

Quantitative Detection of Cathepsin B Activity in Neutral pH Buffers Using Gold Microelectrode Arrays: Toward Direct Multiplex Analyses of Extracellular Proteases in Human Serum

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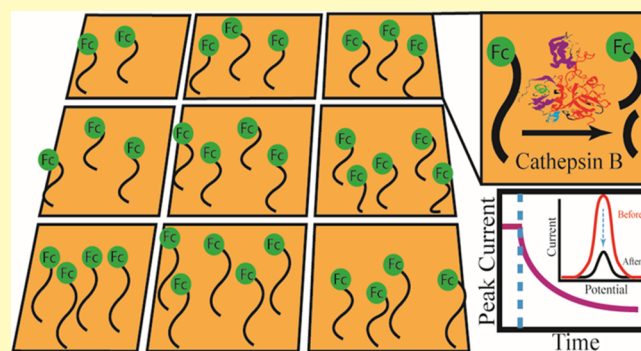
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ABSTRACT: Proteases are critical signaling molecules and prognostic biomarkers for many diseases including cancer. There is a strong demand for multiplex bioanalytical techniques that can rapidly detect the activity of extracellular proteases with high sensitivity and specificity. This study demonstrates an activity-based electrochemical biosensor of a 3×3 gold microelectrode array for the detection of cathepsin B activity in human serum diluted in a neutral buffer. Proteolysis of ferrocene-labeled peptide substrates functionalized on $200 \times 200 \mu\text{m}$ microelectrodes is measured simultaneously over the nine channels by AC voltammetry. The protease activity is represented by the inverse of the exponential decay time constant ($1/\tau$), which equals to $(k_{\text{cat}}/K_{\text{M}})[\text{CB}]$ based on the Michaelis–Menten model. An enhanced activity of the recombinant human cathepsin B (rhCB) is observed in a low-ionic-strength phosphate buffer at pH = 7.4, giving a very low limit of detection of $8.49 \times 10^{-4} \text{ s}^{-1}$ for activity and 57.1 pM for the active rhCB concentration that is comparable to affinity-based enzyme-linked immunosorbent assay (ELISA). The cathepsin B presented in the human serum sample is validated by ELISA, which mainly detects the inactive proenzyme, while the electrochemical biosensor specifically measures the active cathepsin B and shows significantly higher decay rates when rhCB and human serum are activated. Analyses of the kinetic electrochemical measurements with spiked active cathepsin B in human serum provide further assessment of the protease activity in the complex sample. This study lays the foundation to develop the gold microelectrode array into a multiplex biosensor for rapid detection of the activity of extracellular proteases toward cancer diagnosis and treatment assessment.

KEYWORDS: cathepsin B, enzyme-linked immunosorbent assay, extracellular protease analysis, human serum, microelectrode array, multiplex detection, protease activity profiling



Proteases play important roles as protein-degrading enzymes in many metabolic processes, including immune response, wound healing, food digestion, cell cycle, and protein recycling.¹ Proteases hydrolyze proteins based on recognition of specific peptide sequences. They also act as key signaling molecules in progression of many diseases such as cancers, neurodegenerative diseases, cardiovascular diseases, and other inflammatory diseases.¹ For example, aberrant overexpression of proteases has been reported in breast cancer, colorectal cancer, gastric cancer, and prostate cancer.^{2–4} It is well-known that proteases play diverse roles in tumor growth, invasion, and metastasis.^{2,4} Several matrix metalloproteinases (MMPs) – a type of proteases, such as MMP-2 and MMP-9, can affect signaling pathways and growth factors to enhance tumor growth.³ Numerous proteases, including cathepsins, kallikreins, and other serine proteases, facilitate the spread of cancer to distant organs by degrading the extracellular matrix.^{5–8} For example, proteases can degrade E-cadherin, a tumor suppressor

and an essential protein in the formation of adherent junctions to bind cells with each other.⁹ Small-molecule drugs targeting protease inhibition have attracted great attention for cancer treatment.¹⁰ However, it remains a great challenge to understand the complex protease signaling and develop specific inhibitors. First, there are about 600 known proteases in humans, and they interact with each other in a complex network.^{1,10} Second, individual proteases have very limited indicative value because they only represent one aspect of carcinogens.¹¹ Third, the protease levels in humans are

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extremely low. Hence, there is a strong demand for developing highly sensitive and highly specific bioanalytical techniques that can detect a group of protease biomarkers in parallel rather than a single one each time. Particularly, the activity of proteases in extracellular space is significantly reduced and subject to rapid inactivation.¹² Developing new techniques that can directly detect extracellular protease activity is critical for disease diagnostics.^{3,13} Here, we demonstrate an electrochemical method based on a peptide-functionalized gold microelectrode array (MEA) for direct detection of extracellular cathepsin B activity in human serum (HS) in a neutral pH buffer, which has the potential to analyze multiple protease activities simultaneously.

Cathepsin B is a member of the cysteine cathepsin family consisting of 11 lysosomal hydrolases. It is linked to general protein degradation in lysosomes. Initially, cathepsin B is synthesized on the rough endoplasmic reticulum as a proenzyme consisting of 339 amino acids with a signal peptide of 17 amino acids. After post-translational modification, proenzyme cathepsin B undergoes autocatalytic activation (normally under mild acidic conditions) and converts into mature cathepsin B by proteolytic cleavage and dissociation of the blocking peptide.^{1,12,14} Increased cathepsin B levels have been observed in many types of cancer such as prostate cancer, melanoma cancers, and breast cancer.^{15–18} Its activity is essential for tumor migration, invasion, and metastases.^{17,19–21} However, the link between the cathepsin B concentration and activity in these studies is not clear. In light of the above studies, developing a rapid, sensitive, and specific method for detecting cathepsin B activity is critical for cancer diagnosis and therapeutic efficacy assessment.

Currently, protease detection can be classified into two broad categories, i.e., affinity-based and activity-based techniques.²² The affinity-based technique detects the protease concentration by capturing proteases using the specific probe-target affinity such as enzyme-linked immunosorbent assay (ELISA) and aptamer sensors. Although ELISA has very high selectivity and sensitivity, it is time-consuming, expensive, and can only be operated in the laboratory by skilled personnel. Furthermore, it does not provide the activity of target proteases because both the proenzyme and mature enzyme may have common epitopes that can bind to the antibodies. Missing information of the important biological functions of the proteases limits its effectiveness in cancer diagnosis. The activity-based analyses primarily focus on detecting the biological function of proteases, i.e., the proteolysis rate of the peptide substrate by the cognate proteases. This category of analysis is more relevant to cancer progression. Activity-based analyses preclude proenzymes that do not have any preformed active site or whose preformed active sites are blocked by a peptide unit.¹ Only the active mature proteases with the binding pocket exposed can be detected. For instance, a group of sensors known as activity-based probes (ABPs) can covalently bind with a protease's active site through an addition or displacement of a warhead of the substrate peptide, which are useful for *in vivo* imaging or immunoblotting analyses.^{23,24} Fluorogenic techniques based on fluorescence resonance energy transfer (FRET) are another type of activity-based biosensor for protease detection. A pair of fluorescence donor (fluorophore) and acceptor (quencher) is covalently attached within a short distance (<10 nm) at the opposite sides of the cleavage site in the peptide substrate.^{22,24} FRET occurs when the emission spectrum of the donor overlaps with the

absorption spectrum of the acceptor. The acceptor and donor are separated when the peptide substrate is hydrolyzed by proteases and thus the fluorescence emission of the donor is restored. The rise of the fluorescence intensity can be recorded in real-time to reveal the kinetics of the proteolysis process. Such fluorogenic techniques are highly sensitive and selective. However, the fluorescence signal can be easily affected by auto-fluorescence or quenching of indigenous molecules, especially in a complex biological sample such as plasma or cell lysates. Moreover, the broad spectra of fluorophores easily overlap with each other and limit the capability for multiplex detections.

