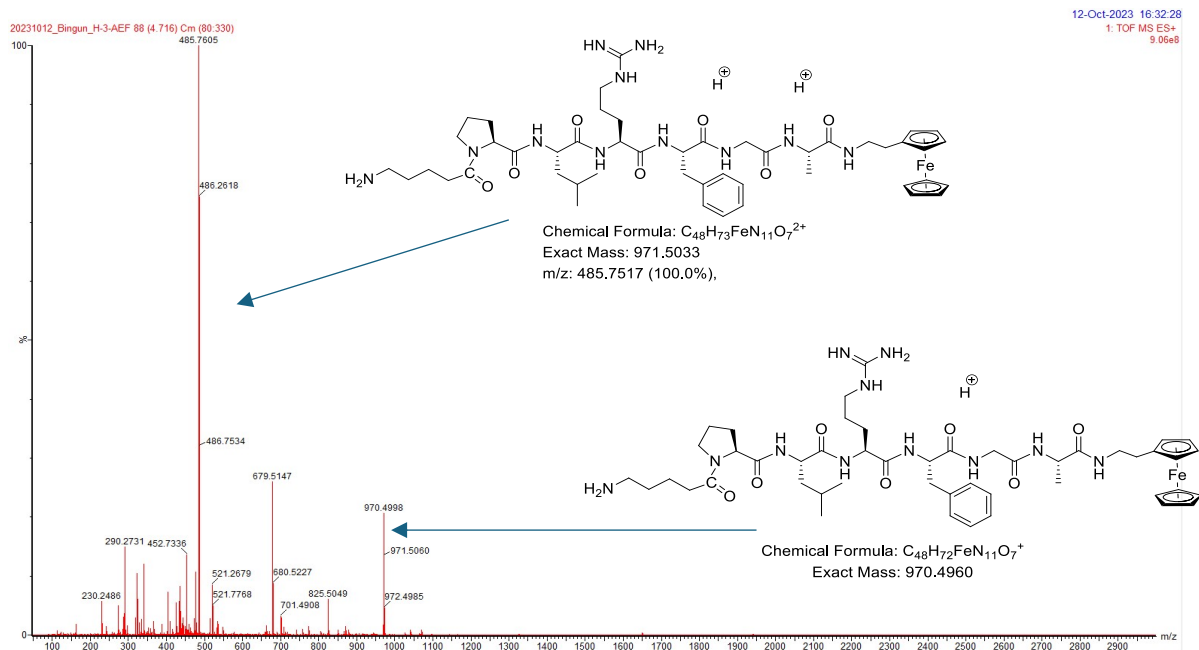
Column: PROTO 300-C18 10  $\mu\text{m}$  10 x 250 mm; Phases C4 and C18, Particle Sizes 4.5 and 10  $\mu\text{m}$ , pore size 300Å, pore volume 0.9  $\text{cm}^3/\text{g}$ ; %Carbon (w/w) C4 = 3%, C18 = 8%; gradient elution 0%  $\text{CH}_3\text{CN}$  to 95%  $\text{CH}_3\text{CN}$  within 60 min or up to 80 % within 50 min. HRMS spectrometer: Waters Xevo G2/XS ToF mass spectrometer.

#### H-3-AEF [ $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Arg-Phe-Gly-Ala-NH-CH}_2\text{-CH}_2\text{-Fc}$ ]:

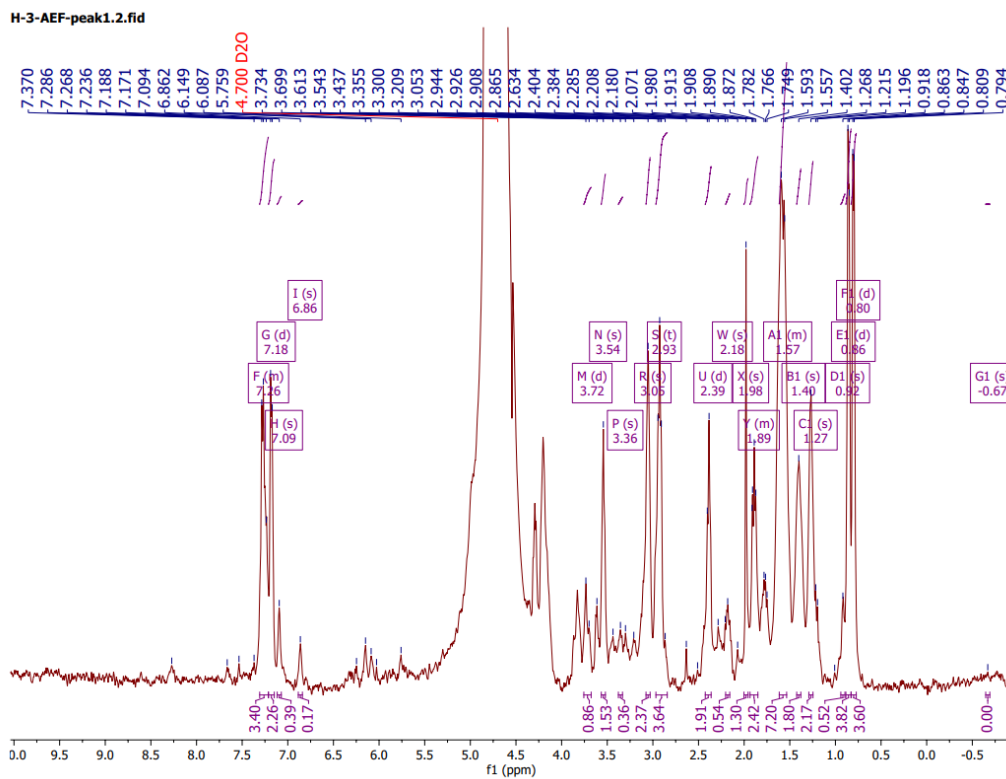
**HPLC:** Peak at 42.0-min is a singly protonated peptide, while peak at 33.7-min is a doubly protonated peptide.



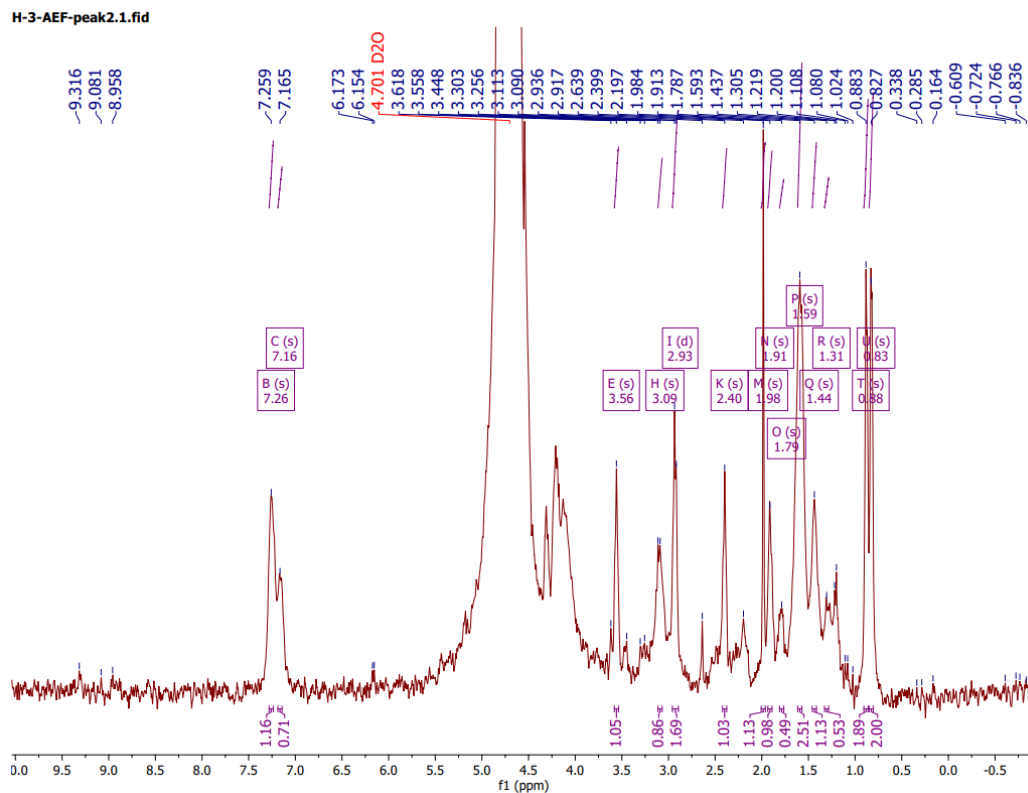
## HRMS:



## $^1H$ NMR (400 MHz) (in $D_2O$ ) of 42.0-min peak:

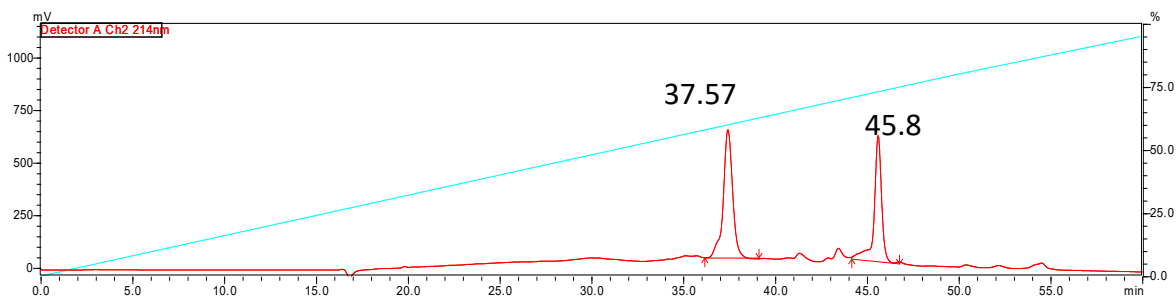


**$^1\text{H}$  NMR (400 MHz) (in  $\text{D}_2\text{O}$ ) of 33.7-min peak:**

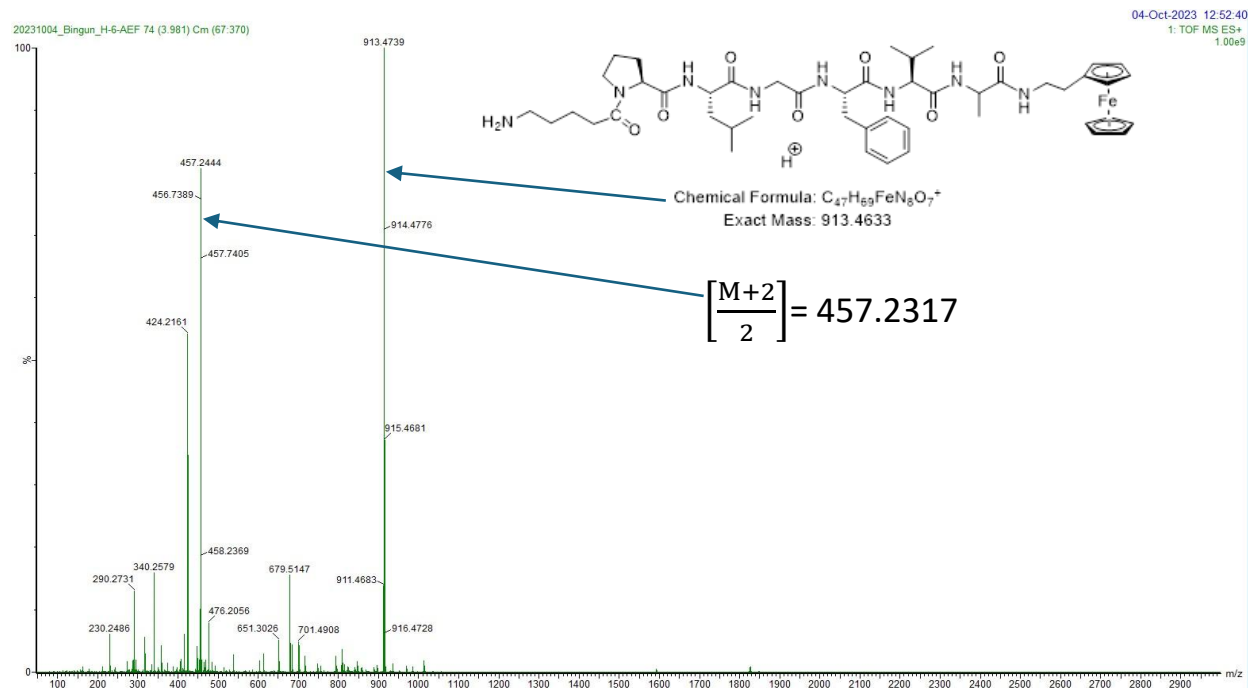


**H-6-AEF  $[\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}]$ :**

**HPLC:** Peak at 45.8-min is singly protonated peptide, while peak at 37.5-min is doubly protonated peptide.  $^1\text{H}$  NMR spectra of these two isolated pure materials show similarly.

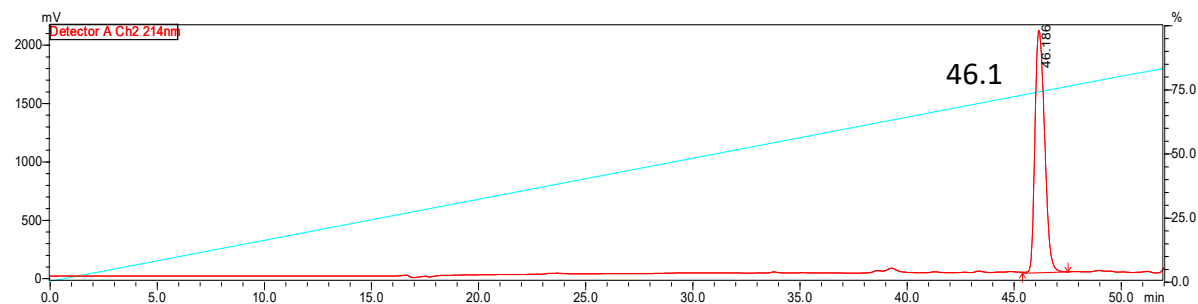


## HRMS:

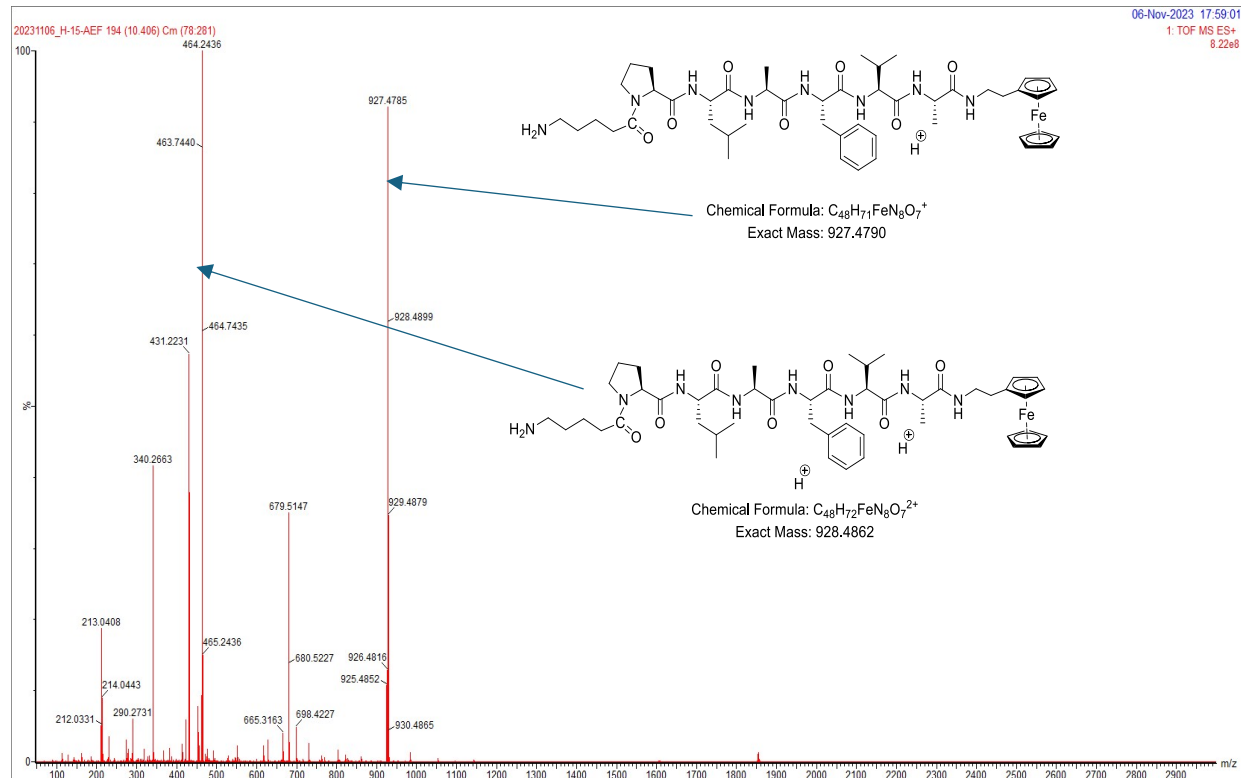


**H-15-AEF [ $H_2N-(CH_2)_4-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH_2-CH_2-Fc$ ].**

**HPLC: Peak at 46.18-min is singly protonated peptide. Only one peak was observed, and no doubly protonated peptide was found. Beside the  $-NH_2$  group, there is no other basic nitrogen for protonation.**



## HRMS:



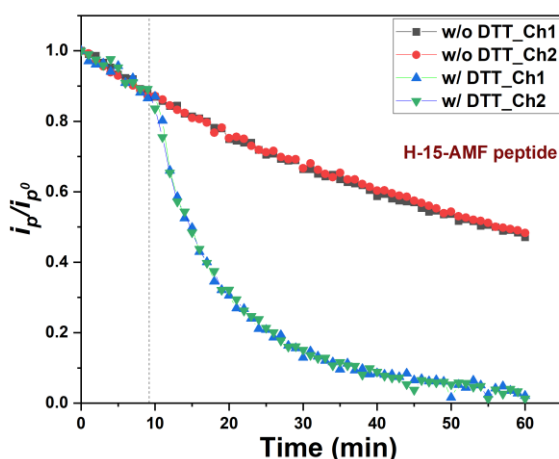
### A.4 Au electrode surface functionalization

- Surface functionalization **protocol-1** used in previous studies.

