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Quantitative Electrochemical Detection of Cathepsin B Activity in Complex Tissue Lysates Using Enhanced AC Voltammetry at Carbon Nanofiber Nanoelectrode Arrays

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Abstract

The proteolytic activity of a cancer-related enzyme cathepsin B is measured with alternating current voltammetry (ACV) using ferrocene (Fc) labeled tetrapeptides attached to nanoelectrode arrays (NEAs) fabricated with vertically aligned carbon nanofibers (VACNFs). This combination enables the use of high AC frequencies (~1 kHz) with enhanced electrochemical signals. The specific proteolysis of the Fc-peptide by cathepsin B produces decay in the ACV peak current versus the reaction time. The exponential component of the raw data can be extracted and defined as the “extracted proteolytic signal” which allows consistent quantitative analyses using a heterogeneous Michaelis-Menten model. A “specificity constant” $k_{\text{cat}}/K_M = (3.68 \pm 0.50) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for purified cathepsin B was obtained. The detections of cathepsin B activity in different concentrations of whole lysate of human breast tissue, tissue lysate spiked with varied concentrations of cathepsin B, and the tissue lysate after immunoprecipitation showed that there are ~13.4 nM higher cathepsin B concentration in $29.1 \mu\text{g}\cdot\text{mL}^{-1}$ of whole tissue lysate than the immunoprecipitated sample. The well-defined regular VACNF NEAs by e-beam lithography show a much faster kinetics for cathepsin B proteolysis with $k_{\text{cat}}/K_M = 9.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. These results illustrate the potential of this technique as a portable multiplex electronic system for cancer diagnosis by rapid protease profiling of serum or blood samples.

Keywords

nanoelectrode; AC voltammetry; protease; enzyme activity; detection

1. Introduction

Cathepsin B is a lysosomal cysteine protease which is highly expressed during cancer invasion and progression (Fujise et al., 2000; Saleh et al., 2006; Tumminello et al., 1996). Increased level of cathepsin B in tumors or in extracellular fluids can serve as prognostic factors for cancer patients (Duffy, 1996; Kos and Lah, 1998). It has been reported that

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cathepsin B is overexpressed in many cancer types, such as laryngeal cancer, oral cancer, breast cancer and colorectal cancer, compared to that in normal tissue (Hirai et al., 1999; Kandalaft et al., 1993; Kawasaki et al., 2002; Lah et al., 2000; Macabeo-Ong et al., 2003). Therefore simple and rapid detection of the expression level of cathepsin B, particularly quantitatively determine its enzymatic activity, is highly demanded for early diagnosis and treatment of cancers (Turk, 2006).

Traditional methods to measure protease activities include spectroscopy, gel electrophoresis, enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) (Obrenovitch et al., 1983; Orosco et al., 2006; Shimura et al., 1998; Tang et al., 2011; Vestling et al., 1990). However, these methods suffered from various limitations. For example, optical methods are not readily applicable in colored fluids or complex turbid solutions such as whole blood and plasma samples. ELISA requires specific antibodies and HPLC requires specially synthesized peptides. These methods also demand sophisticated instrumentation which is costly, time-consuming, and typically limited in operation in central labs. Radioimmunoassay (RIA) is simple but needs to use reagents that are notorious to human health and environment (Hook et al., 1996). Simple and rapid electrochemical detection technology has received much attention nowadays due to its direct electronic readout, ease in miniaturization and multiplexing, low cost, and the potential to be developed into portable systems for point-of-care diagnosis of diseases.

Liu et al. first demonstrated electrochemical detection of proteolytic cleavage of a ferrocene (Fc) labeled helical peptide with nine amino acids that was immobilized on a gold electrode by matrix metalloproteinase-7 (MMP-7) (Liu et al., 2006). The proteolysis was found to be highly specific to the selected peptide sequence by MMP-7 over other matrix metalloproteinases such as MMP-2 and MMP-3. Following this, more electrochemical techniques have been employed to measure proteolysis of Fc-labeled peptides immobilized on macroscopic gold electrodes. The enzymatic properties of proteases including trypsin, thrombin and plasmin have been quantitatively analyzed with the kinetic Fc signal after adding the proteases (Adjemian et al., 2010; Ohtsuka et al., 2009). However, electrochemical detection and quantitative analyses of protease activities in more complicated samples such as whole tissue lysates have not been reported.