Electrochemical biosensors are recognized as a cost-effective sensor platform with high sensitivity, fast response, and the capability for high-degree miniaturization and multiplex detection.^{25,26} Electrochemical detection of proteases has been demonstrated in both affinity and activity formats.^{22,27,28} Liu *et al.* first demonstrated electrochemical detection of MMP-7 through its specific proteolytic cleavage of a ferrocene (Fc)-labeled helical peptide consisting of nine amino acids that was immobilized on a gold electrode.²⁸ Detection of other proteases such as MMP-9,²⁹ trypsin,³⁰ plasmin,³¹ caspase 3,³² etc. has also been reported using peptide-functionalized electrodes. In a series of studies based on Fc-labeled short peptides functionalized on nanoelectrode arrays made of embedded vertically aligned carbon nanofibers (VACNFs), we demonstrated detection of cathepsin B in pure buffer solutions,^{33,34} tissue lysates,³⁵ and cell lysates.³⁶ More importantly, a heterogeneous Michaelis–Menten model was developed to fit the experimentally measured kinetic proteolysis curves and derive the fundamental kinetic constants.^{34,35} This algorithm enables determination of the effective activity of cathepsin B in addition to measurement of the concentration as reported in other electrochemical studies.

Recently, we have fabricated a 3 × 3 gold MEA and demonstrated its capability for multiplexed detection of cathepsin B activity using three specific peptide substrates functionalized on separate microelectrodes.³⁷ Highly consistent proteolysis results have been obtained over nine independent channels simultaneously. In principle, the Au MEA can be applied for multiplex detection of distinct proteases by functionalizing highly selective peptide substrates on designated microelectrodes. Here, we report further development of this Au MEA platform toward measuring the activity profile of multiple extracellular cancer-related proteases in human serum (HS). A big challenge toward this goal is that different protease families require very different conditions such as the pH value and buffer composition. For example, the optimal condition for cathepsin B detection in our previous studies^{33,34} was 25 mM MES buffer with pH = 5.0, while the optimal condition was pH = 9.0 for MMP-9 and pH = 7.0 for MMP-7.²⁸ Here, we demonstrate that it is possible to detect cathepsin B activity in a neutral phosphate buffer (pH = 7.4). This physiology-compatible condition is attractive for directly measuring multiple extracellular proteases in HS without significantly alternating their intrinsic activities. By lowering the ionic strength in the buffer, a significantly higher cathepsin B activity was measured, leading to a more sensitive detection with a limit of detection (LOD) down to 57.1 pM, which is comparable to ELISA and is sufficient for measuring cathepsin B in diluted human serum. The electrochemical method was directly compared with the traditional affinity-based ELISA in measuring diluted HS and that spiked with cathepsin B. The

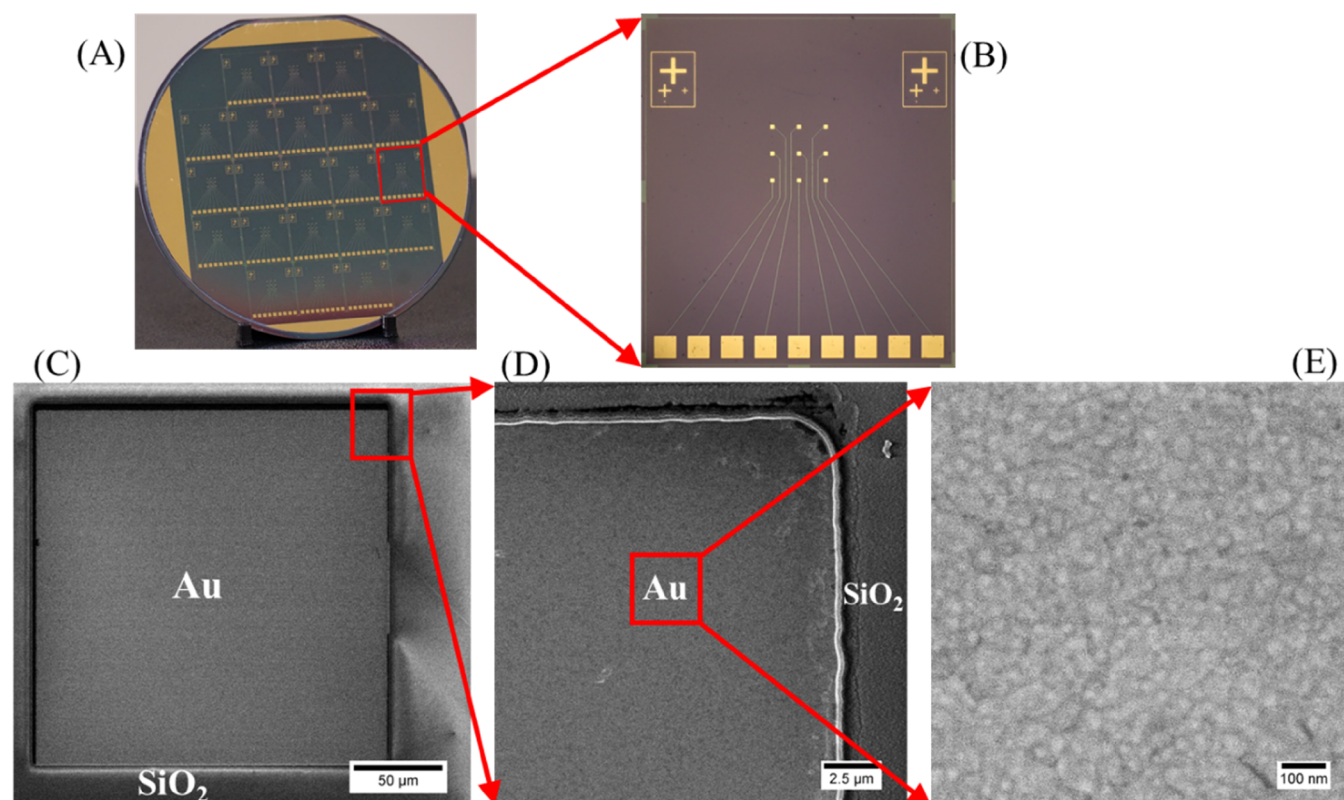


Figure 1. Optical images of (A) 4" Si wafer consisting of 21 Au MEA chips and (B) single Au MEA chip in which the size of the contact pads at the bottom is 1.0 mm $\times$ 1.0 mm. SEM images of (C) a full microelectrode (the one at the center with the contact line at the right side), (D) the corner of the Au microelectrode whose edges are encapsulated under the surrounding insulating SiO₂ layer, and (E) the microstructure of the Au electrode surface. The scale bars in panels (C–E) are 50 μ m, 2.5 μ m, and 100 nm, respectively.

results show that the electrochemical method can consistently measure cathepsin B activity based on the proteolytic kinetics. In contrast, ELISA mainly measures the inactive proenzyme and its signal is significantly suppressed in activated cathepsin B. These two techniques provide complementary information regarding the properties of cathepsin B in the complex HS, which is critical toward disease diagnosis based on detecting activity profiles of extracellular proteases.