To functionalize the MEA with the peptide-Fc substrate, the chip was initially incubated in a solution of 1.0 mM 6-mercapto-1-hexanol and 0.2 mM 6-mercapto-1-hexanoic acid in deionized water for 40 minutes, forming a densely packed self-assembled monolayer (SAM) due to thiol adsorption on the gold surface. This alkanethiol layer acts as an insulator to minimize background current, while providing a surface ratio of approximately 1:5 between  $-COOH$  and  $-OH$  groups. The electrode was then incubated with a 2.0 mM peptide-Fc substrate solution, containing 0.2 g/L 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.2 g/L N-hydroxysulfosuccinimide (Sulfo-NHS) as coupling agents, for 2 hours at room temperature. This enabled the formation of amide bonds between the amine group of the peptide-Fc substrate and

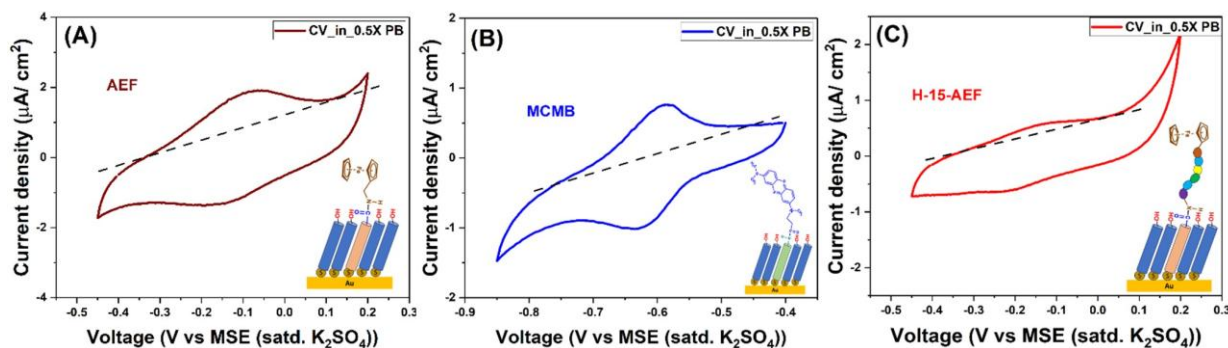
the carboxyl groups on the SAM surface. The presence of mixed  $-\text{COOH}$  and  $-\text{OH}$  groups in the SAM was crucial for reducing peptide-Fc density on the gold surface, thus lowering steric hindrance in subsequent proteolysis measurements. Finally, the functionalized chip was rinsed with deionized water for 30 seconds to remove any physically adsorbed molecules.

### A.5 Nonspecific decay observed upon the addition of dithiothreitol (DTT)



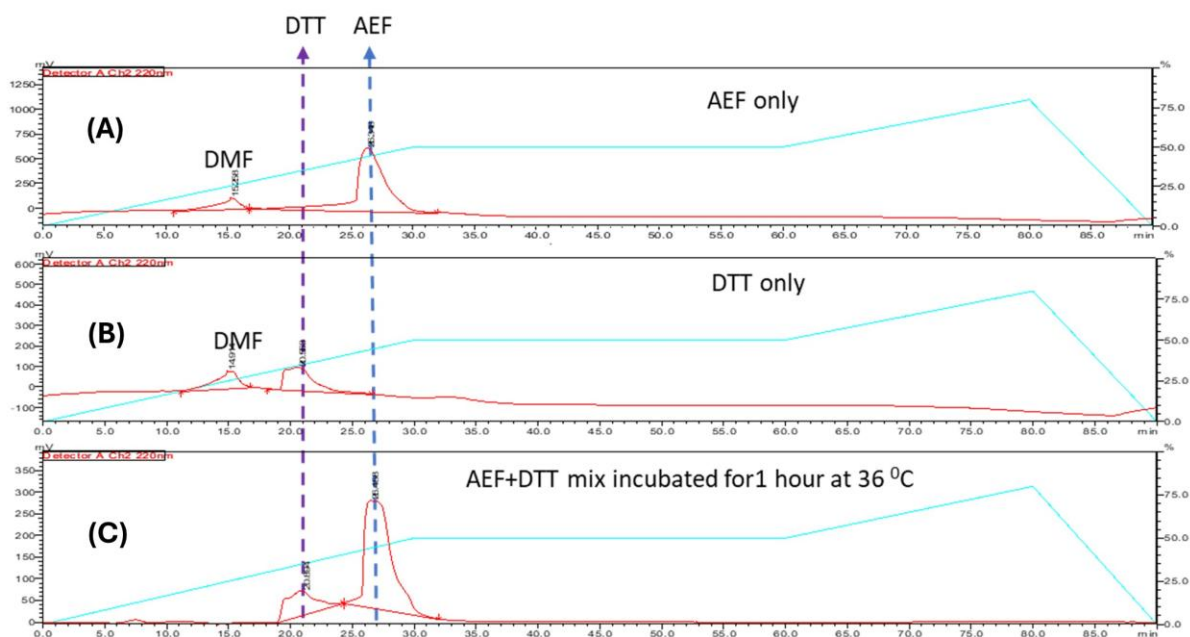
**Appendix Figure A.1** Evolution of normalized ACV peak current over time for the peptide substrate, H-15-AMF ( $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Ala}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{Fc}$ ) functionalized on SAM-covered Au MEA. The stability was checked during continuous ACV cycling in presence and absence of DTT ( $20\ \mu\text{M}$ ) in  $0.5\text{X PB}$  at  $36^\circ\text{C}$ . Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

### A.6 Surface Coverage Determination

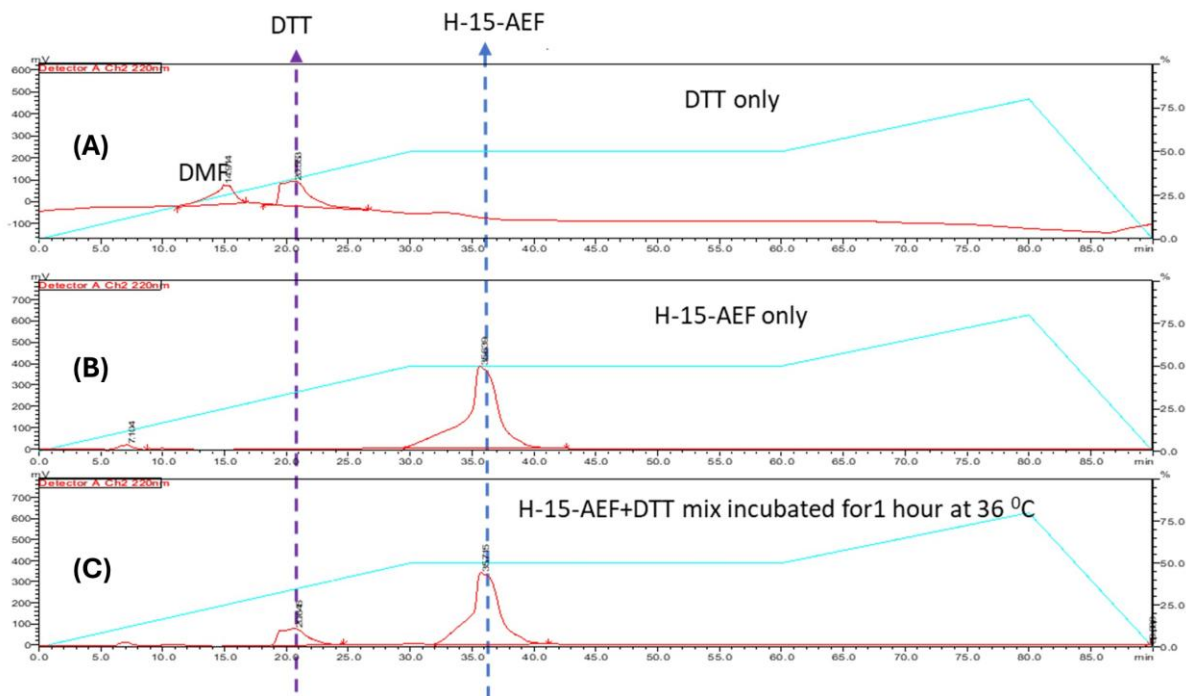


**Appendix Figure A.2** Cyclic voltammograms of (A) AEF (B) MCMB (C) H-15-AEF on SAM-covered Au disk electrode ( $2.0\ \text{mm}$  in diameter) in  $0.5\text{X PB}$  at  $36^\circ\text{C}$ . Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

## A.7 Impact of DTT on AEF and peptide H-15-AEF chemical stability

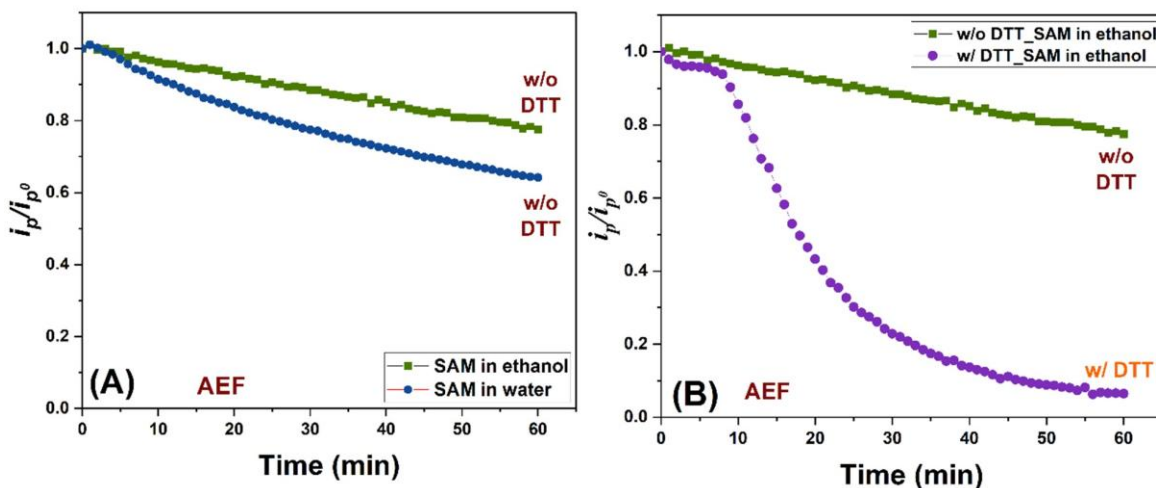


**Appendix Figure A.3** HPLC chromatograms of (A) 5.0 mM amino ethyl ferrocene, (B) 5.0 mM dithiothreitol, and (C) 5.0 mM dithiothreitol and 5.0 mM AEF incubated for 1 hour at 36°C. Reprinted (adapted) from *Biosensors and Bioelectronics*, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.



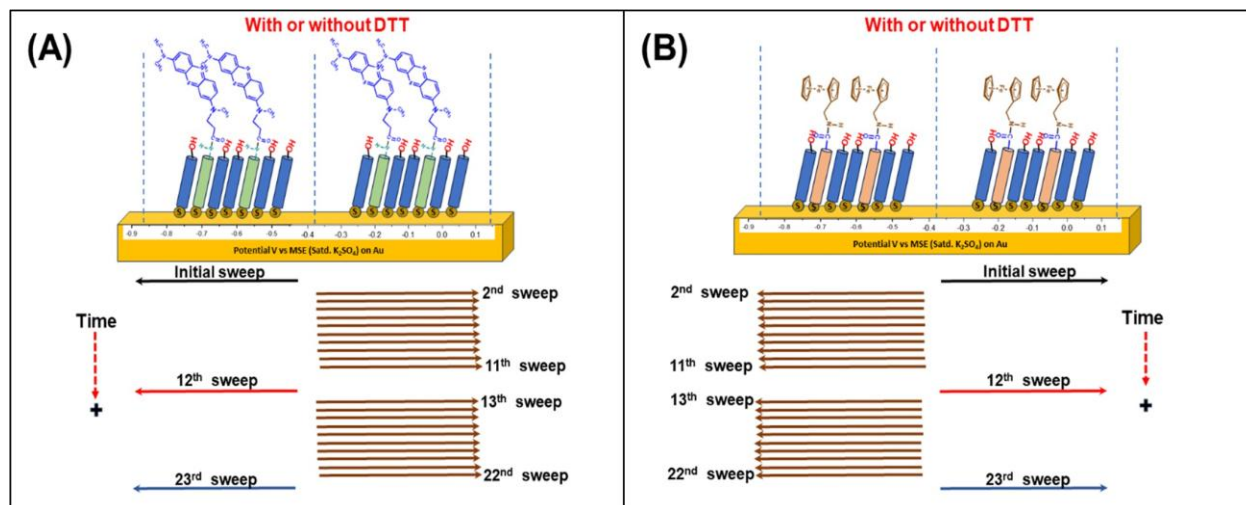
**Appendix Figure A.4** HPLC chromatograms of (A) 5.0 mM dithiothreitol, (B) 5.0 mM H-15-AEF, and (C) 5.0 mM dithiothreitol and 5.0 mM H-15-AEF incubated for 1 hour at 36 °C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

## A.8 Impact of self-assembled monolayer stability and SAM formation protocol

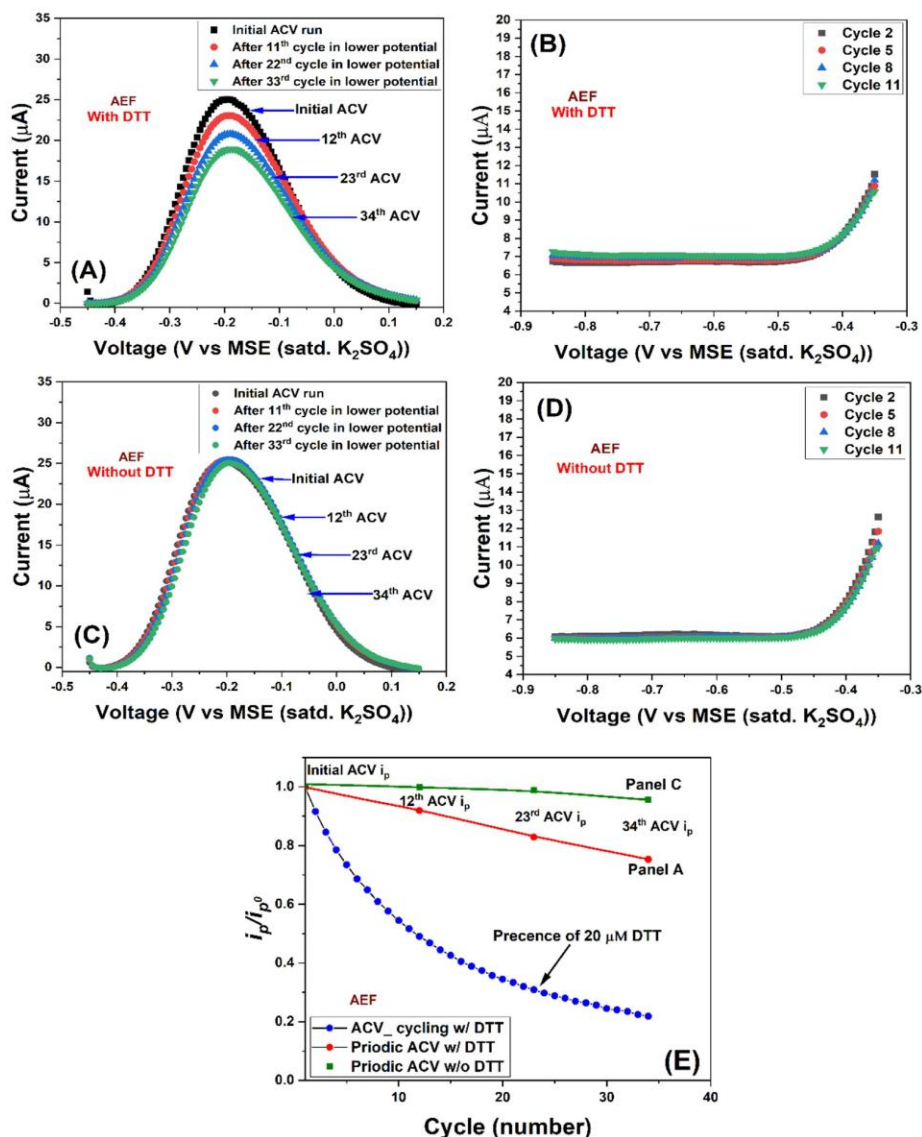


**Appendix Figure A.5** (A) The kinetic curve of normalized peak current over time for AEF functionalized on SAM-covered Au disk electrodes using different protocols. Blue: SAM functionalization was done in water for 50 min and AEF was coupled using EDC/Sulfo NHS chemistry. Green: SAM functionalization was done in anhydrous ethanol for 12 hours and AEF was coupled using EDC/Sulfo NHS chemistry followed by  $\text{NH}_2\text{OH}$  treatment. The stability was checked with continuous ACV cycling in 0.5X PB at 36 °C. (B) The stability was checked after adding 20  $\mu\text{M}$  DTT during continuous ACV cycling in 0.5X PB at 36 °C to see stability improvement upon addition of DTT after using the improved SAM formation protocol-2. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