Recently, we demonstrated a new approach by applying alternating current voltammetry (ACV) on nanoelectrode arrays (NEAs) fabricated with embedded vertically aligned carbon nanofibers (VACNFs) to measure the activities of two cancer related proteases, legumain and cathepsin B (Swisher et al., 2013). The proteolytic kinetics of both proteases can be approximately analyzed with a heterogeneous Michaelis-Menten model (Li et al., 2012a; Li et al., 2012b; Swisher et al., 2013). Cathepsin B is particularly interesting since its k_{cat}/K_M value was derived as $(4.3 \pm 0.8) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, about four times of that of legumain, and hence can be reliably measured in a wider concentration range.

The ACV technique involved superimposing a sinusoidal alternating current (AC) voltage on the direct current (DC) potential ramp, which can enhance the redox current from Fc moieties immobilized at the electrode surface by shuffling the electrons back and forth many times between the Fc moieties and the electrode (Creager et al., 1999; Syed et al., 2011). Particularly, applying ACV on the VACNF NEAs enabled the use of much higher AC frequencies than on conventional macroscopic glassy carbon electrodes (GCEs) (Syed et al., 2011). For Fc-appended tetrapeptide on VACNF NEAs, the maximum ACV peak current was at $\sim 1,000 \text{ Hz}$, about 40 times of that on macro-GCEs (Swisher et al., 2013). The enhanced electrochemical signal and higher temporal resolution ($< 1 \text{ min}$) are critical for measuring the fast enzymatic kinetics in this study.

However, the previous method (Swisher et al., 2013) was found unable to consistently compare data collected from different electrodes due to the variation in electrochemical signals. Here we report a refined method for analyzing the kinetic ACV data of proteolysis of Fc-peptide attached on VACNF NEAs. Consistent quantitative analyses of cathepsin B activity at varied concentrations have been established. In addition, two critical steps toward rapid multiplex cancer diagnoses have been achieved. First, we demonstrate that this detection method is applicable on the whole tissue lysate of normal human breast samples, the tissue lysate spiked with varied concentrations of purified cathepsin B and the tissue lysate after immunoprecipitation. The relative cathepsin B concentrations in these complex samples are clearly correlated with the exponential rate. Second, a 3×3 microarray containing nine independently addressed regular VACNF NEAs by e-beam lithography showed substantially enhanced performance than the previously used non-patterned random VACNF NEAs. The measured activity of cathepsin B appeared to be much higher due to the smaller and well-defined CNF tips. These results demonstrated the great potential for this nanoelectronic method as a portable multiplex technique for simultaneously detecting a large number of distinct proteases in a minute drop of serum or lysate sample. This may enable quick analyses of a patient's protease profile, which is critical for rapid cancer diagnosis and treatment monitoring.

2. Materials and methods

2.1. Apparatus, Reagents, and Synthesis of Fc-appended Tetrapeptide

The electrochemical measurements were conducted with an electrochemical analyzer (Model 440A, CH Instruments, Austin, TX). Fluorescence assay was performed on GloMax-Multi+ Microplate Multimode Reader (Promega, Madison, WI). Purified recombinant human cathepsin B (molecular weight of 29 kDa) was acquired from R&D Systems Inc. (Minneapolis, MN). Human breast whole tissue lysate (adult normal) with the stock concentration of $4.9 \text{ mg}\cdot\text{mL}^{-1}$ was purchased from Novus (Littleton, CO). In order to obtain full activity, cathepsin B and the tissue lysate needed to be activated in 5 mM dithiothreitol (DTT) and 25 mM 2-(4-Morpholino)ethane sulfonic acid (MES) buffer (pH 5.0) right before used for proteolytic reactions. The details of other reagents are listed in the Supplementary Information. The synthesis of Fc-appended tetrapeptides including the specific tetrapeptide $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Leu}-\text{Arg}-\text{Phe}-\text{Gly}-\text{NH}-\text{CH}_2-\text{Fc}$ and “negative control” tetrapeptides as the substrates for testing enzymatic proteolysis by cathepsin B is described in the Supplementary Information (Figs. S1 & S2).