EXPERIMENTAL SECTION

The details of the used materials, chemicals, reagents, and instruments are described in the [Supporting Information](#) (SI). Only two critical biological reagents are described here: (1) the carrier-free recombinant human cathepsin B (rhCB) ($\sim$ 60% 37 kDa proenzyme and $\sim$ 40% 29 kDa mature enzyme) from R&D Systems Inc. (Minneapolis, MN) was used as a surrogate human cathepsin B for demonstrating the activity detection, and (2) a "pooled human serum off the clot" (catalog no. ISER10ML, Innovative Research, Novi, MI) was used to validate the detection of extracellular cathepsin B. This HS sample was derived by allowing the whole blood to clot followed by processing into serum via centrifugation. The sample was frozen immediately after processing by the vendor. The received HS sample was stored in a -80°C freezer in aliquots and only a small aliquot was taken out and used in each experiment.

Fabrication of the Au MEA Chip. The procedure to fabricate the Au MEA follows our previous report,³⁷ and more details are provided in [Section 2](#) of the SI. Briefly, the Au MEA chip was fabricated on a thermally oxidized 4" Si(100) wafer. A stack of Ti/Au/Ti films was then deposited on the SiO₂/Si wafer sequentially. A positive photoresist film was coated and patterned through a photomask. The exposed Ti/Au/Ti was etched by a combination of HF/H₂O solution and a Transene TFA Au etchant. Only the microelectrodes

(200 μ m $\times$ 200 μ m) in the 3 $\times$ 3 MEA, the nine electrical contact pads (1 mm $\times$ 1 mm), and connection lines between them were protected by the unexposed photoresist and thus preserved in the etching process. After stripping the photoresist, the whole chip was deposited with a 1 μ m SiO₂ layer followed by the second positive photolithography and reactive ion etching to expose the 3 $\times$ 3 microelectrodes and the nine contact pads. Finally, the top Ti layer in the MEA was etched with HF/H₂O solution to expose the clean Au surface. [Figure S1](#) illustrates the structure and layout of the whole 4" SiO₂/Si wafer consisting of 21 individual Au MEA chips. The edge-to-edge distance of adjacent microelectrodes was set at 1 mm to ensure enough space for spotting reagents on each microelectrode during peptide functionalization while avoiding cross contaminations.

ELISA Measurements. Cathepsin B concentrations were validated using an ELISA kit from R&D Systems in a 96-well plate. The schematic of the ELISA procedure is illustrated in [Figure S2](#) and detailed in [Section 3](#) of the SI. [Figure S3](#) demonstrates the ability of ELISA in measuring cathepsin B in diluted HS. A linear curve can be obtained in a range of 0.5–5% HS. It begins to deviate from the linear line above 5% HS. Thus, 2.5% HS was used in later ELISA experiments of spiked rhCB. A series of activated and non-activated rhCB samples were first prepared in 0.5 $\times$ phosphate buffer (0.5 $\times$ PB) consisting of $\sim$ 3.6 mM Na₂HPO₄ and $\sim$ 2.3 mM NaH₂PO₄, pH = 7.4. About 1% BSA was added in the 0.5 $\times$ PB to reduce non-specific adsorption. Another series of solutions were prepared by spiking different amounts of activated and non-activated rhCB into the solution containing 2.5% HS besides the 0.5 $\times$ PB and 1% BSA. The rhCB activation was done by first incubating the high-concentration rhCB in 25 mM 2-(4-morpholino) ethanesulfonic acid (MES) buffer containing 5 mM dithiothreitol (DTT) at room temperature for 15 min and then diluting into desired rhCB concentrations in proper solutions. For the series involving spiking activated rhCB into 2.5% HS solutions, the HS was activated under the same conditions, i.e., in 25 mM MES (pH = 5.0) and 5 mM DTT, before mixing with the

activated rhCB and diluting to the final 2.5% HS solution. After completing the ELISA procedure, the optical density (OD) in the developed wells of the 96-well plate was measured at a 450 nm wavelength with an EL311 microplate autoreader (Biotek, Winooski, VT). Figure S4A shows the typical 96-well plate design of our ELISA measurements with only the buffer solution (1% BSA in 0.5× PB buffer) (blue-colored cells) and those after adding 2.5% HS (orange-colored cells). Figure S4B shows an image of a developed 96-well plate after completing the ELISA procedure.

Gold MEA Functionalization. The diced Au MEA chip was sonicated in acetone for 5 min to remove the photoresist protection layer followed by rinsing with methanol and subsequently with isopropanol for 30 s. The Au MEA was then rinsed with deionized water and blow-dried with N₂. Prior to use, the Au MEA chip was mounted in a homemade electrochemical cell described in our previous report³⁷ and electrochemically cleaned by cyclic voltammetry (CV) between −0.60 and 0.70 V in 0.10 M phosphate buffer (0.038 M NaH₂PO₄ and 0.061 M Na₂HPO₄, pH = 7.4) vs a mercury/mercurous sulfate reference electrode (MSE) filled with a saturated K₂SO₄ solution (CH Instruments, Austin, TX). The cleaned Au MEA was then rinsed with deionized water and blow-dried with N₂. All electrochemical protease detection measurements in this study used an in-house synthesized hexapeptide attached with a ferrocene (Fc) redox tag, i.e., H₂N-(CH₂)₄-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH₂-Fc, as the substrate (referred to as “peptide-Fc” in this study). This peptide substrate was selected from about 30 synthesized peptides (named H-15 in our previous study³⁷) and has shown the highest proteolytic activity by cathepsin B. It was specifically cleaved by cathepsin B between Leu and Ala residues as determined by HPLC-MS.³⁷ To functionalize the MEA with this peptide-Fc substrate, the chip was first incubated in 1.0 mM 6-mercapto-1-hexanol mixed with 0.2 mM 6-mercapto-1-hexanoic acid in deionized water for 40 min to form a close-packed self-assembled monolayer (SAM) due to thiol adsorption on the Au surface. The formed alkanethiol layer serves as an insulator to reduce the background current while providing a ratio of about 1:5 between −COOH and −OH groups at the top surface. The electrode was then incubated in a 2.0 mM peptide-Fc substrate mixed with 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 h at room temperature to form amide bonds between the amine group in the peptide-Fc substrate and the carboxylic acid group on the SAM surface. The mixed −COOH and −OH groups in the SAM surface were found to be critical in lowering the peptide-Fc density on the Au surface to reduce the steric hindrance in the subsequent proteolysis measurements. The functionalized chip was rinsed with deionized water for 30 s to remove the physically adsorbed molecules.