## A.9 Impact of redox potential window of ferrocene (relatively higher potential window)



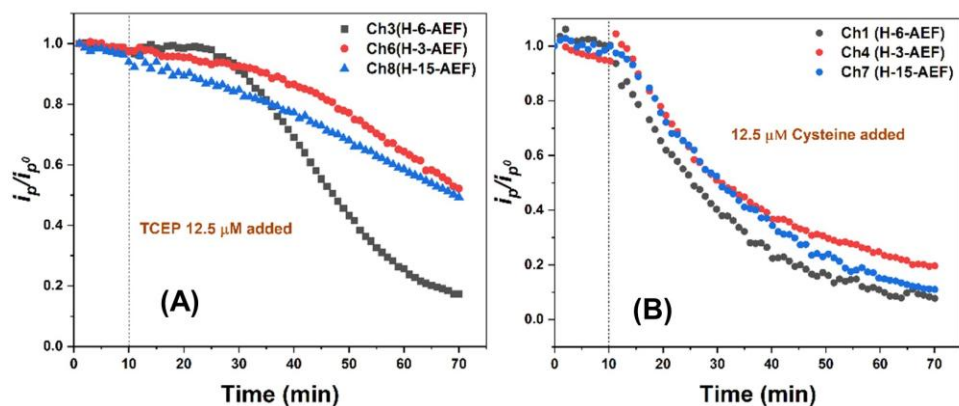
**Appendix Figure A.6** The schematic representation shows how the experimental ACV scans were performed: (A) Periodic ACV probing of MCMB/SAM/Au at (-0.85 to -0.40 V vs MSE) during continuous ACV cycling simulating the conditions of AMF and AEF measurements at higher potentials (-0.40 to 0.15 V vs MSE) in presence and absence of 20  $\mu$ M DTT in Figure 2.4; (B) Periodic ACV probing of AEF/SAM/Au at (-0.45 to 0.15 V vs MSE) during continuous ACV cycling simulating the conditions of MCMB measurements at lower potentials (-0.85 to -0.35 V vs MSE) in presence and absence of 20  $\mu$ M DTT in Figure 2.5. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.



**Appendix Figure A.7** (A) Periodic ACV probing of AEF/SAM/Au at the high potential window (-0.45 to 0.15 V vs MSE) during continuous ACV cycling simulating the conditions of MCMB measurements at lower potentials (-0.85 to -0.35 V vs MSE) in the presence of 20  $\mu\text{M}$  DTT. (B) Selected ACV curves showing no AEF peak during continuous ACV cycling at the MCMB redox potential region (-0.85 to -0.35 V vs MSE) in the presence of DTT. (C) No decay in ACV peak current in similar experiments as panel (A) in the absence of DTT, confirming that ACV at high potentials (-0.40 to 0.15 V vs MSE) in the presence of DTT caused the signal decay. (D) Selected ACV curves showing no AEF peak during continuous ACV cycling at the MCMB redox potential region (-0.35 to -0.85 V vs MSE) without DTT. (E) Comparison of the rapid decay in normalized peak current of AEF/SAM/Au in 20  $\mu\text{M}$  DTT during continuous ACV cycling at high potentials (-0.45 V to 0.15 V vs MSE, blue curve) to the much slower decay shown by periodical probing at this potential range during continuous ACV cycling at the low potentials (-0.85 V to -0.35 V vs MSE). All measurements are carried out in 0.5X PB at 36  $^\circ\text{C}$  on Au disk electrodes. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

## A.10 Investigating any interference from alternative activation agents

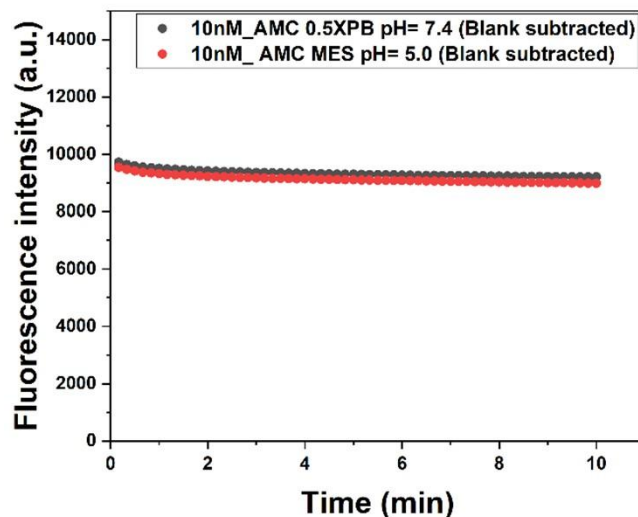
### (TCEP, Cysteine)



**Appendix Figure A.8** The kinetic curve of the normalized peak current over time during continuously repeated ACV cycling for H-3-AEF, H-6-AEF, and H-15-AEF. (A) TCEP (12.5  $\mu$ M) was added at the 10<sup>th</sup> cycle. (B) Cysteine (12.5  $\mu$ M) was added at the 10<sup>th</sup> cycle, functionalized on the SAM-covered Au MEA. The measurements were in 0.5X PB at 36 °C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

TCEP has been extensively used in a wide range of applications to reduce disulfide bonds, to maintain free sulfhydryl groups (Han and Han, 1994). Although TCEP could activate CB enzyme in mild conditions, TCEP showed interference with the SAM-covered peptide functionalized sensor platform, as shown in **Fig A.8A**. The exact reason for interference is not clear in this study. However, a few reports have been published discussing its side reactions with peptides and other complex proteins, resulting in cleaved peptide fragments (Peiran Liu and Yunping Huang). Cysteine exhibited a similar interference pattern (**Fig A.8B**) to DTT due to the presence of a sulfhydryl group, which can potentially replace SAM at higher potentials (-0.40 to 0.15 V vs MSE).

## A.11 Investigating the influence of buffer on fluorescence intensity from AMC



**Appendix Figure A.9** Investigating the influence of the buffer (0.5X PB (pH = 7.4) and 25 mM MES (pH = 5.0)) on fluorescence intensity from amino methyl coumarin (AMC) fluorophore utilized in FRET assay in Figure 2.4 panel (A). Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

## A.12 Optimization of DTT removal by ultrafiltration at different temperatures

Amicon ultra centrifugal filters were used to remove small DTT molecules from the CB solution. Ultrafiltration using centrifugal filters is widely used for rapid sample processing and facilitates high sample recoveries, which are particularly effective even with dilute samples <sup>173, 174</sup>. Filter-aided sample preparation (FASP) and similar techniques are becoming increasingly popular for proteomic sample preparation <sup>175</sup>. These filters range from different volumes and different molecular weight cutoffs from MilliporeSigma. To achieve the effective removal of DTT from activated CB samples, a suitable filter should be chosen, and the methodology should be developed.

It is important to investigate the role of DTT in CB activation before its removal. In most cases, disulfide bonds can be reduced by reagents like DTT, and their formation can be inhibited by metal-chelating agents such as EDTA or by removing metal ions <sup>176, 177</sup>. Unlike CB, some enzymes, such as deoxyribonucleases (DNases), become inactive once their disulfide bridges are reduced. One study observed that after 24 hours of exposure to air oxidation at pH = 7.5 in the presence of 1 mM EDTA, 95% of the disulfide bridges of DNase remained in the cysteine form <sup>178</sup>. Based on the literature, it was decided to remove DTT in the presence of EDTA to maintain CB in its active state. This approach ensures that CB remains active after the reduction of disulfide bridges, and enzyme activity can be validated using fluorescence assays.

For the removal of DTT from the activated CB sample, 3K molecular weight cut-off (MWCO), 0.5 mL filter, and 10K MWCO, 0.5 mL filter were tried as possible candidates due to its MWCO values, that could retain the activated CB (29 kDa) molecules in the residue. From these two filters, the 10K device was a better candidate due to its proven efficiency than the 3K filter by the vendors. The optimum filter to remove DTT from the activated CB sample within a lesser number of continuous centrifugation rounds at 14000 g was checked at different temperatures to keep CB in activated form as shown in **Appendix Table A.2**.

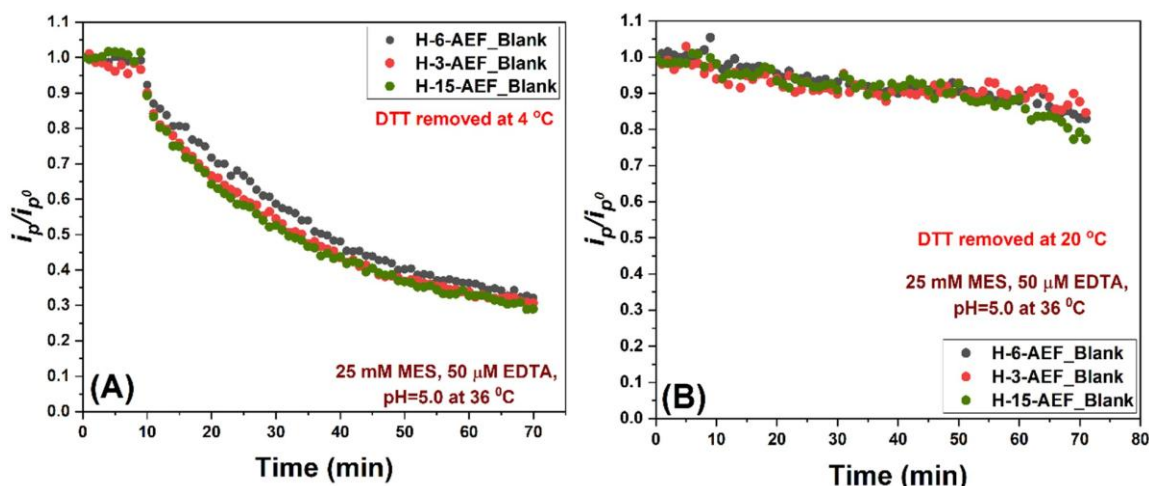
**Appendix Table A.2** The time taken to reach 50  $\mu$ L from 500  $\mu$ L using 3k and 10k Amicon ultracentrifugation filters at different temperatures (4 °C and 20 °C).

| Time taken to reach 50 $\mu$ L from 500 $\mu$ L | 3K device | 10K device |
|-------------------------------------------------|-----------|------------|
| At 4 °C                                         | 20 min    | 10 min     |
| At 20 °C                                        | 10 min    | 5 min      |

The optimum DTT removal was done using a 10k Amicon Ultra (0.5 mL) device at 20 °C. This device consumed a minimum amount of time to remove DTT from the activated CB sample. As shown in **Fig. A.10A**, DTT removal was not sufficient at 4 °C using the same filter device. The current rapidly came down for all three peptides upon the addition of DTT removed sample at 4 °C.

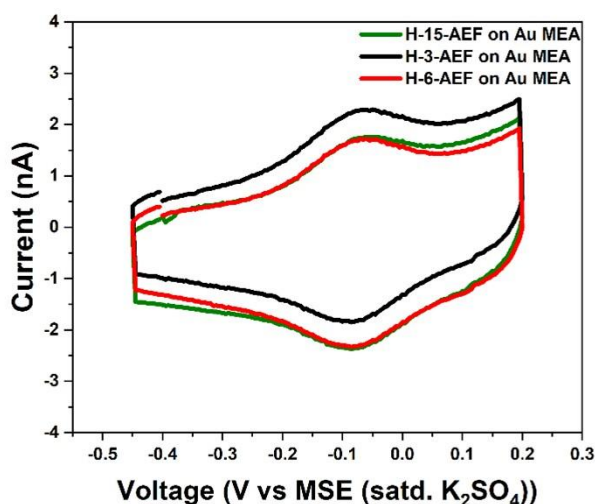
During the optimization of the MWCO filtration, it was determined that the 10K filter effectively removed a significant amount of DTT, after 2 centrifugation rounds (total time taken 10 min), resolving the interference issue. This was confirmed by adding the DTT-removed blank solution to the peptide-functionalized sensor platform in a 25 mM MES (pH = 5.0), 50  $\mu$ M EDTA buffer as the electrolyte. The blank sample was prepared by following the same procedure to remove DTT without the CB enzyme. There was no considerable decay after the addition of DTT-removed samples on ferrocene-tagged peptide substrates during ACV cycling, as shown and described in **Fig. A.10B**.

The final DTT removal procedure is as follows. A known concentration of CB was activated in 1 mM DTT, 25 mM MES (pH = 5.0) for 15 min at RT (total volume 23  $\mu$ L). A Clean Amicon ultra 10K, 0.5 mL filter was filled with 477  $\mu$ L of 25 mM MES (pH = 5.0), 50  $\mu$ M EDTA dilution buffer, and the activated CB sample was added (total volume = 500  $\mu$ L). The device spun down at  $14,000 \times g$  for 5 min in a 40° fixed angle rotor at 20°C until 50  $\mu$ L. After that, another 450  $\mu$ L of dilution buffer was added and spun down to 50  $\mu$ L using the same procedure. The remaining residue was taken as the final activated CB sample to do proteolysis experiments in 25 mM MES, 50  $\mu$ M EDTA buffer. The blank was prepared by following the same procedure without CB.



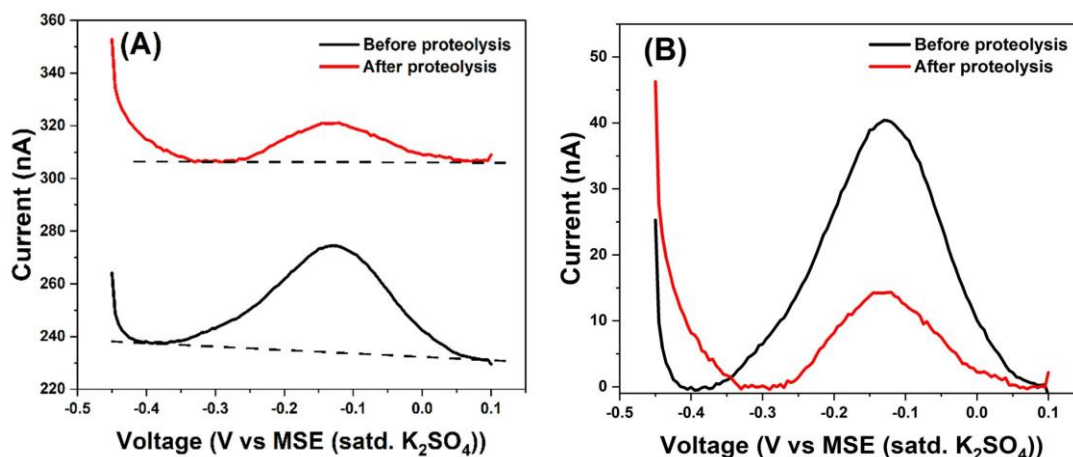
**Appendix Figure A.10** Evolution of normalized peak current over time for (A) Addition of DTT removed (at 4°C) sample on H-6-AEF, H-3-AEF and H-15-AEF functionalized SAM-covered Au MEA (B) Addition of DTT removed sample (at 25 °C) on H-6-AEF, H-3-AEF and H-15-AEF functionalized on SAM-covered Au MEA. All electrochemical measurements were done in 25 mM MES buffer containing 50.0  $\mu$ M EDTA, pH=5.0 at 36 °C. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

### A.13 Representative CV curves for H-6-AEF, H-3-AEF, H-15-AEF functionalized on Au MEA in 25 mM MES, 50 $\mu$ M EDTA, pH= 5



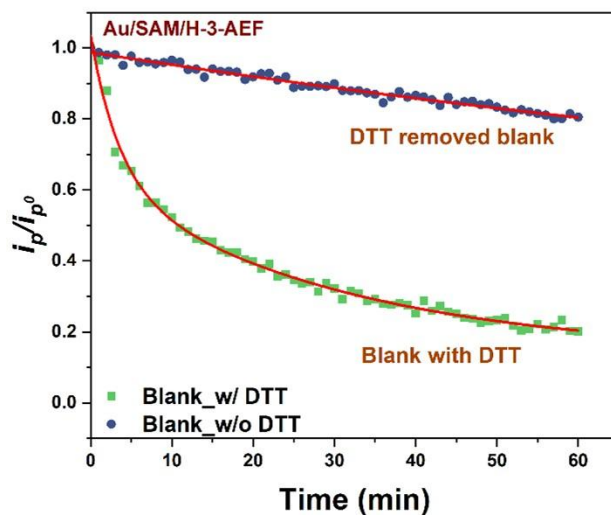
**Appendix Figure A.11** Representative CV curves for H-6-AEF, H-3-AEF, H-15-AEF functionalized on SAM-covered Au MEA in 25 mM MES, 50  $\mu$ M EDTA, pH = 5.0. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

#### A.14 Representative ACV curves for H-6-AEF functionalized on Au-MEA in 25 mM MES, 50 $\mu$ M EDTA, pH= 5 before and after proteolysis.