2.2. Fabrication of VACNF NEA Chips

Two types of VACNF NEAs were employed in this study, including (1) single NEA chips consisting of randomly distributed VACNFs in a forest-like configuration and (2) 3×3 multiplex NEA chips consisting of nine independently addressed regular VACNF arrays defined by e-beam lithography. The random NEA chips were readily produced and thus used for the extensive studies on method development. Limited by the high cost, e-beam patterned NEA chips were only used in experiments to demonstrate the capabilities of high-performance multiplex detection. The fabrication procedures follow previous reports for random (Li et al., 2003; Li et al., 2005; Siddiqui et al., 2010; Swisher et al., 2013) and regular (Arumugam et al., 2009; Siddiqui et al., 2010) VACNF NEAs and are detailed in the Supplementary Information.

Importantly, a typical random NEA chip consisted of a VACNF forest embedded in the SiO_2 matrix as illustrated in the insert of Fig. 1A. All carbon nanofibers (CNFs) were directly attached to a 100-nm thick Cr film on a Si(100) wafer. Only a length of ~20 nm to 300 nm of the longest CNFs were exposed by protruding over the SiO_2 matrix (see Fig. S3).

The diameter of the VACNFs varied between ~100 nm to ~250 nm and the average spacing was around 0.5 to 2 μm , corresponding to a surface density of $(3 - 50) \times 10^7$ CNF tips per cm^2 .

The 3×3 multiplex NEA chip consisted of regular VACNF arrays grown from e-beam lithographically patterned Ni catalyst dots (~100 nm in diameter) forming a $1.0\text{-}\mu\text{m}$ square lattice on the nine individually addressed $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ Cr microelectrode pads on a 500-nm SiO_2 covered Si(100) wafer predefined with UV-lithography (see Fig. S4). The produced CNF diameter was uniformly around ~100 nm. The exposed CNF length was also uniformly controlled around 150 nm. Overall, the e-beam patterned regular VACNF NEAs present more uniform structure and more consistent electrochemical properties. They can be mass-produced to lower the cost for future applications.

As detailed in the Supplementary Information, the SiO_2 surface of VACNF NEAs was passivated with ethylene glycol to reduce nonspecific adsorption of biomolecules. The VACNFs were activated by electrochemical pretreatments. The Fc-appended tetrapeptides were then covalently linked to the VACNF NEA by forming an amide bond between the $-(\text{CH}_2)_4\text{NH}_2$ linker and the $-\text{COOH}$ group at the exposed CNF tip. In order to achieve stable electrochemical signals, the unreacted $-\text{COOH}$ sites at the VACNF NEA was further reacted with 6-aminohexanol as diluent agents inserted between tetrapeptides.

2.3. Electrochemical Measurements

The electrochemical measurements with random VACNF NEAs were performed in a TEFLON cell with a total volume of 250 μL that was sealed against the VACNF NEA chip with a 3-mm i.d. O-ring. The measurements with e-beam patterned multiplex chips were carried out with a larger polycarbonate cell sealed against the chip with an 8-mm i.d. O-ring. All nine microelectrode pads were exposed to the solution. All measurements used three-electrode configuration with a VACNF NEA working electrode, an Ag/AgCl (3M KCl) reference electrode, and a coiled Pt wire counter electrode. The electrolyte solution for proteolytic measurements was 25 mM MES (pH = 5.0). The ACV was measured by applying a 800 Hz and 150 mV amplitude AC voltage on the DC staircase waveform sweeping from -0.1 V to $+0.75\text{ V}$ at $10\text{ mV}\cdot\text{s}^{-1}$ scan rate.

2.4. Validating Enzyme Activities with Fluorescence Assays

The activities of the enzymes vary significantly from batch to batch and are very sensitive to the environment and storage conditions. To reduce errors, the enzyme activities were first validated with a commercial fluorescence-based biochemical method using a substrate benzyloxycarbonyl-Leucine-Arginine-7-amino-4-methylcoumarin (Z-Leu-Arg-AMC) (Bachem, Torrance, CA) before each electrochemical experiment. The cleavage to the substrate at the site between arginine and AMC dye released AMC from quenching by the carbonyl group and thus gave increased fluorescence intensity.