Electrochemical Experiments. Electrochemical experiments were performed on a specially designed electrochemical cell mounted on a large copper block for temperature control by circulating thermostatic silicone liquid through the internal channels. The optimal temperature setting at the thermal circulator (Julabo, Allentown, PA) was 38.6 °C based on our previous results.³⁴ Two buffer solutions, 0.5× PB (pH = 7.4) and 25 mM MES (pH = 5.0), were used. In some experiments, the rhCB was activated by incubation in 25 mM MES buffer (pH = 5.0) containing 5 mM DTT for 15 min. About 10 μL of activated cathepsin B solution was added to the electrochemical cell containing 815 μL of buffer solution (i.e., 0.5× PB with pH = 7.4) as the electrolyte. The Fc signal on the Au surface was detected by alternating current voltammetry (ACV) with an AC frequency of 300 Hz and a voltage amplitude of 100 mV on a DC ramp from −0.45 to 0.20 V vs MSE. Electrochemical measurements were performed using an IVIUM n-Stat potentiostat (Eindhoven, The Netherlands), which allows measurement of up to 10 independent working electrodes simultaneously versus a common MSE reference electrode and a common Pt coil counter electrode.

RESULTS AND DISCUSSION

MEA Characterization. Figure 1 shows optical and scanning electron microscopy (SEM) images to illustrate the features from the whole 4" wafer to the local surface of the Au microelectrodes. A 4" wafer consists of 21 Au MEA chips (Figure 1A). A diced individual chip (Figure 1B) consists of nine microelectrodes (200 μm × 200 μm each) in a 3 × 3 array, each of which is independently connected to a contact pad (1 mm × 1 mm) that is lined at the bottom of the chip. The exposed contact pads are connected to the multi-channel IVIUM potentiostat through pogo pins. As shown in Figure 1C,D, the edges of each Au microelectrode (about a few microns) and the connection lines are buried under a 1.0 μm-thick insulating SiO₂ layer. The Au surface consists of ~30–50 nm grains, which provide a higher effective surface area than a flat Au surface. This MEA is highly robust and can be repeatedly used multiple times after cleaning and refunctionalization with the procedure described in the Experimental Section.

Principle of the Electrochemical Detection of Protease Activity. The sensing principles are illustrated in the abstract figure and detailed in our previous reports.^{34,37} As shown in the cyclic voltammogram in Figure S5, the Fc tag in the peptide-Fc functionalized at the Au microelectrode surface can be oxidized into ferrocenium (Fc⁺), giving a pair of well-defined redox waves centered at −0.15 V (vs MSE). These peaks disappeared after the peptide-Fc substrate was cleaved by proteolysis. The surface concentration of peptide-Fc substrate Γ_s can be derived from the integrated area under the oxidation peak to give Γ_s = 3.1 pmol/cm², corresponding to an average nearest-neighbor distance of 7.9 nm between the peptide-Fc. This low density allows easy access by the protease. As further shown in Figure S6, the weak faradaic signal can be amplified using ACV, i.e., applying an AC voltage on top of a DC potential ramp from −0.45 to 0.20 V (vs MSE). The optimal AC conditions were found to be 300 Hz in frequency and 100 mV in voltage amplitude for H-15 peptide-Fc functionalized on the Au electrode. An ACV peak was observed at the same potential (−0.15 V) as in CV, confirming that it is attributed to the specific faradaic signal from the functionalized peptide-Fc. The capacitive baseline can be subtracted from the raw ACV curves to extract only the faradaic ACV peak current *i*_p, which is proportional to the amount of attached peptide-Fc. In the presence of cathepsin B, some of the peptide-Fc substrates were proteolyzed by cathepsin B between Leu and Ala (as determined by HPLC-MS in our previous study³⁷), causing the Fc redox tag to diffuse away from the electrode surface. Consequently, the anodic peak current *i*_p in ACV decreased over time. Such kinetic proteolysis curves, typically at a time interval of 30 s and recorded continuously for 60–80 min, can be fit with the following function:³⁷

$$i_p = a[\exp(-t/\tau)] + bt + c \quad (1)$$

where the exponential term $a[\exp(-t/\tau)]$ corresponds to the proteolysis reaction, and the linear term $(bt + c)$ accounts for the background drift. The exponential decay time constant τ represents the proteolysis reaction rate, i.e., a smaller τ value indicates the faster reaction and higher protease activity.

The proteolysis kinetics can be accurately described by the heterogeneous Michaelis–Menten model as demonstrated in our previous studies^{33–35,37} and detailed in the SI for reference.

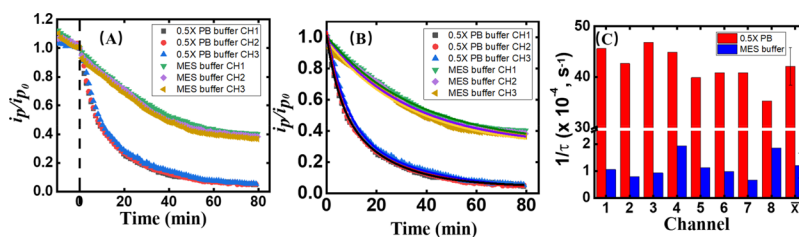


Figure 2. (A) Representative normalized kinetic proteolysis curves for Au MEA modified with peptide substrate $H_2N-(CH_2)_4-CO-Pro-Leu-Ala-Phe-Val-Ala-NH-CH_2-Fc$ (peptide-Fc) by 1.0 nM activated cathepsin B in 0.5× PB (pH = 7.4) and 25 mM MES buffer (pH = 5.0), respectively. (B) Fitting of the extracted kinetic proteolysis curves. (C) Bar graph showing the fitted values of $1/\tau$ from eight individual channels, with the average value and the standard deviations (as the error bars) of these eight channels shown at the far right.

Importantly, the protease activity can be represented by the inverse of the exponential decay time constant τ as

$$\frac{1}{\tau} = \left(\frac{k_{cat}}{K_M} \right) [E]_0, \quad (2)$$

where k_{cat}/K_M is a specific constant revealing the intrinsic catalytic efficiency of the protease to the specific peptide substrate and $[E]_0$ is the concentration of the active protease. The τ value depends on both the catalytic efficiency and the protease concentration. The traditional affinity-based measurements (such as ELISA) only indirectly reveal $[E]_0$ but not k_{cat}/K_M . In this study, we focus on deriving the protease catalytic activity by fitting the kinetic exponential decay curves that give the value of $(k_{cat}/K_M)[E]_0$. As will be shown in later sections, the (k_{cat}/K_M) value strongly depends on the measuring conditions such as peptide sequence, buffer composition, and temperature as well as the properties of proteases. This carries rich information related to the biological function of the protease, which cannot be obtained from the concentration of the protease determined by affinity-based analytical techniques, such as ELISA.