**Appendix Figure A.12** Representative ACV curves for H-6-AEF functionalized on SAM-covered Au MEA in 25 mM MES, 50  $\mu$ M EDTA, pH = 5.0. (A) Raw data and linear baselines (dashed lines) of ACV obtained before (black) and after (red) proteolysis. (B) The processed ACV curves after baseline subtraction for before (black) and after (red) proteolysis. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

#### A.15 Representative blank curves (normalized) for H-3-AEF functionalized on Au MEA



**Appendix Figure A.13** Evolution of normalized ACV peak current over time for the peptide substrate, H-3-AEF functionalized on SAM-covered Au MEA. The blank run after incorporating DTT removal protocol (blue curve) and blank run without DTT removal (green curve) in 25 mM MES (pH = 5.0), 50  $\mu$ M EDTA for comparison. Reprinted (adapted) from Biosensors and Bioelectronics, 273 (2025), 117193. Copyright 2025 Elsevier Ltd.

## Appendix B - Supplementary Information for Chapter 3

### B.1 Materials and Instruments

The peptide sequences (H-3:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Arg-Phe-Gly-Ala-COOH}$ , H-6:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Gly-Phe-Val-Ala-COOH}$ , and H-15:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Pro-Leu-Ala-Phe-Val-Ala-COOH}$ ) were purchased from Biomatik USA LLC (Wilmington, DE). The peptide sequence H-32:  $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Nle-Leu-Gly-Phe-Val-Ala-COOH}$  was synthesized in-house. (2-Aminoethyl) ferrocene (AEF) redox tag was purchased from Ambeed USA Inc (Buffalo, IL). The synthetic procedure for attaching the amine-terminated ferrocene redox tag to the substrate peptides and the characterization of the peptide sequence are described in later sections. 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) was bought from Chem-Impex International, Inc. (Wood Dale, IL). *N*-Hydroxysulfosuccinimide (Sulfo-NHS), 6-mercapto-1-hexanol, 6-mercapto-1-hexanoic acid, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate dibasic heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ), sodium phosphate monobasic monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ), dithiothreitol, and 2-(4-morpholino)ethanesulfonic acid (MES) were purchased from Fisher Scientific (Hampton, NH). Carrier-free recombinant human cathepsin B (rhCB) (~60% 37 kDa proenzyme, ~40% 29 kDa mature enzyme), cathepsin B-specific fluorogenic peptide substrate (Z-Leu-Arg-AMC) were acquired from R&D Systems Inc. (Minneapolis, MN). Other cathepsin B-specific fluorogenic peptide substrates (Z-Lys-Arg-AMC, Z-Nle-Arg-AMC, Z-Leu-Lys-Arg-AMC) were custom-synthesized and purchased from Biomatik USA LLC (Wilmington, DE). Z-Nle-Lys-Arg-AMC substrate was purchased from

MedChemExpress LLC (Monmouth, NJ). Cathepsin B solutions were activated in 25 mM MES buffer containing 1 mM or 5 mM DTT for 15 min to activate the proenzyme.

Cathepsin B enzyme activity was validated using a fluorogenic assay with a GloMax Multi+ Microplate Multimode reader (Promega, Madison, WI) in 96-well plates. Electrochemical measurements were carried out using an IVIUM n-Stat potentiostat (Eindhoven, The Netherlands), which allowed for simultaneous measurement of up to 10 independent working electrodes with a shared mercury mercurous sulfate, saturated K<sub>2</sub>SO<sub>4</sub> (MSE) reference electrode, and a common platinum coil counter electrode.

HPLC was performed on a Waters Breeze 2 system consisting of a 1525 pump and 2998 photodiode array detector. High-resolution MS (HRMS) was recorded on a Waters Xevo G2-XS QToF mass spectrometer equipped with a NanoLockSpray exact mass ionization source. Analytical HPLC analysis was performed using a SHIMADZU UFLC system.

## **B.2 Utilized Peptide-Fc substrates for cathepsin B**

We have designed, synthesized, and tested a small library of peptide-Fc substrates, each consisting of 6-8 amino acids, in previous studies, to be cleaved by cathepsin B based on analyses of specific recognition sequences reported in the literature. The activity of cathepsin B on these peptide-Fc substrates was evaluated using a combination of electrochemical measurements (employing Au disk electrodes and VACNF nanoelectrode arrays), fluorogenic assays, and HPLC-MS analysis. From the studies, three cathepsin B-selective peptides, H-3, H-6, and H-15, were chosen from the library for further study due to their higher proteolysis rates by cathepsin B compared to other candidates after tagging them with (2-aminoethyl)ferrocene (Fc) redox reporter. The cleavage sites, identified in our previous reported studies, are highlighted in pink and cyan for reference. Depending on the fluorogenic data in this study, we have modified the H-6 peptide

substrate to the H-32 peptide substrate, as shown in **Appendix Table B.1**, and the cleavage site was determined using HRMS analysis after proteolysis by activating cathepsin B.

**Appendix Table B.1 Peptide sequences screened for cathepsin B in previous and this study, with the cleavage sites between the purple and cyan highlighted amino acids.**

| Substrate | Peptide substrate sequence                                                                                                                                   |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| H-3-Fc    | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Arg}-\text{Phe}-\text{Gly}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |
| H-6-Fc    | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |
| H-15-Fc   | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Pro}-\text{Leu}-\text{Ala}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |
| H-32-Fc   | $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Nle}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{Fc}$ |

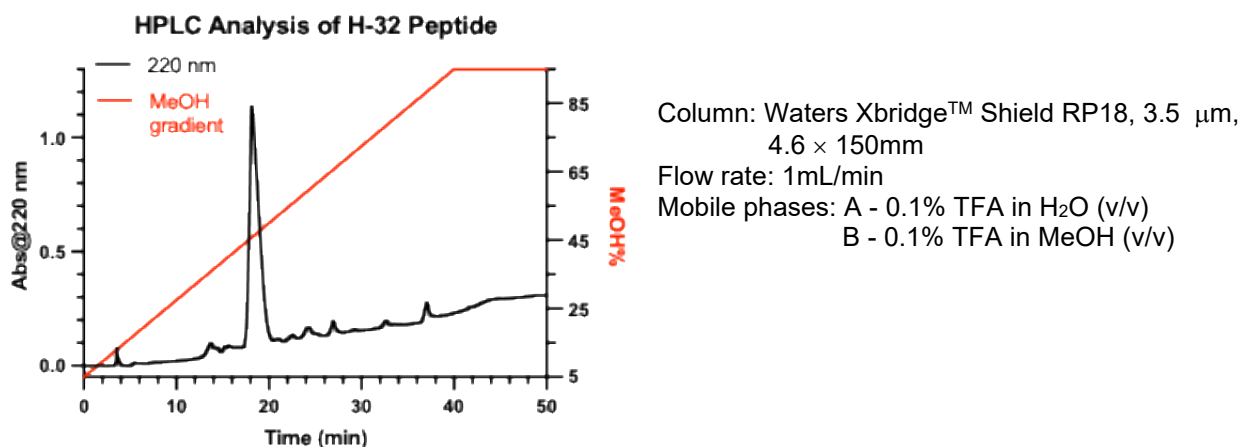
### B.3 Peptide Synthesis and Characterization

- Synthesis protocol of H-32 peptide.

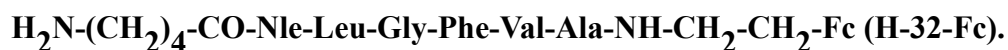


H-32 peptide was prepared by solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin (2.0g, 1.2 mmol/g, Aapptec®, catalog # RTZ001) pre-swollen in dichloromethane (DCM, 10 mL/g) for 30 min. After DCM was removed by aspiration, the resin was added Fmoc-Ala-OH (2.0 equiv.) and diisopropylethylamine (DIPEA, 4.0 equiv.) pre-dissolved in DCM. Reaction was carried out at room temperature with agitation for 2 hours. After the solution was discarded by aspiration, the resin was added 20 mL of capping mixture consisting of DCM:MeOH:DIPEA (17:2:1, v/v) and kept agitated for at least 30 minutes. Subsequently, the resin was filtered, washed with DMF, and dried with DCM. The loading percentages of the resin were determined to be 60% (0.72mmol/g) per the protocol at <https://www.peptideweb.com/loading-protocols>. SPPS was performed at a 0.5 mmol scale using Fmoc chemistry on a Liberty Blue 2.0 HT4 peptide synthesizer (CEM Corporation) following manufacturer's protocol. Cleavage of the H-32 peptide was achieved by first washing the resin 3 times with DCM and then suspending it in

6 mL 95% (v/v) TFA/DCM for 30 minutes at room temperature. The resin was filtered and washed with a small amount of TFA. The filtrates were collected, combined and added to cold ether (8-10-fold volumes) to precipitate out the peptide. The peptide precipitated was collected by centrifugation, washed with cold ether, and used for the next step without further purification. Purity of H-32 peptide was determined to be ~90% by HPLC and its identity was confirmed by HRMS ( $m/z$   $[M+H]^+$ : Calcd.  $C_{36}H_{60}N_7O_8^+$ , 718.4498; Found: 718.4549).



- Synthesis protocol for attaching AEF to in-house synthesized H-32 peptide substrate as a representative coupling methodology. H-3-Fc, H-6-Fc, and H-15-Fc were similarly synthesized. Characterization of H-3-Fc, H-6-Fc, and H-15-Fc can be found in our previous report<sup>31</sup>, Supporting information S-6, section 4.2.



Firstly, a flask containing 0.020 g (0.0280 mmol) of  $\text{H}_2\text{N}(\text{CH}_2)_4\text{CO}-\text{Nle}-\text{Leu}-\text{Gly}-\text{Phe}-\text{Val}-\text{Ala}-\text{OH}$  (MW = 717.4 g/mol) was dried under vacuum and maintained under argon. After that, 0.5 mL of DMF and 20  $\mu$ L (0.16 mmol) of triethylamine were added, and the solution was stirred for 5 min., followed by the addition of a solution of 0.01 g (0.042 mmol) of di-*tert*-butyl dicarbonate ( $\text{Boc}_2\text{O}$ ) in 0.5 mL of DMF using a syringe under argon. The solution was stirred at room

temperature for 1 h, and HPLC analysis of an aliquot of the solution showed the absence of starting material peptide and only the presence of the desired Boc-protected peptide. The solution was concentrated under reduced pressure for 3 h to give the Boc-protected crude product, which was used without purification in the following reaction. To the aforementioned *N*-Boc-HN(CH<sub>2</sub>)<sub>4</sub>CO-Nle-Leu-Gly-Phe-Val-Ala-OH under argon, a solution of 0.014 g (0.037 mmol) of HATU in 1 mL of DMF was added, and the mixture was stirred for 1.5 h at 40 °C. A solution of 12.7 mg (0.048 mmol) of (2-aminoethyl)ferrocene (AEF) HCl salt (neutralized with triethylamine) in 1 mL of DMF was added. The solution was stirred at 40 °C for 12 h, and an aliquot of the reaction mixture was removed and analyzed by HPLC, showing the absence of the starting peptide. The solution was concentrated under reduced pressure for 3 h to give the Boc-protected and AEF functionalized crude product. The reaction mixture was filtered through a Chromafil Xtra PTFE-20/25 syringe filter (0.20 μm) and the filtrate was purified by preparative HPLC using deionized water and acetonitrile (0% to 95% acetonitrile gradient in 60 min; 8 ml/min; the deionized water contains 0.1% of TFA) as the running solvent to give *N*-Boc-HN(CH<sub>2</sub>)<sub>4</sub>CO-Nle-Leu-Gly-Phe-Val-Ala-NH-CH<sub>2</sub>-CH<sub>2</sub>-Fc (Fc = ferrocenyl). After removal of the majority of the eluents (acetonitrile and water), the collected sample from HPLC was freeze-dried to obtain a solid product. Freeze-dried powder, *N*-Boc-HN(CH<sub>2</sub>)<sub>4</sub>CO-Nle-Leu-Gly-Phe-Val-Ala-NH-CH<sub>2</sub>-CH<sub>2</sub>-Fc, was diluted with 8% TFA in dichloromethane (DCM) at 0 °C under argon and gradually warmed to room temperature for 1 hour, and DCM and most of the TFA were removed on a rotary evaporator. The final Boc sample was dissolved in water and freeze-dried to give a yellow/green solid. The HRMS charts are shown below for the final product obtained.

- Characterization of H-32-Fc:



#### HPLC, HRMS spectra of H-32-Fc:

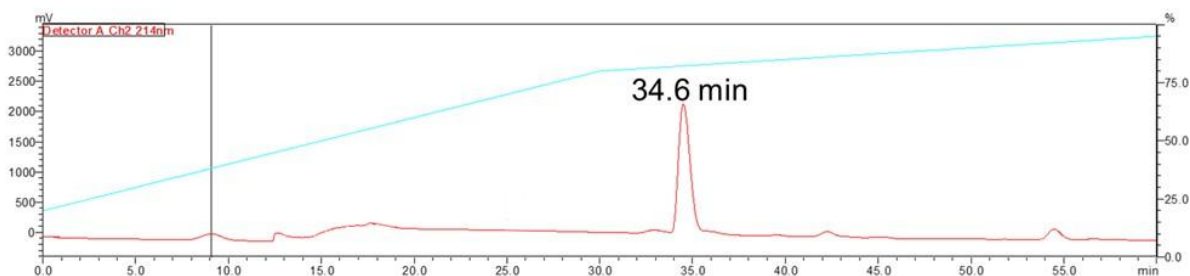
- HPLC column and solvent program used in the analysis of H-32-Fc

Column: PROTO 300-C18 10  $\mu\text{m}$  250 x 10 mm; Phases C4 and C18, Particle Sizes 4.5 and 10  $\mu\text{m}$ , pore size 300Å, pore volume 0.9  $\text{cm}^3/\text{g}$ ; %Carbon (w/w) C4 = 3%, C18 = 8%; gradient elution 0%  $\text{CH}_3\text{CN}$  to 80%  $\text{CH}_3\text{CN}$  within 30 min and up to 95 % within 60 min at 1.0 mL/min flow rate.

- HRMS spectrometer: Waters Xevo G2/XS ToF mass spectrometer.



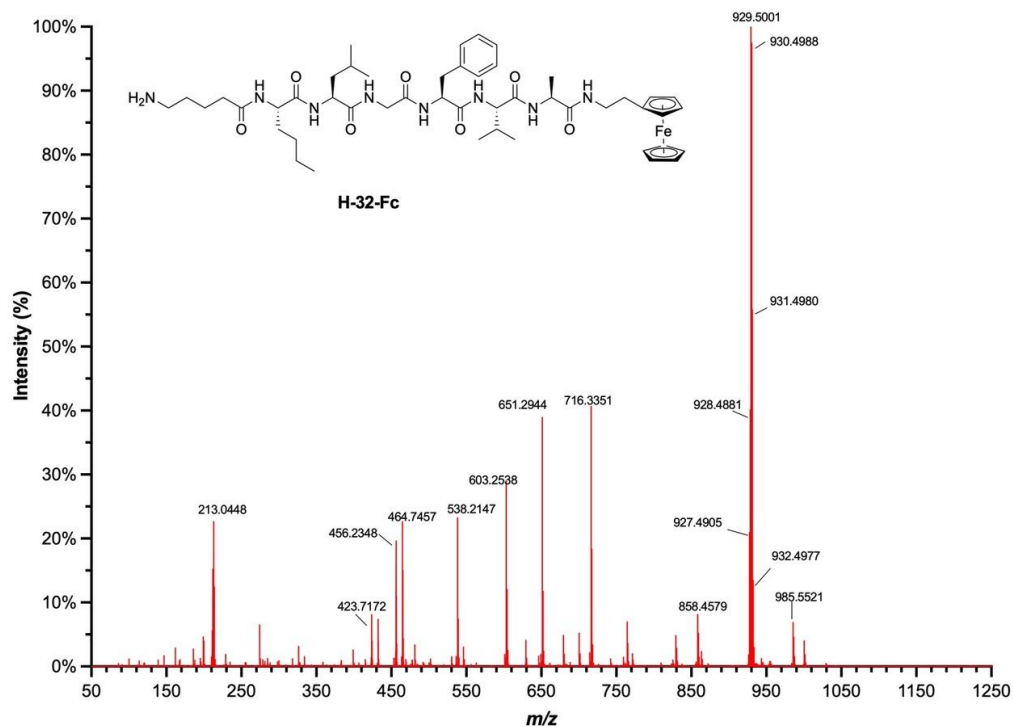
**HPLC: Peak at 34.6 min is H-32-Fc peptide.**



**Appendix Figure B.1** Analytical HPLC chromatogram of the H-3-Fc peptide substrate showing a single major peak at a retention time of 34.6 minutes, confirming the purity and identity of the synthesized peptide.