3. Results and Discussion

3.1. Protease Sensor Design and Principles

The insert of Fig. 1A illustrates the principles of electrochemical assay for measuring the enzymatic activity of cathepsin B using VACNF NEAs. The tetrapeptides Leu-Arg-Phe-Gly labeled with electroactive Fc at the distal end are immobilized on the VACNF NEAs. Fc provides a reliable oxidation peak at $\sim 0.25\text{ V}$ versus Ag/AgCl (3 M KCl) in ACV measurements. In our previous report (Swisher et al., 2013), we have confirmed with HPLC and MS analyses that cathepsin B specifically cleaved the tetrapeptide at the site between arginine and phenylalanine. This releases the fragment containing Fc into the bulk solution

and caused the peak current to decrease. The kinetics of the proteolytic reaction is monitored with continuously repeated ACV measurements. As reported in our previous studies (Swisher et al., 2013; Syed et al., 2011), a noteworthy advantage of ACV technique on Fc-attached VACNF NEAs is that the optimum AC frequency (800 to 1,800 Hz) was much higher than that on macro-GCEs (~40 Hz), leading to higher sensitivity and higher temporal resolution.

3.2. Quantitative Analyses of Protease Activities of Purified Cathepsin B

Fig. 1A shows a representative ACV curve of $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Leu-Arg-Phe-Gly-NH-CH}_2\text{-Fc}$ immobilized on a random VACNF NEA. The AC peak current $i_{p,\text{acv}}$ can be derived by subtracting a linear background. Figs. 1B–D show four representative background-subtracted ACV curves corresponding to 0, 20, 40 and 60 min, respectively, after adding the purified cathepsin B solution at three different final concentrations. The background-subtracted peak current $i_{p,\text{acv}}$ clearly decreased with the reaction time while the peak position slowly shifted from ~0.25 to ~0.15 V. Fig. 1E–G illustrate the value of $i_{p,\text{acv}}$ from all ACVs versus the reaction time t . The value of $i_{p,\text{acv}}$ was relatively stable at the beginning. Adding the cathepsin B solution at $t = 0$ caused some disturbance to the $i_{p,\text{acv}}$ signal. Following that, all curves showed an exponential decay versus the reaction time which can be fit with:

$$i_t = i_0 \exp(-t/\tau) + (bt + c). \quad (1)$$

The values of the fitting parameters to Fig. 1E–G are listed in Table S1. Apparently, the value of i_0 correlated with the total amount of surface-functionalized peptide-Fc (Creager et al., 1999; Syed et al., 2011), which varied with the density and exposed length of VACNFs in the NEAs. The term $(bt + c)$, whose nature was not fully understood in our previous study (Swisher et al., 2013), was found due to the Fc residues that were not cleavable by the protease. A large portion of them was attributed to physically trapped peptide-Fc at the Cr or embedded CNF surface due to the defects and pores in the SiO_2 insulating layer. Fig. S5 shows the ACV feature of physically trapped peptide-Fc on a random VACNF NEA by simply soaking the chip in 1.0 mM peptide-Fc solution for 2 hrs. There was also a possibility that some covalently attached peptide-Fc molecules might lie tightly on the VACNF surface and cannot be cleaved by the protease due to the steric hindrance. Both factors may lead to the negative shift of ACV peak potential in Fig. 1B–D.

Two “negative control” experiments were carried out to ascertain that the exponential decay of the $i_{p,\text{acv}}$ signal was attributed to specific proteolysis of cathepsin B by using a non-relevant peptide-Fc substrate $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Ala-Ala-Asn-Leu-NH-CH}_2\text{-Fc}$ and a peptide-Fc substrate with the specific cleavage site (i.e P1) –Arg– replaced by –Asn–, i.e. $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Leu-Asn-Phe-Gly-NH-CH}_2\text{-Fc}$. As shown in Fig. S6A and S6B, the peak current $i_{p,\text{acv}}$ of these two tetrapeptides did not show the characteristic exponential decay while the activated cathepsin B was added into the electrochemical cell.

Apparently, the exponential decay of the $i_{p,\text{acv}}$ value after adding the protease represents the proteolysis kinetics. However, though each curve in Fig. 1E–G can be analyzed separately, the raw $i_{p,\text{acv}}(t)$ values cannot be directly compared due to the variations in Fc-peptide quantity and background level from sample to sample. In order to normalize these varying factors, we define a new function S , referred as “extracted proteolytic signal”, with

$$S = [i_t - (bt + c)]/i_0 = \exp(-t/\tau). \quad (2)$$

The extracted function S is essentially the exponential component of the kinetic data, whose decay time constant τ is directly related to the protease activity, as will be discussed later. As shown in Fig. 2, the proteolytic measurements of different random VACNF NEAs at three cathepsin B concentrations are normalized to the same fractional scale using S , with all curves passing through $(t = 0, S = 1.0)$. This allows clear view of the decay rate from different samples. The increase in cathepsin B concentration caused the function S to decay faster, giving smaller decay time constant τ and higher protease activity. This new method is critical for later studies requiring quantitative comparison of data from a large set of electrodes.