Electrochemical Detection of Cathepsin B Activity Close to Physiological Conditions. According to the literature, the optimal cathepsin B assay buffer is 25 mM MES buffer (pH = 5.0), an acidic buffer.³⁸ This is also the assay buffer recommended by the vendor (R&D Systems Inc.) and was used in our previous studies.^{33–37} Figure S7 shows the typical kinetic proteolytic curves of 1.0 nM activated cathepsin B in 25 mM MES buffer from nine microelectrodes in the Au MEA functionalized with the same peptide-Fc substrate. The average value of $1/\tau$, i.e., the measured protease activity, is $(1.21 \pm 0.46) \times 10^{-4} s^{-1}$. Results from nine microelectrodes in the Au MEA are highly consistent, with the curves overlapping with each other. However, the long-term goal of our study is to develop the Au MEA as a cancer diagnostic technique for simultaneously measuring the activity of multiple cancer-related extracellular proteases in HS samples. The representative proteases that have been reported to be related to cancer development include cathepsin B, ADAM-10, ADAM-17, MMP-9, uPA, etc., which may require very different pH values for optimal proteolysis. For example, the recommended assay buffer for ADAM-10 and ADAM-17 is 25 mM Tris, 2 μ M $ZnCl_2$, and 0.005% (w/v) Brij35 with pH = 9.0 by the vendor (R&D Systems, catalog number: 936-AD). This pH value is 4 units higher than the optimal MES buffer (pH = 5.0) for cathepsin B. To facilitate future multiplex detections, we investigated the possibility for electrochemical detection of proteolysis in a common buffer for all extracellular proteases in HS, i.e., at the neutral pH = 7.4. This pH value is compatible

with the physiological condition of HS and is the optimal condition for some proteases, such as MMP-7 measured in tricine buffer (pH = 7.4).²⁸

To achieve the above goals, we first tried the commonly used phosphate buffer saline (PBS) (pH = 7.4). Figure S8 shows the kinetic proteolysis curves measured with the Au MEA functionalized with peptide-Fc in 1.0 nM activated cathepsin B dispersed in 1× PBS, 0.5× PBS, and 0.2× PBS, respectively. Clear exponential decay was observed in all three buffers. The inverse decay constants ($1/\tau$), i.e., the measured protease activity, are $(4.2 \pm 1.1) \times 10^{-4} s^{-1}$, $(11.44 \pm 0.64) \times 10^{-4} s^{-1}$, and $(7.65 \pm 0.42) \times 10^{-4} s^{-1}$ for 1×, 0.5×, and 0.2× PBS, respectively. To our surprise, all these activity values are higher than that in MES buffer, i.e., $1.21 \times 10^{-4} s^{-1}$. In addition, the proteolysis rate strongly depends on the ionic strength of the buffer. The catalytic proteolysis reaction in 0.5× PBS is faster than those in both 1× and 0.2× PBS. The buffer concentration also had an impact on the stability of the Au MEA as reflected in the linear ACV peak current drifting in the period before adding cathepsin B, i.e., before $t = 0$ min. It is clear that the drift rate is higher at a higher PBS concentration. It is likely that the chloride ions in high-concentration PBS may have caused roughening and dissolution of the Au surface, as reported previously.³⁹

To improve the stability of the Au electrode, we modified the common PBS by removing sodium chloride and potassium chloride. The reformulated homemade buffer only consists of the proper ratio of two phosphate salts, i.e., sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) to give pH = 7.4, referred to as “1× PB” in this study. The buffer was diluted to 0.5× prior to use in order to obtain a high proteolysis activity. Figure S9 shows the representative raw proteolytic curves and the fitting to the extracted exponential portion from nine sets of data obtained simultaneously from the same Au MEA. Again, the measurements from different microelectrodes are highly consistent and overlapping with each other. A striking feature is that the peak current decays much faster than those in both MES buffer and 0.5× PBS.

Figure 2A provides direct comparison of the normalized kinetic proteolysis curves of the peptide-Fc-modified Au MEA by 1.0 nM activated cathepsin B in 0.5× PB (pH = 7.4) and the 25 mM MES buffer (pH = 5.0), respectively. Since the ACV peak currents vary among different Au MEA chips, they are normalized to the value right before adding cathepsin B to give i_p/i_{p0} so that the proteolytic curves can be directly compared. The exponential decay portions are plotted and fitted in Figure 2B. Figure 2C shows the fitted $1/\tau$ values from eight channels of the Au MEA in the two buffers (with one channel omitted due to loose contact). The average measured

activities ($1/\tau$) in 0.5× PB and 25 mM MES buffer are $(42.1 \pm 3.7) \times 10^{-4} \text{ s}^{-1}$ and $(1.21 \pm 0.46) \times 10^{-4} \text{ s}^{-1}$, respectively. The activity is nearly 40 times higher in 0.5× PB. It is noteworthy that these two sets of electrochemical experiments were carried out on the same Au MEA chip, with the same preparation procedure, and under the same measurement conditions (300 Hz ACV frequency, 100 mV ACV voltage amplitude, and 38.6 °C temperature setting). This finding strongly indicates that switching the assay buffer from 25 mM MES to 0.5× PB is feasible. Thus, all our electrochemical measurements in later sections were carried out in 0.5× PB.

Assessment of the LOD in Measuring Activated Cathepsin B in 0.5× PB. Since the proteolysis is much faster in 0.5× PB than in 25 mM MES buffer as shown in Figure 2, a much lower LOD can be expected. To assess the LOD in 0.5× PB, a series of proteolysis measurements were carried out at different activated rhCB concentrations using Au MEA chips functionalized with the peptide-Fc substrate. As shown in Figure 3A, the proteolytic curve decays faster as the

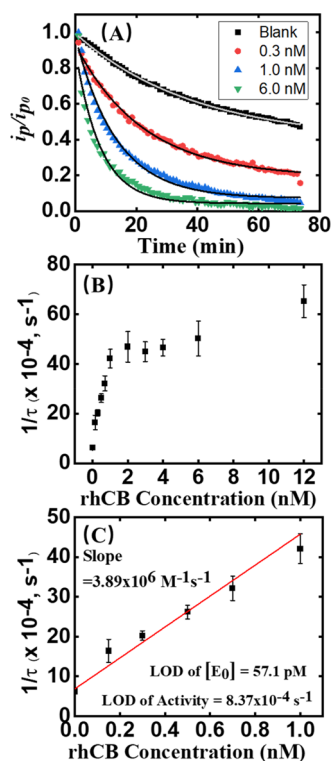


Figure 3. (A) Representative normalized kinetic proteolysis curves and the fitting lines for Au MEA modified with the peptide-Fc substrate at activated rhCB concentrations of 0, 0.3, 1.0, and 6.0 nM in 0.5× PB (pH = 7.4). (B) Scatter plot of $1/\tau$ vs rhCB concentration. The error bars are the standard deviation of eight replicates at each concentration. (C) Linear calibration curve of cathepsin B protease activity in the activated rhCB concentrations from 0 to 1.0 nM.