#### HRMS:

HRMS spectrum is shown below. HRMS (ESI) calculated for  $\text{C}_{48}\text{H}_{73}\text{FeN}_8\text{O}_7^+ [\text{M} + \text{H}]^+$ :  
929.4946, found 929.5001.



**Appendix Figure B.2** High-resolution mass spectrum (HRMS) of the intact H-32-Fc peptide substrate, confirming its expected molecular weight and verifying the identity and purity of the peptide before enzymatic cleavage.

## B.4 Structural Modeling and Molecule Docking

A search of the Protein Data Bank revealed no experimentally resolved structures of active human cathepsin B at near-neutral pH (pH 7.3); structures available at this pH correspond only to the inactive proenzyme and are unsuitable for substrate docking. Therefore, the crystal structure of mature cathepsin B (PDB ID: 1CSB), resolved under acidic conditions (pH  $\approx$  5.0), was used and re-protonated to pH 7.3 following the protocol described below. In addition, a mature cathepsin B structure was generated using AlphaFold2, which represents a more general conformation of the enzyme. This model was likewise prepared with protonation states corresponding to pH 7.3 and used for comparative docking to account for potential pH- and conformation-dependent effects. Structure was predicted using AlphaFold2 implemented via

AlphaFold-ColabFold (version 2.3.5) <sup>153</sup>. The amino acid sequence corresponding to the mature form of cathepsin B was obtained from R&D Systems, consistent with the recombinant human cathepsin B (rhCB) enzyme used in experimental studies. AlphaFold2 prediction yielded a structure with high local confidence, exhibiting a mean predicted Local Distance Difference Test (pLDDT) score of 95, with approximately 90% of residues showing  $pLDDT \geq 90$ , indicative of very high model reliability. Importantly, residues comprising the catalytic triad and core structural regions were located within high-confidence regions ( $pLDDT \geq 90$ ), suggesting accurate backbone and side-chain positioning. Five structural models were generated sequentially, and the highest-ranked model was selected and saved in PDB format for further analysis. Regions at the N- and C-termini, along with several surface-exposed loops, displayed lower confidence ( $pLDDT < 70$ ), consistent with intrinsic flexibility or disorder; these regions were excluded from detailed structural interpretation.

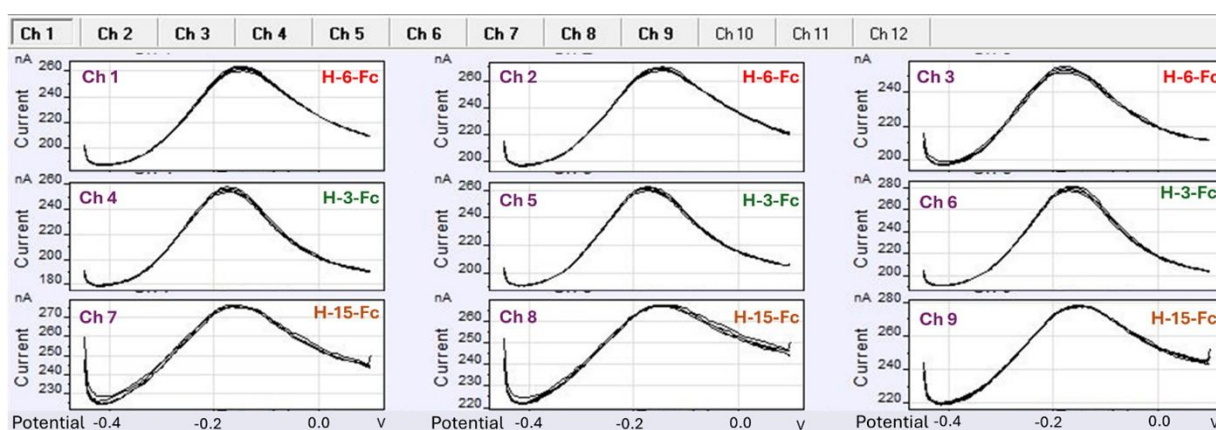
The predicted cathepsin B structure was validated by structural alignment with the crystal structure of active human cathepsin B (PDB ID: 1CSB) <sup>152</sup>. Structural superposition yielded a root-mean-square deviation (RMSD) of 0.405 Å, demonstrating high structural agreement between the 1CSB and predicted model. Both the 1CSB crystal structure (resolution 2.00 Å) and the AlphaFold2-predicted structure were subsequently prepared for docking using the Dock Prep tool in UCSF ChimeraX (version 1.10) <sup>179</sup>.

Further, protonation states and pKa values of ionizable residues were calculated using the H++ web server (version 4.0) <sup>180</sup> at pH 7.3, followed by the protonation and deprotonation, optimization of hydrogen positions, and assignment of atomic charges appropriate for the specified pH. The resulting prepared protein structures were downloaded in PDB format for docking simulations.

The peptide substrate (*Nle-Leu-Gly-Phe-Val-Ala*) was modeled using Avogadro (version 1.2.0)<sup>181</sup> and subjected to energy minimization employing the General AMBER Force Field (GAFF). The minimized ligand structure was saved in mol2 format.

Molecular docking simulations were performed using the CB-Dock2<sup>154</sup> server for both the experimental 1CSB structure and AlphaFold2-predicted cathepsin B. CB-Dock2 identifies potential binding cavities by clustering solvent-accessible surface regions and conducts blind, structure-based docking using the AutoDock Vina algorithm. Docking results were analyzed by visual inspection of ribbon and surface representations. Final molecular graphics and surface-rendered views were generated using UCSF Chimera (version 1.16)<sup>182</sup>.

## B.5 Obtaining multiplex ACV signals from Ivium-Soft v2.561 software

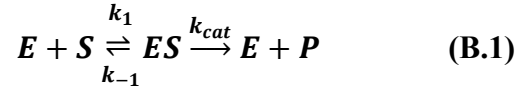


**Appendix Figure B.3** Representative multiplex alternating current voltammetry (ACV) data collected from nine independent microelectrodes on the Au microelectrode array (MEA) chip. The H-3-Fc, H-6-Fc, and H-15-Fc were immobilized on separate electrodes, enabling simultaneous measurement of their electrochemical responses. ACV experiments were performed using an IVIUM n-Stat potentiostat with IviumSoft v2.561, and the resulting voltammograms illustrate the reproducible and well-defined ferrocene redox peaks from each modified electrode.

## B.6 Homogeneous Michaelis-Menten Model for Fluorescence Data Analysis

To describe the time-dependent fluorescence signal generated during proteolytic cleavage of fluorogenic peptide probes, we derived a simplified kinetic model starting from the classical Michaelis–Menten mechanism in homogeneous solution as reported in a previous study<sup>134</sup>.

Proteolytic cleavage of the peptide substrate  $S$  by the enzyme  $E$  is described by the standard mechanism:



Here,  $ES$  is the enzyme–substrate complex, and  $P$  is the fluorescent cleavage product. The rate constants for the associated reactions are  $k_1$ ,  $k_{-1}$ , and  $k_{cat}$ . Using the steady-state assumption, the reaction rate can be expressed as:

$$v = \frac{k_{cat}[E]_0 [S]}{K_M + [S]} \quad (\text{B.2})$$

Assume throughout the time-course the substrate concentration stays much smaller than  $K_M$ :

$$v = \frac{k_{cat}[E]_0 [S]}{K_M} \quad (\text{B.3})$$

But  $[S] = [S]_0 - [P]$ :

$$\frac{dP}{dt} \approx \frac{k_{cat}}{K_M} [E]_0 ([S]_0 - [P]) \quad (\text{B.4})$$

And,

$$k_{obs} \approx \frac{k_{cat}}{K_M} [E]_0 \quad (\text{B.5})$$

After integrating the equation, we have

$$P = S_0 [1 - \exp(-k_{obs} \cdot t)] \quad (\text{B.6})$$

Because the concentration of product  $P$  is directly proportional to the measured fluorescence intensity  $F$ , and the initial substrate concentration  $S_0$  corresponds to the maximum possible fluorescence signal  $F_\infty$  (i.e., the intensity obtained when all substrate molecules are fully cleaved), the exponential solution can be written in its final form as:

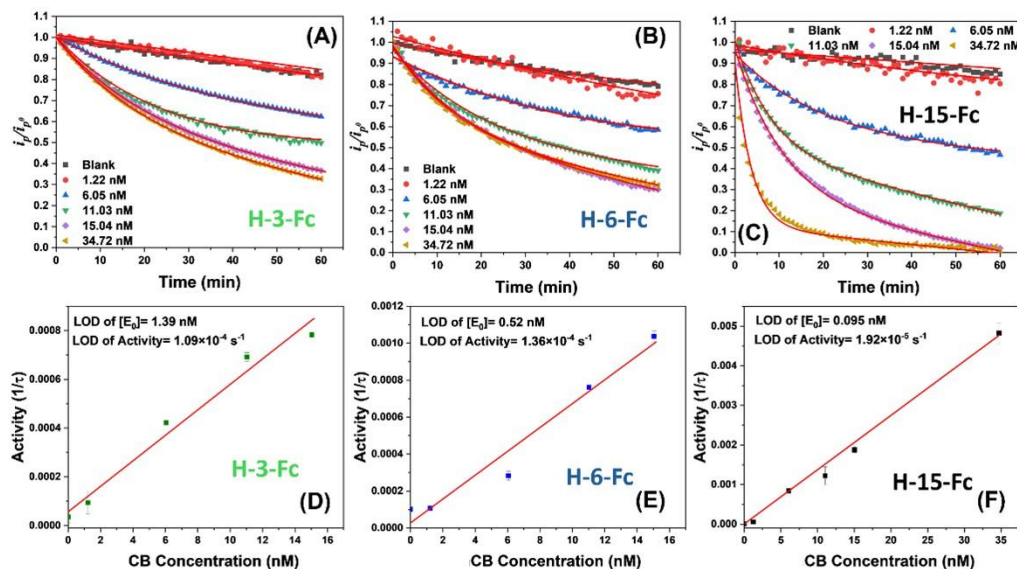
$$F = F_\infty [1 - \exp(-k_{obs} \cdot t)] \quad (\text{B.7})$$

Which can be rearranged as:

$$F = F_{\omega} [1 - \exp(-t/\tau)] \quad (\text{B.8})$$

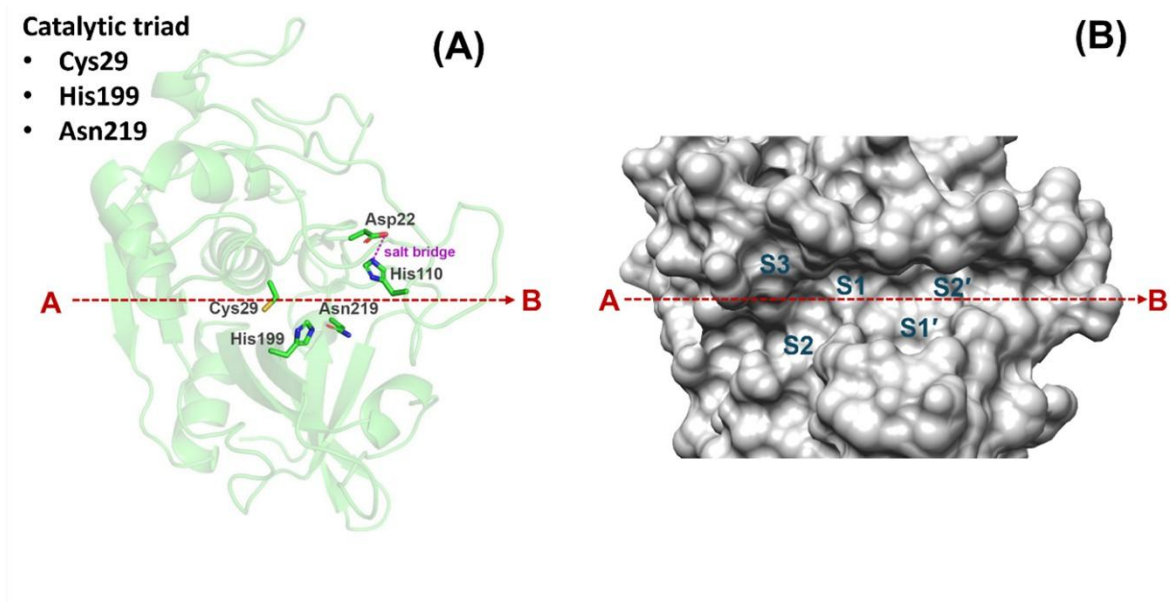
This equation is used to fit the fluorescence intensity data to derive  $1/\tau$  value using the Box-Lucas 1 function in Origin-Pro 2025b software.

## B.7 Assessment of the Limit of Detection in Measuring CB at pH = 5.0 with H-3-Fc, H-6-Fc, and H-15-Fc on Au MEA



**Appendix Figure B.4** (A-C) The representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the hexapeptide-Fc substrates H-3-Fc, H-6-Fc and H-15-Fc, respectively in activated CB (DTT removed after activation), concentrations of 0, 1.22, 6.05, 11.03, 15.04 and 34.72 nM in 25 mM MES, 50  $\mu\text{M}$  EDTA (pH = 5.0). (D-F) The linear calibration curves of CB protease activity in the activated CB with the three hexapeptide-Fc substrates H-3-Fc, H-6-Fc, and H-15-Fc. The error bars represent the standard deviation of three replicates for each measurement.

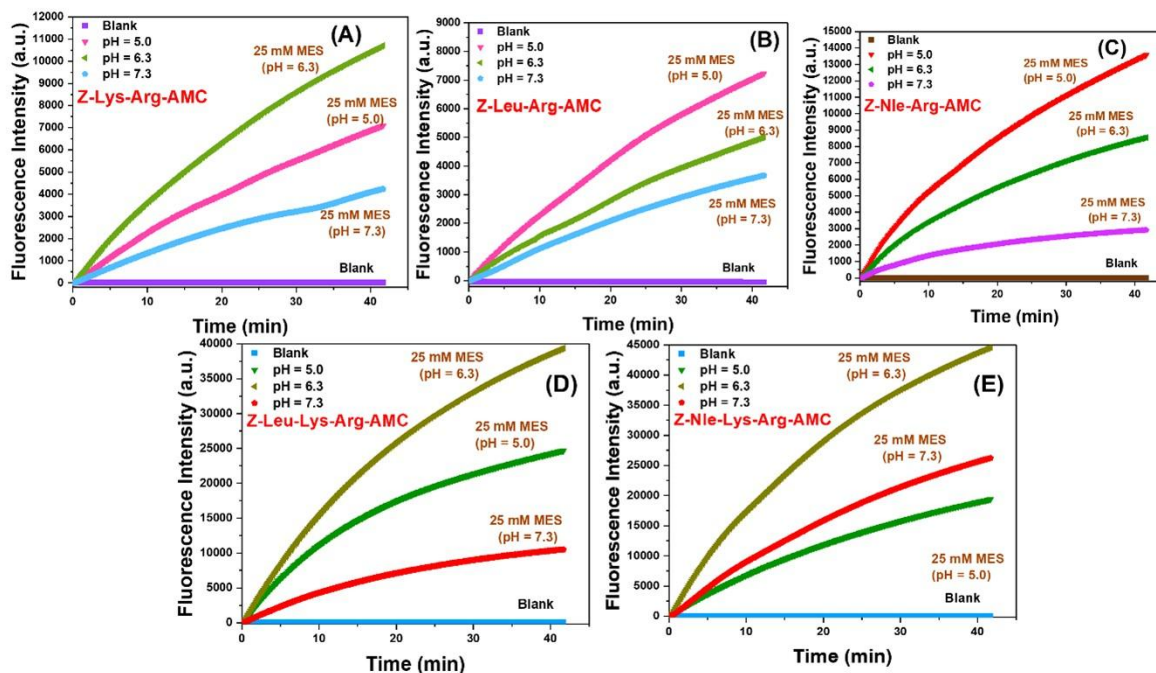
## B.8 Cathepsin B: Active Site Representation



**Appendix Figure B.5** (A) Illustrative representation of catalytic triad (Cys29, His199, Asn219) of active human cathepsin B and possible salt bridge formation between His110 and Asp22 (B) Surface view of the active site cleft of active human cathepsin B showing its S3, S2, S1, S1' and S2' subsites that interact with substrate's P3, P2, P1, P1' and P2' residues respectively.