From our previous report (Swisher et al., 2013), the proteolysis kinetics can be analyzed with a modified Michaelis-Menten model for heterogeneous enzymatic reactions (Gutiérrez et al., 2004):

where E , S_s , ES_s , P_s and P represent the enzyme, surface-bound peptide substrate, enzyme-substrate complex on the electrode surface, surface-attached product, and product released to solution, respectively. The reaction rate v can be defined as

$$v = -\frac{d\Gamma_{S_s}}{dt} = \frac{k_{cat} [E_0] \times \Gamma_{S_s}}{K_M + [E_0]}, \quad (4)$$

where Γ_{S_s} represents the surface density of active peptide substrate, k_{cat} is the dissociation rate constant and $K_M = (k_{cat} + k_{-1})/k_1$ is the Michaelis-Menten constant, respectively. Since “extracted proteolytic signal” S is proportional to the surface densities of Fc-peptide substrates (i.e. $\Gamma_{S_s} \propto S$), Equation 4 can be rewritten as Equation 5 and, at low enzyme concentrations ($[E_0] \ll K_M$), an approximate relationship can be obtained as

$$v \propto -\frac{dS}{dt} = \frac{k_{cat} [E_0] \times S}{K_M + [E_0]} \approx \frac{k_{cat} [E_0] \times S}{K_M}, \quad (5)$$

The value of $(-dS/dt)$ can be derived from Fig. 2 and plot versus S in Fig. 3A. Combining Equations 2 and 5, it gives

$$-\frac{dS/dt}{S} = \frac{k_{cat}}{K_M} [E_0] = \frac{1}{\tau}. \quad (6)$$

Therefore, the measured quantity $(k_{cat}/K_M) \cdot [E_0]$ can be represented by $1/\tau$ or the slope of Fig. 3A, with smaller τ giving larger $(k_{cat}/K_M) \cdot [E_0]$. The statistical value of $(k_{cat}/K_M) \cdot [E_0]$ derived from the slope of each lines in Fig. 3A (i.e. $-dS/dt$ versus S) was $(7.45 \pm 3.53) \times 10^{-4}$, $(1.25 \pm 0.30) \times 10^{-3}$ and $(2.15 \pm 0.37) \times 10^{-3} \text{ s}^{-1}$ from nine experiments with enzyme concentration at 15.5, 30.7 and 62.1 nM, respectively. As shown in Fig. 3B, the derived quantity $(k_{cat}/K_M) \cdot [E_0]$ is linearly proportional to the enzyme concentration $[E_0]$, consistent with the theoretical model. Since $[E_0]$ was known in the measurements shown in Fig. 1 & 2, one can easily derive the concentration-independent “specificity constant” k_{cat}/K_M which is commonly used to represent the fundamental catalytic efficiency of different enzymes.

The calculated k_{cat}/K_M values at three cathepsin B concentrations were very close to each other, giving an average value of $4.11 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and a standard deviation of 6.66×10^3

$\text{M}^{-1}\text{s}^{-1}$. Alternatively, fitting the data points in Fig. 3B with a straight line passing through the origin gave a more accurate $k_{\text{cat}}/K_{\text{M}}$ value of $(3.68 \pm 0.50) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ that incorporated the results from all nine experiments at three discrete cathepsin B concentrations. These $k_{\text{cat}}/K_{\text{M}}$ values are more accurate than our previous analyses (Swisher et al., 2013) based on ACV measurements at a single enzyme concentration. The $k_{\text{cat}}/K_{\text{M}}$ of the same batch of cathepsin B derived from nine fluorescence experiments right before the electrochemical measurements was $(9.6 \pm 4.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, showing much larger standard deviation. However, all values are within the range of $\sim 2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ to $\sim 7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in literature (Almeida et al., 2000; Gutiérrez et al., 2004; Nägler et al., 1997) with a more extensive list showing in Table S2. Importantly, the results confirmed that the $k_{\text{cat}}/K_{\text{M}}$ value by ACV measurements remained constant as the cathepsin B concentration varied, establishing a method to determine the cathepsin B activity in an unknown sample by dividing the measured $(k_{\text{cat}}/K_{\text{M}}) \cdot [\text{E}_0]$ with the above $k_{\text{cat}}/K_{\text{M}}$ value.