rhCB concentration is increased. Figure 3B shows the derived activity ($1/\tau$) vs the rhCB concentration, with the error bars representing the standard deviation of eight replicates for each measurement. The $1/\tau$ value linearly increases from blank to 1.0 nM cathepsin B but is saturated at about $45 \times 10^{-4} \text{ s}^{-1}$ when the rhCB concentration is above 1.0 nM. The saturation may be due to the steric interference between the adsorbed rhCB on the planar Au surface, leading to a significantly decreased slope at a higher rhCB concentration. The fitting

line between 0 and 1.0 nM is shown in Figure 3C and can be described as

$$1/\tau \text{ (in } \text{s}^{-1}) = (3.89 \pm 0.28) \times 10^6 \text{ M}^{-1} \text{ s}^{-1} [\text{rhCB}] + (6.89 \pm 0.83) \times 10^{-4} \text{ s}^{-1}, \quad (3)$$

with $[\text{rhCB}]$ in the unit of M. The slope $m = (3.89 \pm 0.28) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ reveals the specificity constant $k_{\text{cat}}/K_M = (3.89 \pm 0.28) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, which is within the range of 0.928×10^6 to $7.288 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ reported for activated human cathepsin B with different peptide substrates.^{40–42} The LOD of activity can be determined to be $8.49 \times 10^{-4} \text{ s}^{-1}$ by

$$\text{LOD of activity} = 3\sigma_{\text{blank}} + (1/\tau)_{\text{blank}}, \quad (4)$$

where $\sigma_{\text{blank}} = 0.74 \times 10^{-4} \text{ s}^{-1}$ is the standard deviation of blank and $(1/\tau)_{\text{blank}} = 6.27 \times 10^{-4} \text{ s}^{-1}$ is the mean value of blank experiments. The LOD of cathepsin B concentration can be determined as $57.1 \pm 0.41 \text{ pM}$ by

$$\text{LOD of } [\text{rhCB}] = 3\sigma_{\text{blank}}/m. \quad (5)$$

Compared with the 0.32 nM LOD in our previous study using VACNF nanoelectrode arrays in 25 mM MES buffer,³⁴ the LOD of rhCB in this study is about six times lower. Changing the assay buffer from 25 mM MES to 0.5× PB and using the proper density of peptide-Fc on the Au MEA significantly improved the detection sensitivity.

Validation of Cathepsin B in 0.5× PB by ELISA Measurements. ELISA is a highly sensitive and highly selective affinity-based technique for detecting protein analytes through specific binding with the antibodies. The signal is amplified by enzymatic conversion of the colorless substrate to a colored product.⁴³ Here, ELISA is employed to selectively measure the concentration of rhCB and validate the electrochemical activity measurements. Following the procedure described in the SI, we first carried out the ELISA measurements at a series of non-activated rhCB concentrations in 0.5× PB. As shown in Figure 4A (black filled squares), it gives a linear curve in a range of 0–250 pM rhCB. The fitted calibration curve can be expressed as:

$$\text{OD} = (1.29 \pm 0.025) \times 10^{-3} \text{ pM}^{-1} \times [\text{rhCB}] + (0.0082 \pm 0.0037), \quad (6)$$

where the unit of $[\text{rhCB}]$ is pM. We then spiked the same concentrations of non-activated rhCB into diluted HS in 0.5× PB. As explained earlier, 2.5% HS was chosen for the spiking experiments since it is in the middle of the linear range shown in Figure S3. Spiking rhCB into 2.5% HS (red filled circles) also shows a linear curve with the slope similar to that in 0.5× PB, but the OD values consistently upshift by about 0.21. The fitted calibration curve is

$$\text{OD} = (1.54 \pm 0.073) \times 10^{-3} \text{ pM}^{-1} \times [\text{rhCB}] + (0.2169 \pm 0.0103). \quad (7)$$

The upshift of the calibration curve in the 2.5% HS solution can be attributed to the intrinsic cathepsin B in the HS sample. Its concentration in the 2.5% HS can be estimated to be equivalent to $162 \pm 9.5 \text{ pM}$ rhCB by inputting the intercept into 2.5% HS ($\text{OD} = 0.2169 \pm 0.0103$) as the signal in calibration eq 6. This translates into $\sim 6.5 \pm 0.4 \text{ nM}$ equivalent rhCB in 100% HS. Since the rhCB consists of $\sim 60\%$ 37 kDa proenzyme and $\sim 40\%$ 29 kDa active enzyme with an average

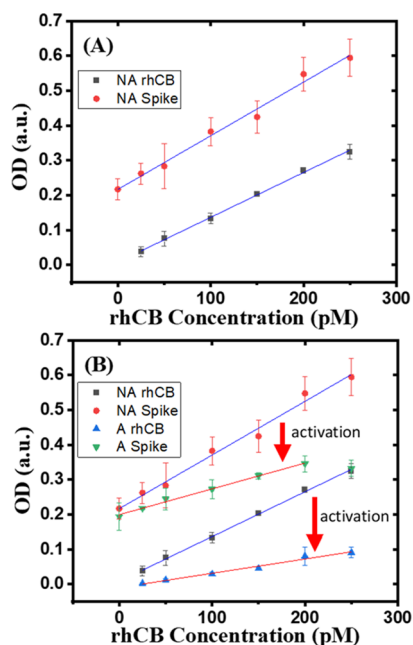


Figure 4. Calibration curves derived from ELISA measurements. (A) OD readings of the developed product generated by varied concentrations of non-activated rhCB in 0.5X PB (black filled squares) and spiking the non-activated rhCB into 2.5% HS in 0.5X PB (red filled circles). (B) Addition of two sets of activation results to panel (A), i.e., activated rhCB in 0.5X PB (blue filled upper triangles) and spiking the activated rhCB into the activated 2.5% HS in 0.5X PB (green filled inverted triangles).

molecular weight of 33.8 kDa, the cathepsin B quantity in 100% HS by our ELISA study can be estimated as $\sim 220 \pm 13$ ng/mL, which is close to the reported values of 13.2–126 ng/mL in HS by ELISA in the literature.^{44–46} From these results, the LOD of cathepsin B concentration by ELISA can be determined as $\sim 32 \pm 0.6$ pM in 0.5X PB and $\sim 59 \pm 2.8$ pM in 2.5% HS in 0.5X PB based on the 3σ definition ($\text{LOD} = 3\sigma/m$), where σ is the standard deviation in measurements of the low-rhCB-concentration sample (25 pM) and m is the slope of calibration eqs 6 and 7, respectively. Thus, the LOD of rhCB concentration by our electrochemical method is comparable to that by ELISA. However, while each set of ELISA measurements followed the same trends as Figure 4, there were variations between different sets due to the semi-quantitative nature of ELISA. This led to a lower LOD of $\sim 18 \pm 1.3$ pM in 0.5X PB and $\sim 24 \pm 0.6$ pM in 2.5% HS in 0.5X PB in a set of repeated ELISA experiments. Nevertheless, the main conclusions do not change.