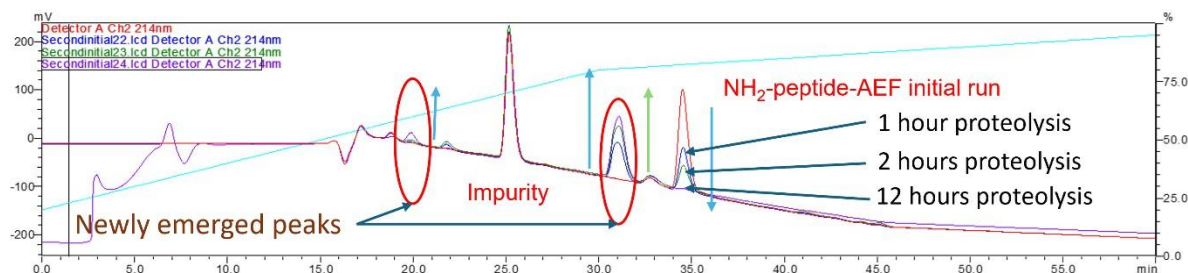
## B.9 Fluorogenic assays with Z-peptide-AMC substrates to monitor cathepsin

### B activity, in different pH conditions

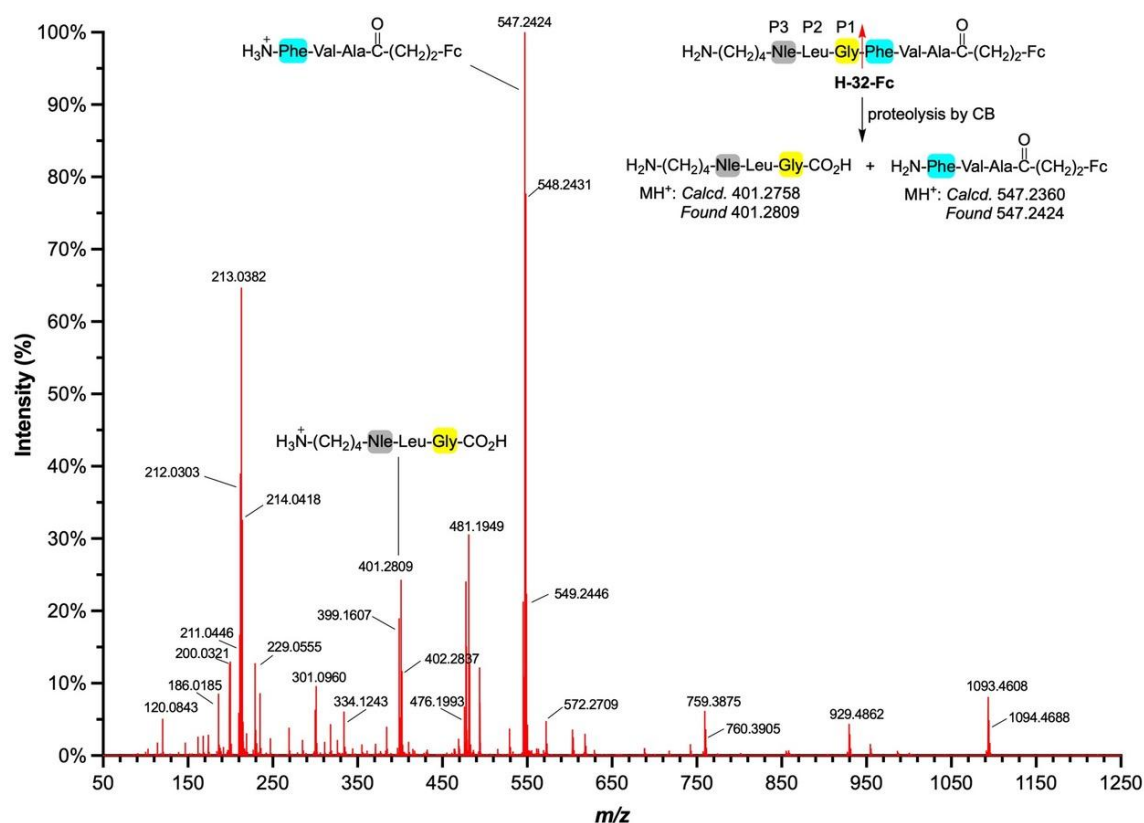


**Appendix Figure B.6** Z-Peptide-AMC (100  $\mu$ M) substrates that can monitor CB (3.45 nM) activity, illustrated by fluorescence intensity in different pH conditions. Cathepsin B activity with (A) Z-Lys-Arg-AMC, (B) Z-Leu-Arg-AMC, (C) Z-Nle-Arg-AMC, (D) Z-Leu-Lys-Arg-AMC, and (E) Z-Nle-Lys-Arg-AMC substrates at pH 7.3, pH 6.3, and pH 5.0 in 25 mM MES buffer was evaluated over 40 mins.

## B.10 Proteolysis of H-32-Fc peptide substrate with CB and cleavage site determination

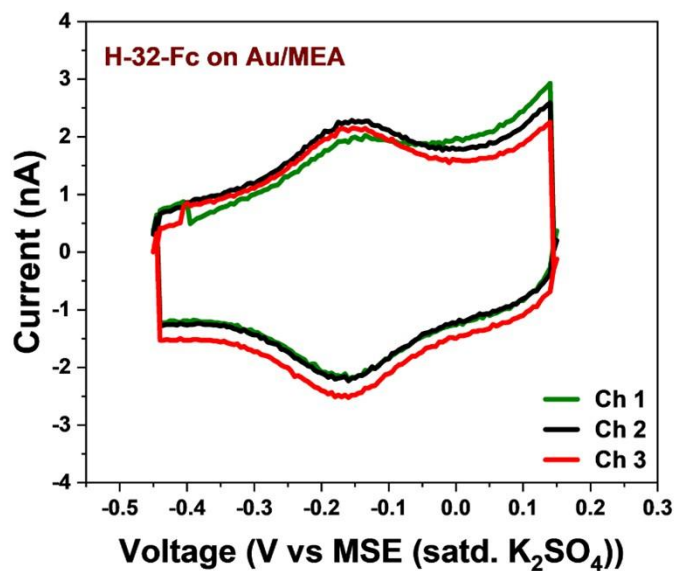


**Appendix Figure B.7** Proteolysis analysis by analytical HPLC for H-32-Fc after mixing with cathepsin B for different proteolysis times. The intact peptide appeared at 34.6 min retention time, and two new peaks appeared at 19.5 min and 31.1 min retention time, corresponding to the cleaved products.



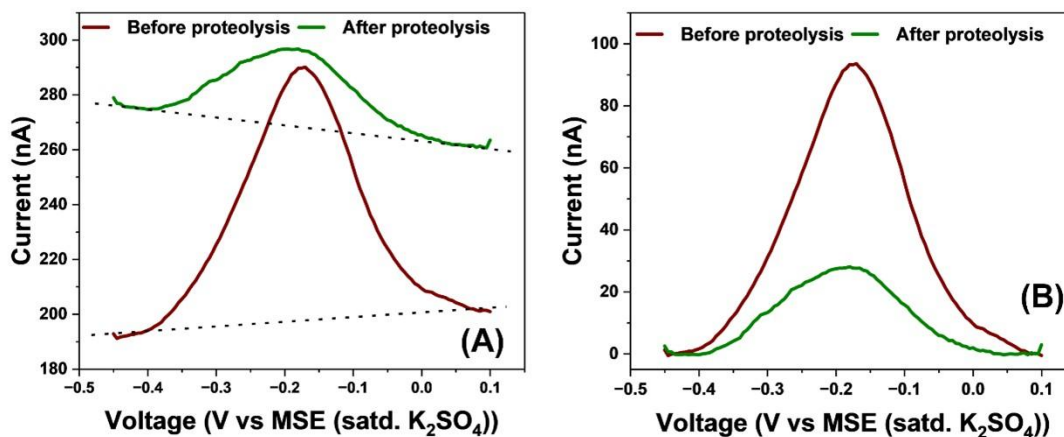
**Appendix Figure B.8** High-resolution mass spectrum (HRMS) of the cleaved crude products generated from H-32-Fc following proteolysis by CB in 25 mM MES buffer (pH 7.3) containing 50  $\mu$ M EDTA. The observed product ions correspond to the expected fragments produced by cleavage at the Gly–Phe bond, confirming the specific proteolytic site within the peptide sequence.

**B.11 Representative CV curves for H-32-Fc functionalized on Au MEA in 25 mM MES, 50  $\mu$ M EDTA, pH = 7.3**



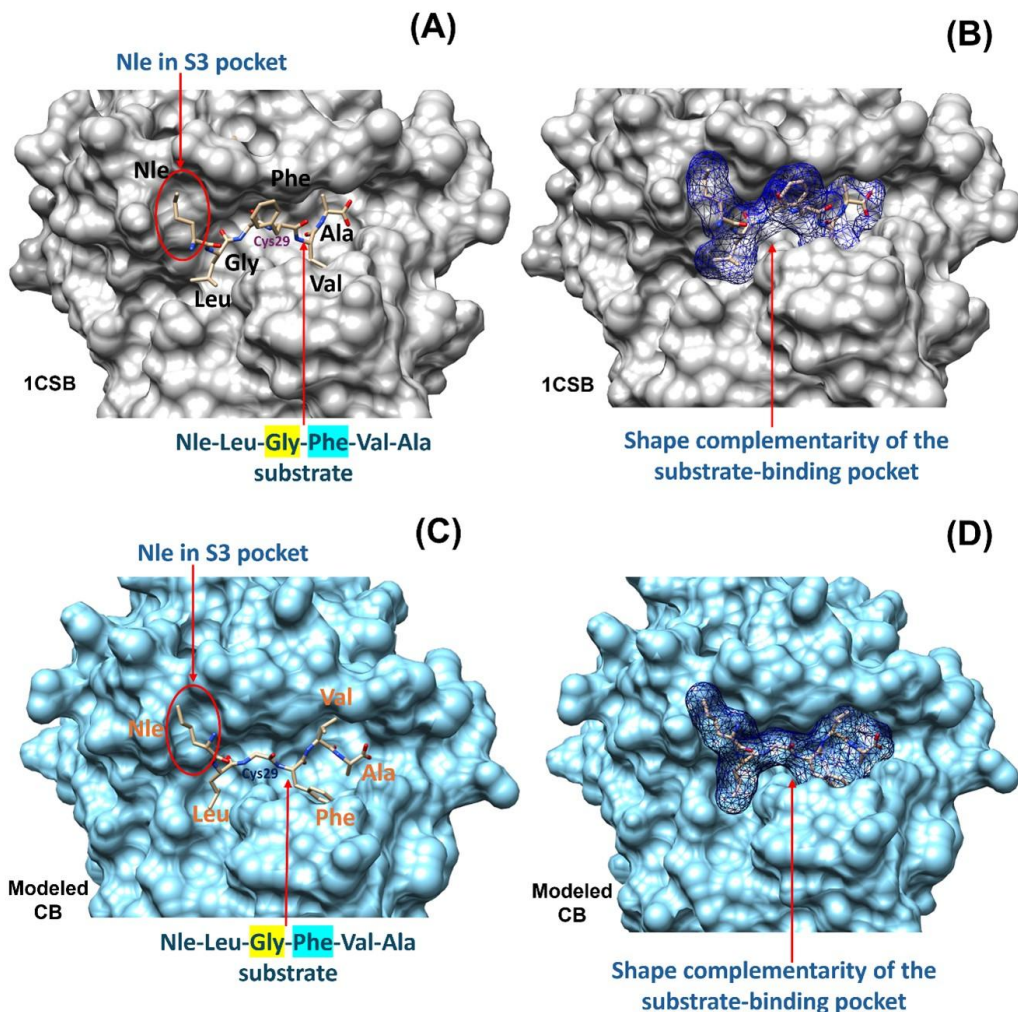
**Appendix Figure B.9** Representative CV curves of H-32-Fc functionalized on SAM-covered Au MEA in 25 mM MES, 50  $\mu$ M EDTA, pH = 7.3.

**B.12 Representative ACV curves for H-32-Fc functionalized on Au-MEA in 25 mM MES, 50  $\mu$ M EDTA, pH = 7.3, before and after proteolysis**



**Appendix Figure B.10** Representative ACV curves for H-32-Fc functionalized on SAM-covered Au MEA in 25 mM MES, 50  $\mu$ M EDTA, pH = 7.3. (A) Raw data and linear baselines (dashed lines) of ACV obtained before (brown) and after (green) proteolysis. (B) The processed ACV curves after baseline subtraction before (brown) and after (green) proteolysis.

## B.13 Molecular docking of cathepsin B protein and Nle-Leu-Gly-Phe-Val-Ala peptide substrate



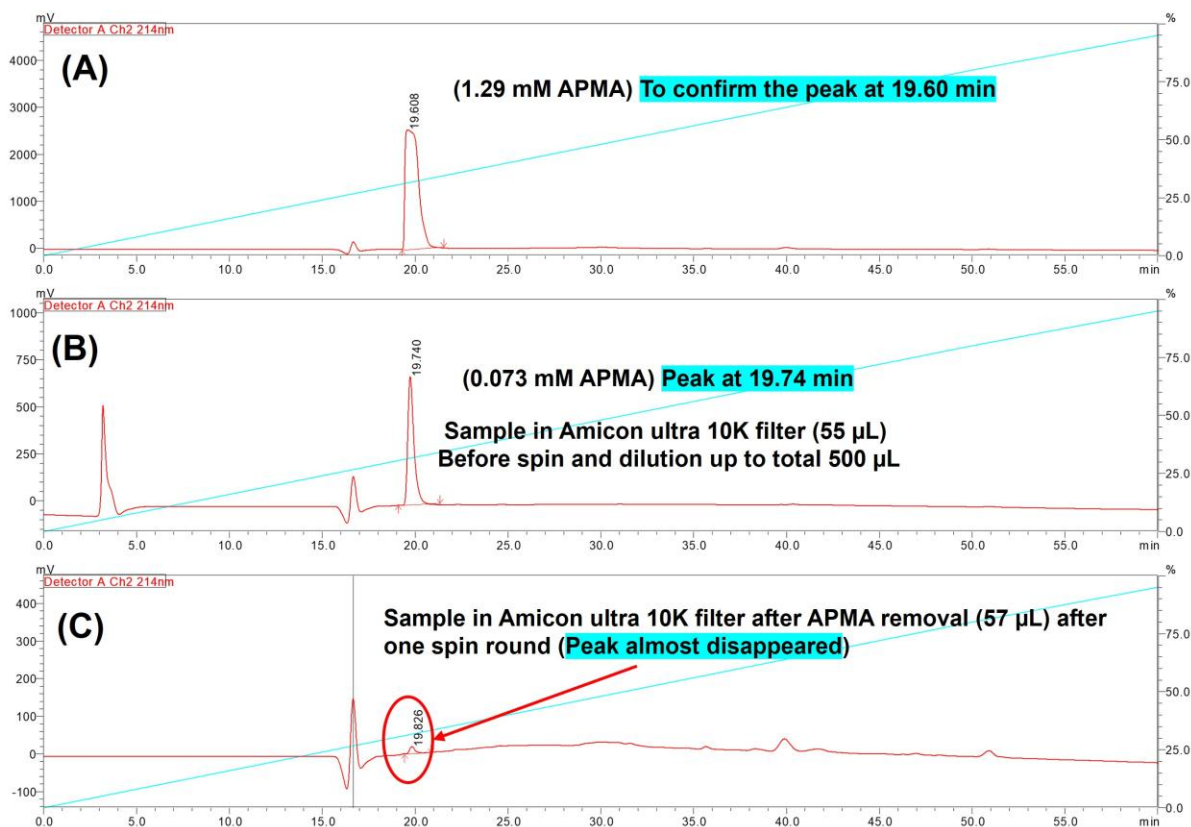
**Appendix Figure B.11** Molecular docking of the peptide substrate Nle-Leu-Gly-Phe-Val-Ala with Cathepsin B. (A) Docking pose of the peptide within the active-site pocket of the 1CSB, shown as a surface representation (gray), with the peptide displayed as sticks. The substrate occupies the catalytic groove and aligns along the S subsites, positioning the putative scissile bond (Gly-Phe) proximal to the catalytic residue Cys29. (B) Surface representation of the 1CSB (gray), highlighting shape complementarity of the substrate-binding pocket; the peptide is shown as sticks with a semi-transparent mesh (blue). (C) Docking pose of the peptide within the active-site pocket of AlphaFold2 modeled cathepsin B, shown as a surface representation (sky blue), with the peptide displayed as sticks and adopting a similar binding orientation along the S subsites. (D) Surface representation of AlphaFold2 modeled cathepsin B (sky blue), highlighting shape complementarity of the substrate-binding pocket, with the peptide shown as sticks and a semi-transparent mesh (blue).