3.3. Detecting Cathepsin B Activity in Complex Tissue Lysates

Normal human tissues may contain many proteases including cathepsin B and their expression at higher level is expected in cancerous tissues. To demonstrate that ACV coupled with VACNF NEAs is viable for detecting protease activities in complex human samples, we have carried out three sets of measurements using lysates from human breast tissue. The cathepsin-B-specific tetrapeptide $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Leu}-\text{Arg}-\text{Phe}-\text{Gly}-\text{NH}-\text{CH}_2-\text{Fc}$ was immobilized on random VACNF NEAs. The human breast whole tissue lysate (adult normal) was first treated with the activation buffer similar to the purified cathepsin B experiments. 25 μL of the activated lysate solution was then added into the electrochemical cell containing 250 μL of 25 mM MES (pH 5.0) while the ACV measurement was continuously repeated. In Fig. 4A, the final concentrations of the lysate were 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$ and 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$, diluted by 245 and 168 fold, respectively from the purchased tissue lysate stock solution. Both lysate samples caused exponential decay after being added into the electrochemical cell, as shown in the “extracted proteolytic signal” $S = (i_t - i_b)/i_0$ with $i_b = bt + c$. The fitted time constants are $\tau_0 = 725 \text{ s}$ and $\tau_0' = 1647 \text{ s}$ at the lysate concentration of 29.1 and 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. The derived $(k_{\text{cat}}/K_{\text{M}}) \cdot [\text{E}_0]$ values are $6.04 \times 10^{-4} \text{ s}^{-1}$ for 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$ tissue lysate and much higher at $1.38 \times 10^{-3} \text{ s}^{-1}$ for 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$ tissue lysate. Using $k_{\text{cat}}/K_{\text{M}} = (3.68 \pm 0.50) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ derived from the purified cathepsin B solutions in Fig. 3, the equivalent cathepsin B concentrations were calculated as $\sim(16.4 \pm 2.2) \text{ nM}$ in the 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$ tissue lysate, and $\sim(37.5 \pm 5.1) \text{ nM}$ in the 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$ tissue lysate. Due to the existence of the experimental deviation and activity variation of biology sample, they did not present a perfect linear relationship but were close.

As 15.5 and 30.7 nM purified cathepsin B were spiked into the 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$ tissue lysate, the decay became faster, giving $\tau_1 = 433 \text{ s}$ and $\tau_2 = 213 \text{ s}$, respectively (Fig. 4B). Clearly, substantial proteolysis was catalyzed by cathepsin B. To further confirm that the measured decay was from the specific proteolysis of peptide-Fc by cathepsin B, an immunoprecipitation (IP) assay was employed to partially remove cathepsin B from the tissue lysate. The exponential decay was indeed much slower, giving $\tau_4 = 1246 \text{ s}$ (Fig. 4C), but some substantial decay feature was retained. It is likely that some cathepsin B still retained in the tissue lysate after four rounds of IP but the level was below the sensitivity of Western blot analysis shown in Fig. 4D. It is also possible that other proteases (particular other cathepsins, such as cathepsin L and V) may cleave the tetrapeptide although the catalytic activity may be lower.

Quantitative estimation of cathepsin B activity in the tissue lysate can be made based on the results shown in Fig. 4B. First, according to Equation 6, we can derive the difference between the 15.5 nM spiked and unspiked samples as:

$$(k_{\text{cat}}/K_{\text{M}}) \cdot ([E_0] + 15.5 \text{ nM}) - (k_{\text{cat}}/K_{\text{M}}) \cdot [E_0] = 1/\tau_1 - 1/\tau_0 = 9.30 \times 10^4 \text{ s}^{-1}, \quad (7)$$

which gives $k_{\text{cat}}/K_{\text{M}} = 6.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. This value is slightly higher than the value $(4.11 \pm 0.67) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ obtained with purified cathepsin B in buffer solutions as discussed above. For the tissue lysates with and without IP procedure, we have

$$(k_{\text{cat}}/K_{\text{M}}) \cdot \Delta[E_0] = 1/\tau_0 - 1/\tau_4 = 5.77 \times 10^{-4} \text{ s}^{-1}. \quad (8)$$