Since the non-activated rhCB includes active form ($\sim 40\%$) and non-active form ($\sim 60\%$), the electrochemical measurements in Figures 2 and 3 were done after activating the rhCB in the MES and DTT solution (pH = 5.0). The activation process is assumed to convert all non-active proenzymes into the active mature form. To assess whether the activation affects the ELISA measurements, we carried out two more sets of ELISA measurements shown in Figure 4B, including adding activated rhCB into the 0.5X PB (blue filled upper triangles) and spiking the same amount of activated rhCB into the preactivated 2.5% HS in 0.5X PB (green filled inverted triangles). Both sets of data show similar slopes (represented by the two parallel lines in Figure 4B), but their values are substantially lower compared to those using non-activated

rhCB. Thus, the detection sensitivity is suppressed. This could be attributed to two possibilities: (1) the activation reagents (MES and DTT) may interfere with the binding of the active rhCB with antibodies during ELISA, and (2) some binding sites may be located on the surface of the cleaved domains, thus leading to lower efficiency in forming the sandwich structure in ELISA measurements. Since MES and DTT are diluted by more than 100 times after activation, the first possibility is unlikely to be the main factor. Based on this observation, ELISA is more sensitive to the inactive proenzyme in the sample and is not suitable in detecting the active rhCB. Interestingly, the intercepts of the curves with activated or non-activated rhCB are about the same in either 0.5X PB or 2.5% HS, while those in 2.5% HS show the similar upshift in OD compared to those in 0.5X PB. The presence of cathepsin B in HS is obviously responsible for this upshift.

Electrochemical Detection of Cathepsin B Activity in Human Serum

To assess the activity of cathepsin B in HS, we compared the kinetic proteolysis curves of 5% HS in 0.5X PB with and without activation by the electrochemical method. The 5% HS concentration is slightly higher than the 2.5% used in ELISA measurements to ensure that a clear exponential decay can be observed in electrochemical experiments. To minimize errors, three Au MEA chips were prepared side-by-side for three sets of experiments, i.e., (i) non-activated 5% HS in 0.5X PB, (ii) activated 5% HS in 0.5X PB, and (iii) the blank 0.5X PB. In experiment (ii), the 5% HS in 0.5X PB was activated by incubating 41.25 μL of stock HS mixed with 3.75 μL of 60 mM DTT in 300 mM MES (pH = 5.0) for 15 min. At $t = 0$ min, all 45 μL of activated solution was added to the electrochemical cell that was prefilled with 780 μL of 0.5X PB. In the final solution, the HS was diluted to 5%. In experiment (i), a proper amount of stock HS was directly added into the prefilled electrochemical cell to dilute the final HS concentration to 5%. Figure 5A shows three sets of representative proteolysis curves for each experiment. Clearly, the kinetic proteolysis rate of the blank 0.5X PB is the slowest followed by a higher rate in non-activated 5% HS and the highest rate in activated 5% HS. Figure 5B is the bar chart plot showing the average value and standard deviation of the corresponding $1/\tau$ value over eight independent channels for each experiment. The cathepsin B activities ($1/\tau$) are $(6.27 \pm 0.74) \times 10^{-4}$, $(12.2 \pm 0.8) \times 10^{-4}$, and $(33.2 \pm 3.0) \times 10^{-4} \text{ s}^{-1}$, respectively. Based on eq 2, the concentration of the active form of cathepsin B in non-activated and the activated sample (equivalent to 100% HS) can be calculated by

$$[\text{CB}]_{100\% \text{ HS}} = \left[\frac{(1/\tau)_{5\% \text{ HS}} - (1/\tau)_{\text{blank}}}{m} \right] \times \frac{100\%}{5\%}, \quad (8)$$

where $(1/\tau)_{\text{blank}} = (6.27 \pm 0.74) \times 10^{-4} \text{ s}^{-1}$ and $m = (3.89 \pm 0.28) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ is the slope of the calibration curve in eq 3. Thus, the concentration of active cathepsin B can be calculated as $3.05 \pm 0.60 \text{ nM}$ in non-activated stock HS and as $13.8 \pm 1.9 \text{ nM}$ in the activated sample. Assuming that the activation process fully converted cathepsin B into the active form, the total cathepsin B concentration in the initial HS sample, including both the active form and inactive form (proenzyme), is 13.8 nM. Thus, it can be estimated that about 22% of the cathepsin B in the stock HS is in the active form.

It is noteworthy that the total cathepsin B concentration (13.8 nM) by the electrochemical method is significantly higher than 6.5 nM derived by ELISA. This could be attributed

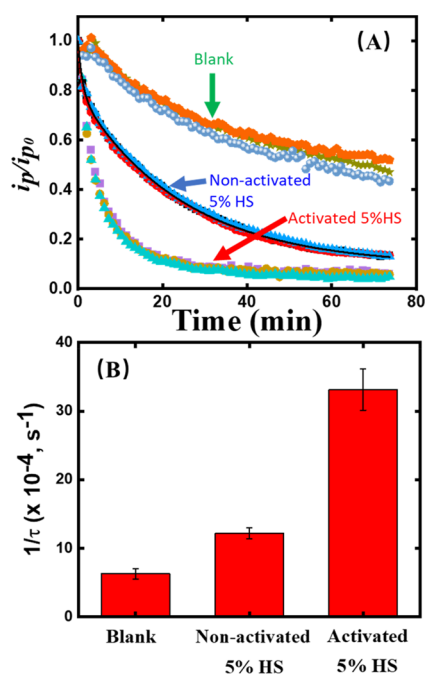


Figure 5. (A) Representative normalized kinetic proteolysis curves and fitting lines (three for each experiment) for peptide-Fc-modified Au MEAs by activated and non-activated 5% HS in 0.5× PB comparing to blank 0.5× PB. (B) Bar chart plot of $1/\tau$ vs different samples (blank, activated, and non-activated 5% HS). The error bars represent the standard deviation from eight replicates ($n = 8$).

to two possibilities: (i) the electrochemical detection involved non-specific cleavage to the peptide-Fc by other proteases in the HS sample, and (ii) the ELISA measurements were suppressed by other compositions in the HS, i.e., the presence of a matrix effect. The most remarkable difference between the electrochemical method and ELISA is that the electrochemical method is highly sensitive to the activity of cathepsin B, i.e., showing a much higher proteolysis rate when it is activated, while ELISA is more sensitive to the inactive form.

Electrochemical Assessment of Effective Cathepsin B Activity Spiked in Human Serum. HS contains very complex components, which may affect the assay sensitivity and reproducibility.^{47,48} To assess the matrix effects, we have carried out the study by spiking different amounts of active rhCB into the non-activated 5% HS in 0.5× PB. Figure S10 shows the raw proteolysis curves of the non-activated 5% HS in 0.5× PB and the corresponding fitting lines. The data were recorded over eight channels of the peptide-Fc-modified Au MEA simultaneously. Figure S11 shows the similar measurements and fitting after spiking 1.0 nM non-activated rhCB into the non-activated 5% HS in 0.5× PB. As specified by the vendor (R&D Systems), the purchased rhCB contains ~40% active cathepsin B. Figure S12 further shows the measurements and fitting after spiking 1.0 nM activated rhCB into the non-activated 5% HS in 0.5× PB. In this experiment, an activation process with 5 mM DTT in 25 mM MES buffer was applied to convert all 1.0 nM cathepsin B into the active form, serving as the condition with 1.0 nM active rhCB spiked into the non-activated 5% HS.