## **Appendix C - Supplementary Information for Chapter 4**

### **C.1 Materials and Instruments**

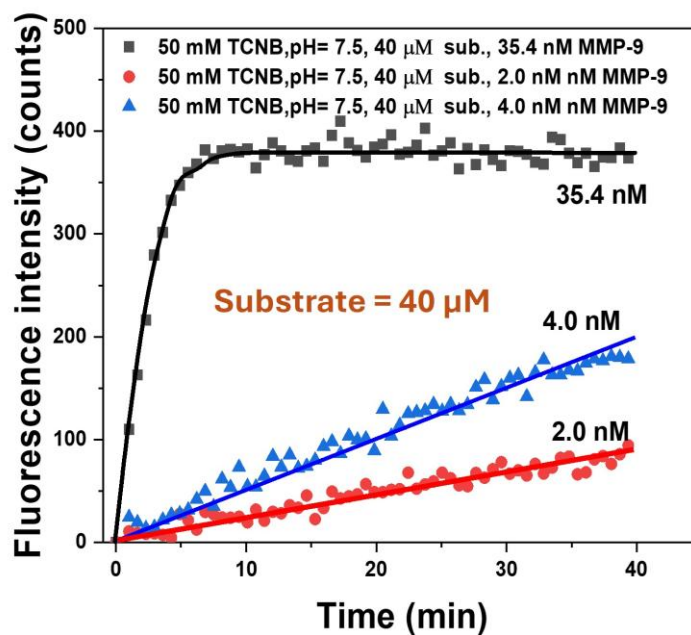
Information on the peptide substrates, common buffer, and modified buffer compositions is provided in **Appendix B (Materials and Methods)**. In addition to those reagents, the following chemicals were used in this study. Carrier-free recombinant human MMP-9 in its inactive form was obtained from R&D Systems Inc. (Minneapolis, MN, USA). A separate batch of activated recombinant human MMP-9, with APMA removed, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The MMP-specific fluorogenic substrate, Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH<sub>2</sub>, was purchased from MedChemExpress (Monmouth, NJ, USA). Pooled human serum obtained from the clot (catalog no. ISER10ML, Innovative Research, Novi, MI, USA) was used for validation studies. The serum was derived from whole human blood by allowing coagulation to occur, followed by centrifugation to separate the serum fraction. The collected supernatant was immediately frozen after processing and stored until use.

## C.2 Confirmation of APMA removal using 10k Amicon Ultra filter



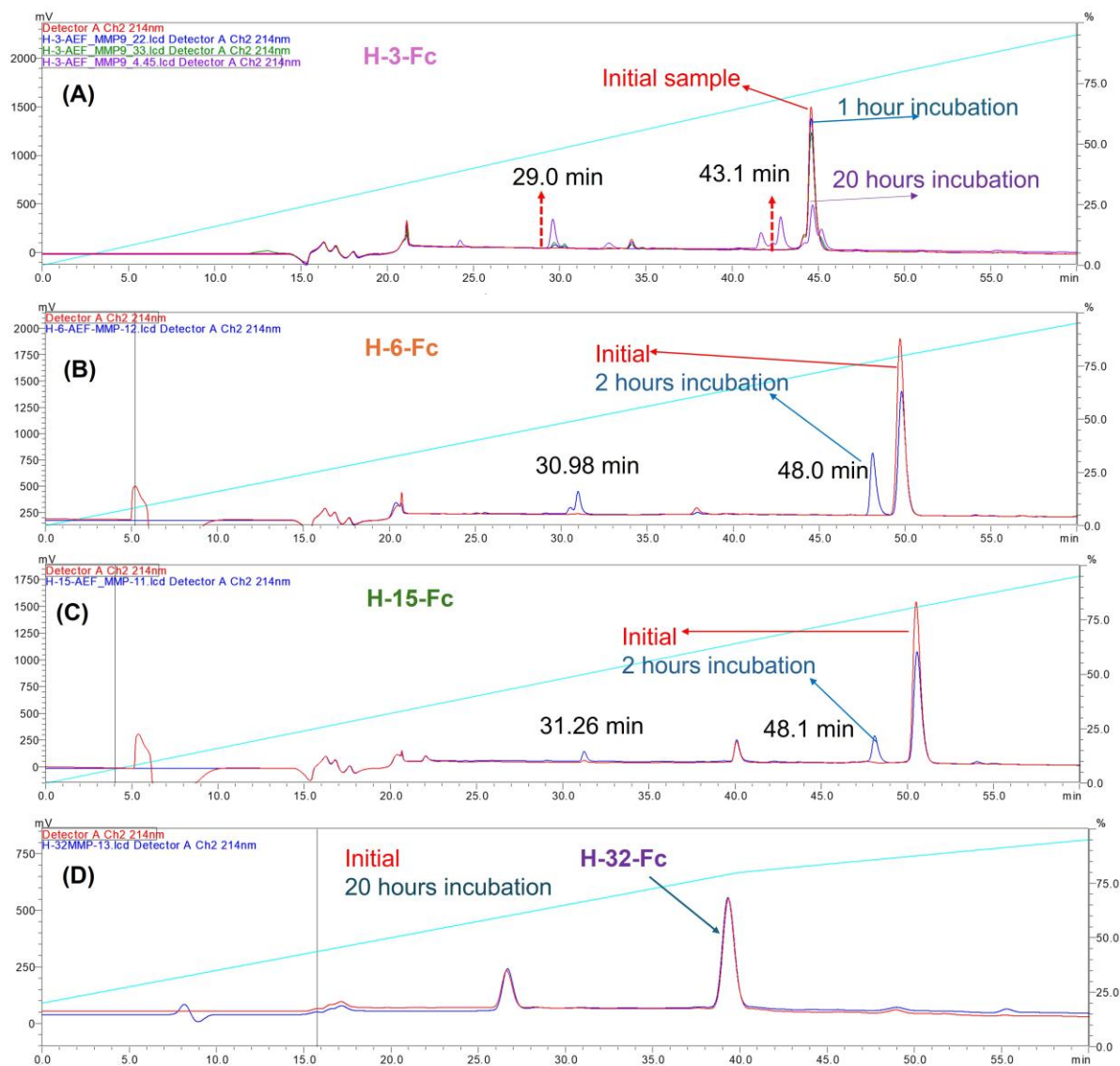
**Appendix Figure C.1** HPLC analysis confirming APMA removal using a 10k Amicon Ultra centrifugal filter. (A) Analytical HPLC chromatogram of 1.29 mM APMA (50  $\mu$ L injection) showing a distinct peak at 19.60 min under a 0–95% acetonitrile gradient (60 min, 1.0 mL min<sup>-1</sup>, detection at 214 nm), establishing the retention time of APMA. (B) Chromatogram of 0.073 mM APMA (50  $\mu$ L injection), corresponding to the concentration used after MMP-9 activation. A clear peak is observed at 19.74 min with an intensity of approximately 700 mV, confirming detectability at lower concentrations. (C) Chromatogram of the same APMA sample after dilution (to 500  $\mu$ L) and centrifugal filtration using a 10k Amicon Ultra filter (14,000 g, 5 min; final volume 57  $\mu$ L). The APMA peak at ~19.7 min is nearly eliminated, with only a trace signal (~35 mV) remaining, demonstrating efficient removal of APMA by the filtration process.

### C.3 Investigation of Apparent Signal Saturation in FRET Assays and Long-Term Activity of MMP-9

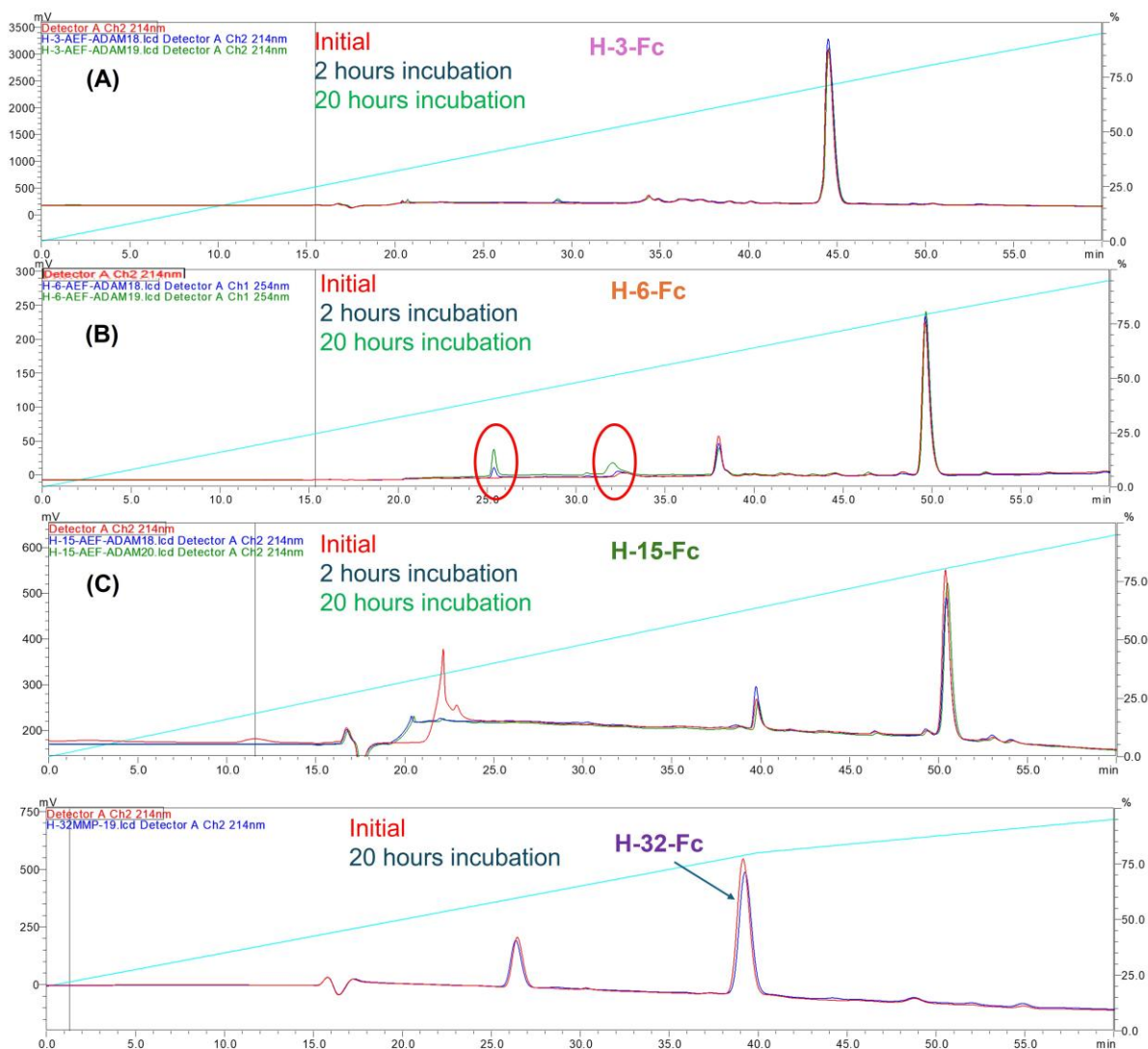


**Appendix Figure C.2** Effect of substrate-to-enzyme ratio on MMP-9 FRET kinetics and signal saturation behavior. Fluorescence intensity as a function of time for cleavage of the FRET substrate (Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub>, 40  $\mu$ M) by MMP-9 at different enzyme concentrations (2.0 nM, 4.0 nM, and 35.4 nM).

## C.4 Investigation of the cleavage sites of H-3-Fc, H-6-Fc, H-15-Fc and H-32-Fc peptide substrates by MMP-9 using analytical HPLC

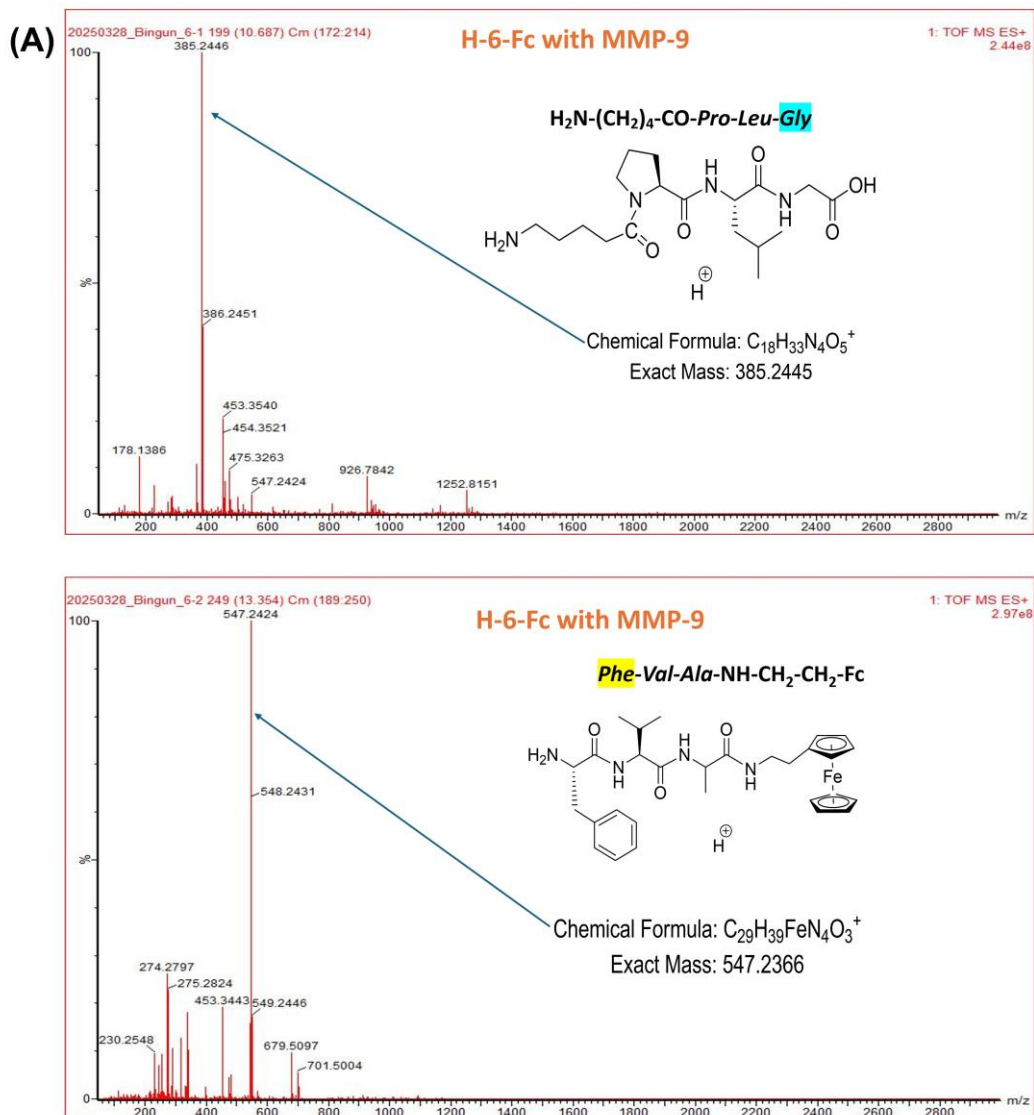


**Appendix Figure C.3** Analytical HPLC chromatograms of peptide substrates after incubation with proteases to identify cleavage sites responsible for the observed electrochemical signal decay. Peptides H-3-Fc (A), H-6-Fc (B), H-15-Fc (C), and H-32-Fc (D) (1 mM) were incubated with MMP-9 (15 nM) in TCNB buffer (pH 7.5). Aliquots collected at the initial time point and after 2 hours of incubation were analyzed by analytical HPLC. Newly emerged peaks corresponding to proteolytic fragments are indicated in the chromatograms, allowing identification of cleavage products for each peptide substrate. Chromatograms for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc are shown in panels A-D, respectively.

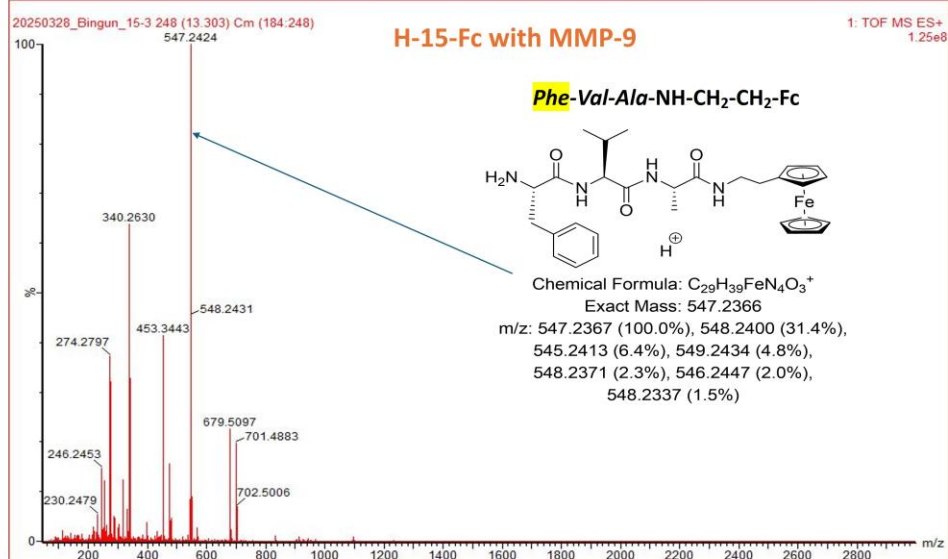
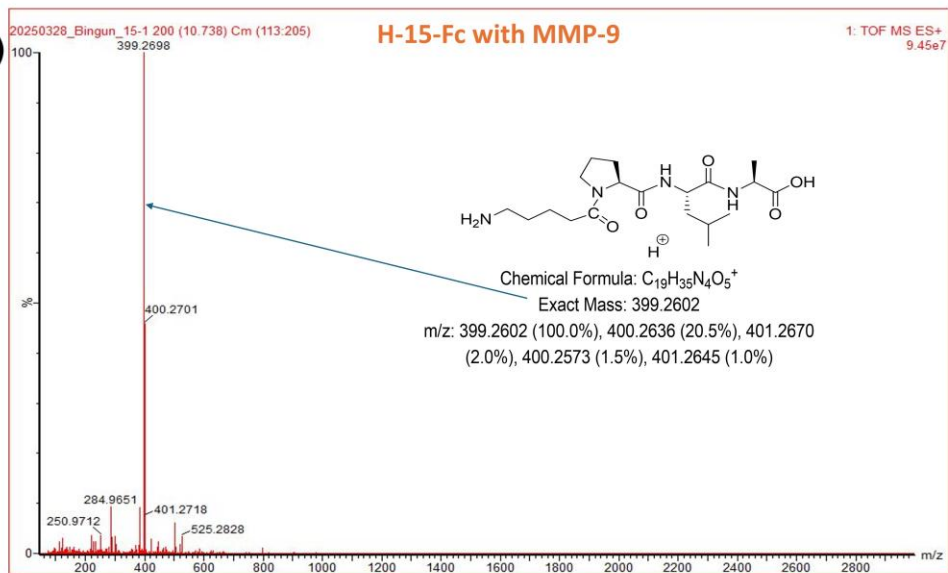


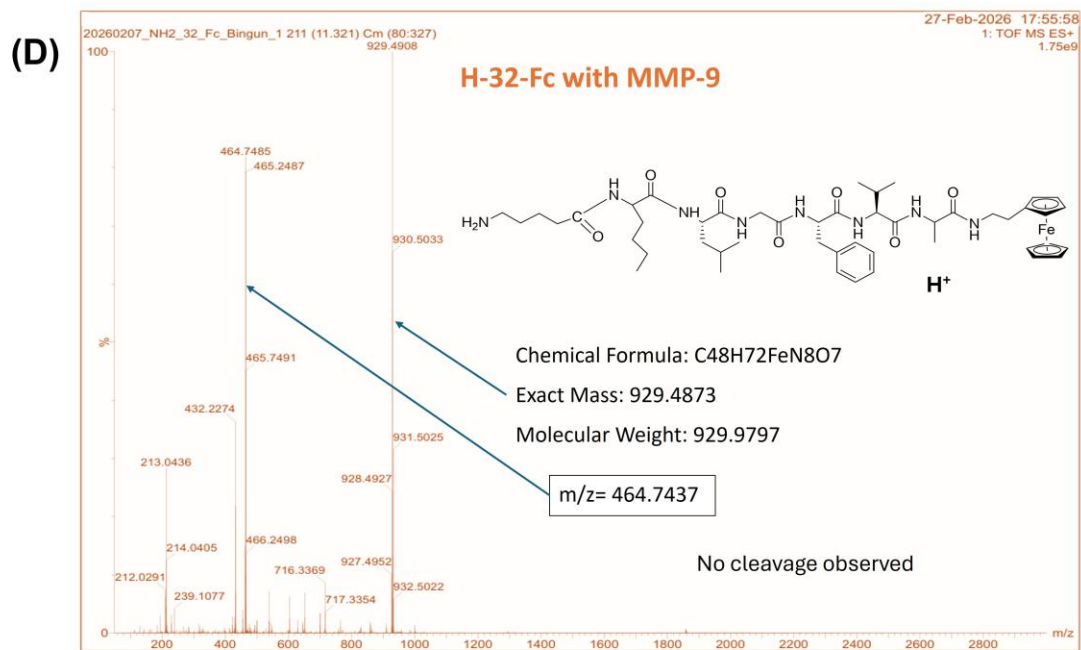
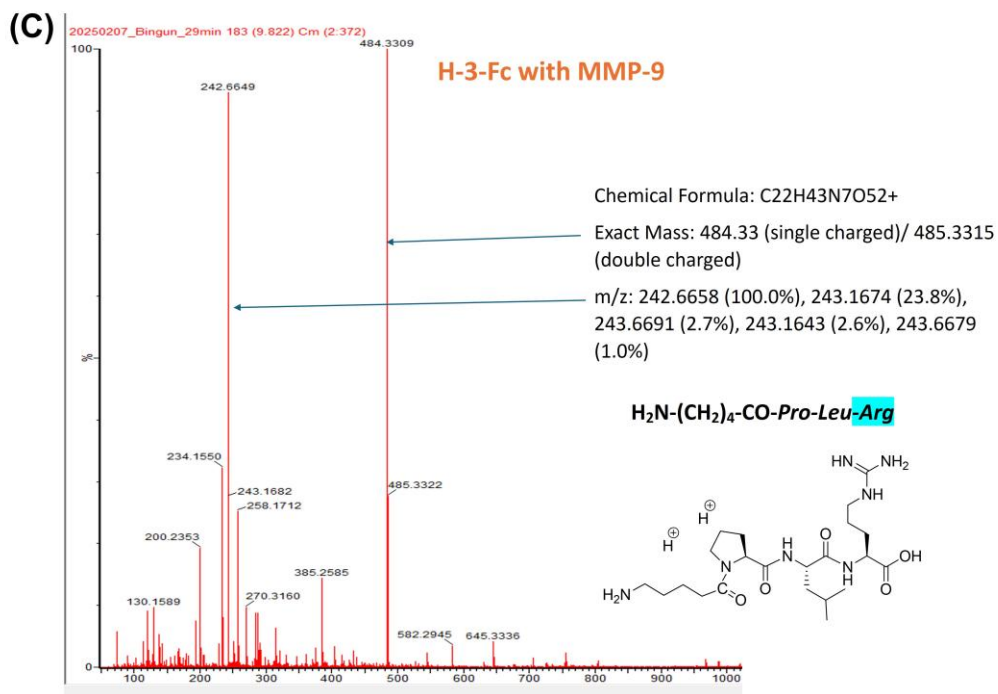
**Appendix Figure C.4** Analytical HPLC chromatograms of peptide substrates after incubation with proteases to identify cleavage sites responsible for the observed electrochemical signal decay. Peptides H-3-Fc (A), H-6-Fc (B), H-15-Fc (C), and H-32-Fc (D) (1 mM) were incubated with ADAM-17 (30 nM) in Tris buffer (pH 9.0). Aliquots collected at the initial time point and after 2 hours of incubation and 20 hours were analyzed by analytical HPLC. Newly emerged peaks corresponding to proteolytic fragments are indicated in the chromatograms, allowing identification of cleavage products for each peptide substrate. Chromatograms for H-3-Fc, H-6-Fc, H-15-Fc, and H-32-Fc are shown in panels A-D, respectively.

## C.5 Investigation of the cleavage sites of H-3-Fc, H-6-Fc, H-15-Fc and H-32-Fc peptide substrates by MMP-9 using HRMS

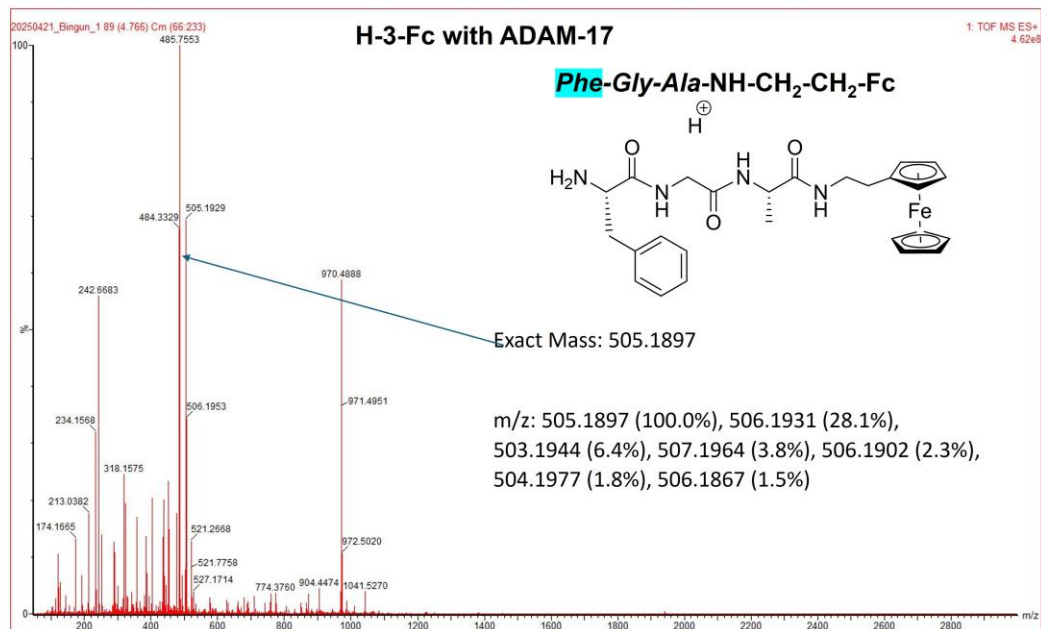
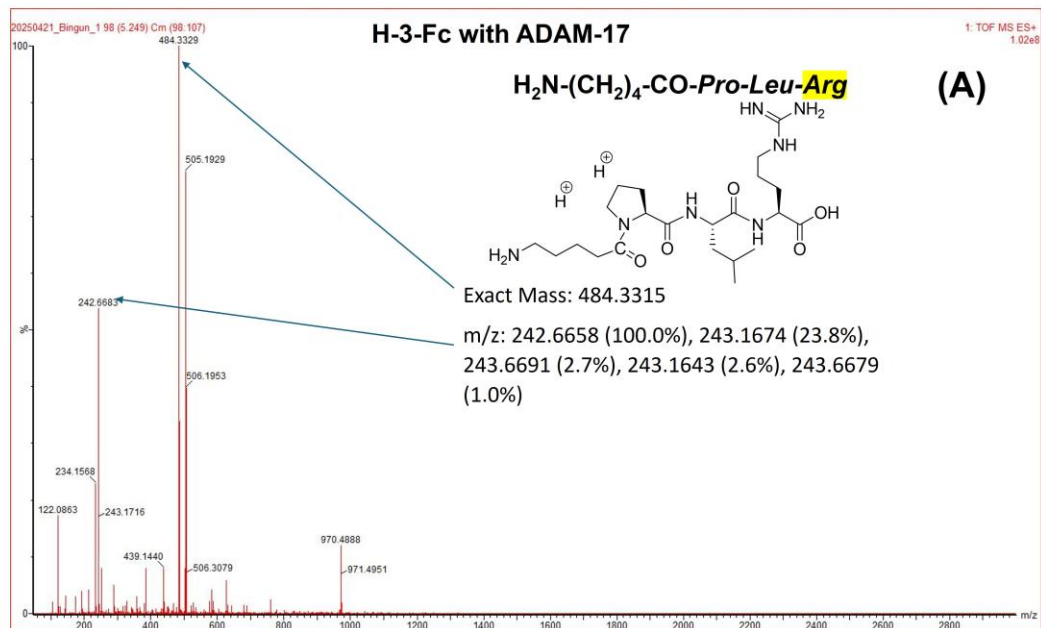


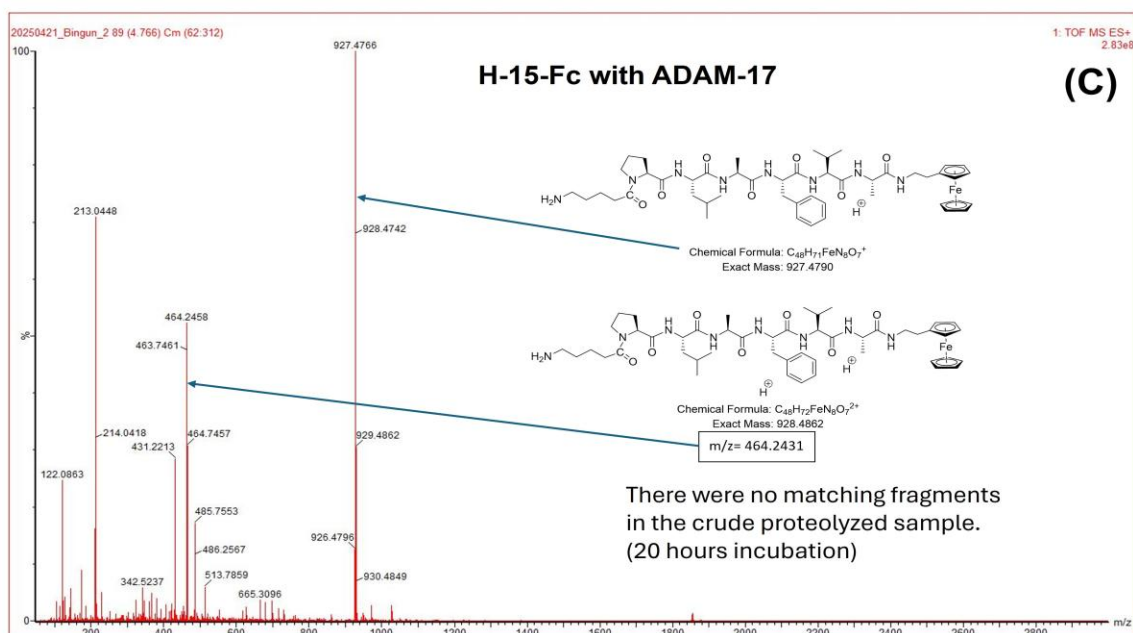
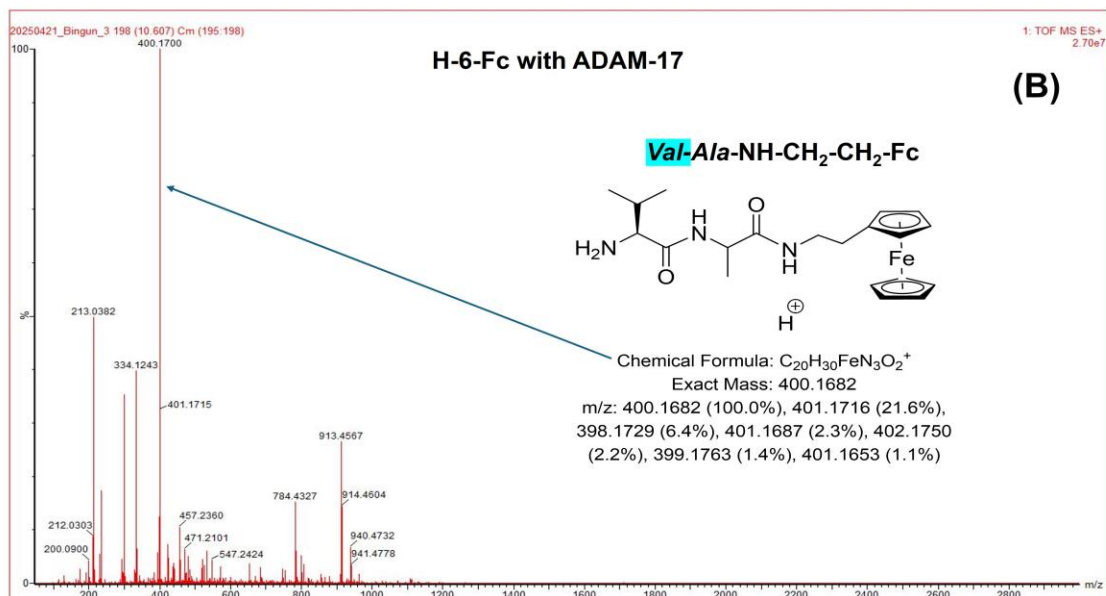
(B)





## C.6 Investigation of the cleavage sites of H-3-Fc, H-6-Fc, H-15-Fc and H-32-Fc peptide substrates by ADAM-17 using HRMS





(D)