Three representative sets of proteolysis curves from Figures S10–S12 are plotted in Figure 6A for direct comparison. Adding 0.40 nM naturally active cathepsin B into 5% HS in 0.5× PB only causes a slightly increased proteolytic rate.

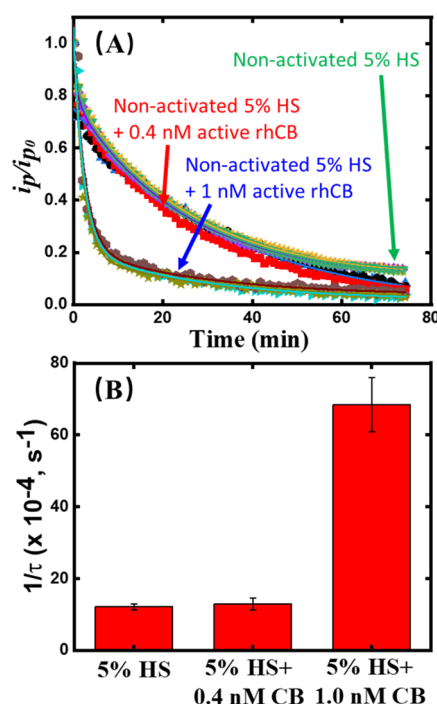


Figure 6. (A) Representative normalized kinetic proteolysis curves and fitting lines for peptide-Fc-modified Au MEA by non-activated 5% HS and those spiked with 0.4 nM and 1.0 nM active cathepsin B, respectively. (B) Bar chart plot of $1/\tau$ in the three samples. The error bars represent the standard deviation from eight replicates ($n = 8$).

However, a much higher proteolytic rate is observed after adding 1.0 nM activated cathepsin B. The bar graph in Figure 6B illustrates the measured $1/\tau$ value for each experiment, which are $(12.15 \pm 0.8) \times 10^{-4}$, $(12.99 \pm 1.66) \times 10^{-4}$, and $(68.4 \pm 7.53) \times 10^{-4} s^{-1}$, respectively. The increased effective rhCB concentration, $\Delta[\text{rhCB}]$, in the spiked 5% HS solution can be calculated based on the difference of the measured $1/\tau$ value in the spiked and non-spiked samples in the following:

$$\Delta[\text{rhCB}]_{5\% \text{ HS}} = \frac{(1/\tau)_{\text{spiked } 5\% \text{ HS}} - (1/\tau)_{5\% \text{ HS}}}{m}, \quad (9)$$

where the slope of the calibration curve $m = (3.89 \pm 0.28) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ is from eq 3. This gives $\Delta[\text{rhCB}]_{5\% \text{ HS}} = 0.0216 \pm 0.047 \text{ nM}$, which corresponds to only $(5.4 \pm 11.8)\%$ recovery percentage of the spiked 0.4 nM naturally active rhCB. The spiked active rhCB was obscured by the HS matrix, making the effect undetectable. In contrast, the $1/\tau$ value after spiking 1.0 nM activated cathepsin B into the 5% HS was much larger, which led to a recovery percentage of $(140 \pm 20)\%$ following the above calculation. It is worth mentioning that these two spiking experiments have critical differences. The cathepsin B activation reagents (such as DTT) used in the latter experiment, though they were diluted to 0.27 mM in the final spiked solution, may interact with the inherent cathepsin B and other cathepsins in the HS. It is known that many of the 11 cysteine cathepsins (B, C, F, H, K, L, O, S, V, X, and W) have largely overlapping specificities and may catalyze proteolysis of the similar peptide substrates.¹² While further studies are needed to develop a better understanding of these results, it is clear that the electrochemical method can sense the small changes in activity of the extracellular cathepsin B in HS.

In summary, the above results are encouraging for detecting cathepsin B activity by the electrochemical method using Au MEAs in neutral phosphate buffers (pH = 7.4). This enables direct detection of the intrinsic activity of human serum under the physiological conditions without alternating the nature of the extracellular proteases. It paves the way for future multiplex detection of different protease families in a common buffer. The electrochemical method is very sensitive to the activity of cathepsin B as demonstrated in the measurements of diluted HS and the spiking experiments with or without applying a preactivation procedure. In contrast, ELISA measurements are more sensitive to the non-activated rhCB in both 0.5× PB and 2.5% HS. Due to the large differences between ELISA and the electrochemical protocols, it is difficult to carry out the activation experiments under the same conditions, making it difficult to draw solid conclusions. However, these techniques reveal different aspects of proteases and in general validate each other. This provides useful new insights into the catalytic properties of extracellular proteases in HS, which may inspire further studies to understand the complex proteolysis problems and to push protease activity profiling toward disease diagnosis.

CONCLUSIONS

We have demonstrated successful electrochemical detection of the activity of rhCB in diluted HS with 0.5× PB buffer at pH = 7.4 using a 3 × 3 Au MEA. The catalytic activity of cathepsin B ($k_{\text{cat}}/K_{\text{M}}$) [CB] is represented by inverse of the exponential decay time constant, i.e., $1/\tau$, which can be derived by fitting of the kinetic proteolysis curve measured with continuously repeated AC voltammetry measurements. The activity was found to be sensitive to the pH value and the ionic strength of the buffer. In low-ionic-strength neutral 0.5× PB, the activity of cathepsin B was surprisingly nearly 40 times of that in the typical optimal buffer, i.e., 25 mM MES with pH = 5.0. A linear calibration curve was obtained in a range of 0–1.0 nM of activated cathepsin B, which led to a very low LOD of rhCB at 57.1 pM. The specificity constant $k_{\text{cat}}/K_{\text{M}}$ was determined to be $(3.89 \pm 0.28) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, comparable to that of human cathepsin B measured by a fluorescence technique in the literature. ELISA measurements validated that the pooled HS sample contained 6.5 nM cathepsin B. The LOD of rhCB concentration by the electrochemical method is comparable to ELISA. However, ELISA results are dominated by proenzymes and the signal is suppressed in measuring active rhCB. In contrast, the electrochemical method is sensitive to the catalytic properties of the active cathepsin B and shows a significantly higher activity when the rhCB and HS are activated in acidic buffer. Spiking 1.0 nM non-activated cathepsin B (containing ~40% naturally active enzyme) and 1.0 nM activated cathepsin B into 5% HS further demonstrates the electrochemical method's ability to detect the activity of cathepsin B in the analytes. Particularly, the gold MEA platform has a great potential for rapid multiplex detection of activities of extracellular proteases toward cancer diagnosis and treatment efficacy assessment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.1c01175>.

Detailed materials and instruments used in this study, Au MEA fabrication procedures, procedures for ELISA measurements, derivation of the Michaelis–Menten model for electrochemical data analysis, and additional electrochemical kinetic proteolysis results of Au MEA in different buffers and in 5% human serum samples (PDF)

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Notes

The authors declare no competing financial interest.

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