Thus the difference in cathepsin B concentration $\Delta[E_0]$ is equivalent to $\sim 14 \pm 2.3 \text{ nM}$ cathepsin B in buffer solution if we use the value $k_{\text{cat}}/K_{\text{M}} = (4.11 \pm 0.67) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ discussed above. Though refinements are needed to reduce the error bar, it is clear that this method is applicable in complex tissue lysate samples. This study established a critical step toward developing meaningful diagnosis tools. *To our knowledge, this is the first time that the activity of a protease has been quantitatively determined by electrochemical methods with complex whole tissue lysates.*

The cathepsin B activity in the normal human breast whole tissue lysate was also validated by the fluorescence method using a short peptide substrate Z-Leu-Arg-AMC. The very small exponential curvature in Fig. S7 gave a much lower value of $(k_{\text{cat}}/K_{\text{M}}) \cdot [E_0]$ ($\sim 4.98 \times 10^{-5} \text{ s}^{-1}$) than that obtained from the electrochemical measurement ($\sim 1.38 \times 10^{-3} \text{ s}^{-1}$), although the lysate concentration was much higher ($0.24 \text{ mg}\cdot\text{mL}^{-1}$ vs. $29.1 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$). But the lysates spiked with 11.9 and 23.9 nM purified cathepsin B showed clear exponential increases in fluorescence signal (Fig. S8) due to proteolysis, which validated that the electrochemical signal was indeed due to cathepsin B.

3.4. Toward Multiplex Detection with Regular VACNF NEAs

In long term, regular VACNF NEAs can be mass-produced as multiplex chips on independently addressed microelectrode pads using UV- and e-beam lithography (Arumugam et al., 2009). Such chips can be integrated into portable electronic systems for profiling a set of proteases. Figs. 5A and S4 show nine $200 \text{ }\mu\text{m} \times 200 \text{ }\mu\text{m}$ microelectrode pads of $\sim 100 \text{ nm}$ -thick Cr film in a 3×3 array on a SiO_2 -covered Si wafer. VACNFs were grown in regular $1 \text{ }\mu\text{m} \times 1 \text{ }\mu\text{m}$ lattice on these microelectrode pads to form embedded NEAs which were independently connected with the measuring circuits through larger ($1 \text{ mm} \times 1 \text{ mm}$) contact pads at the top of Fig. S4A (not shown). Fig. 5B shows the zoom-in image of exposed VACNF tips of $\sim 100 \text{ nm}$ in diameter and $\sim 150 \text{ nm}$ length protruding over the SiO_2 matrix. Fig. S9A shows the CVs of a $50 \text{ mM K}_3\text{Fe}(\text{CN})_6$ solution from all nine VACNF NEAs. Clearly, they all showed the characteristic sigmoidal shape with low background and almost identical limiting current, indicating the high uniformity of these NEAs.

The covalently attached tetrapeptide $\text{H}_2\text{N}-(\text{CH}_2)_4\text{-CO-Leu-Arg-Phe-Gly-NH-CH}_2\text{-Fc}$ on the e-beam patterned regular VACNF NEA present a similar ACV peak at $\sim 0.25 \text{ V}$ as shown in Fig. S9C. The peak shape was much better defined than the random VACNF NEAs. Similar as before, the ACV peak can be extracted by subtracting a linear background. Fig. S9D shows that the ACV peak current monotonically decreased after a purified cathepsin B solution was added into the electrochemical cell to give a final concentration of 30.7 nM . The peak potential also continuously downshifted to $\sim 0.10 \text{ V}$. The completed kinetic curve of raw $i_{\text{p,acv}}$ during proteolysis is presented in Fig. S10A. The $i_{\text{p,acv}}$ amplitude decayed much faster in the initial reaction period but then leveled off after $\sim 35 \text{ min}$. Similar to the random VACNF NEAs, the leveled $i_{\text{p,acv}}$ was attributed to physically trapped peptide-Fc in the SiO_2 matrix as confirmed by the control experiment shown in Fig. S10B. While such

background can be reduced in the future by improving SiO₂ deposition and passivation, it can be easily excluded using the “extracted proteolysis signal” $S = (i_t - i_b)/i_0$ discussed earlier.

The kinetic curves of S vs. the reaction time after adding 30.7 nM purified cathepsin B is shown in Fig. 5C. The fitted exponential decay time constant τ was 353 s, clearly smaller than 800 ± 192 s obtained with random VACNF NEAs at the same cathepsin B concentration. From either the fitted slope of $-dS/dt$ versus S or τ value, $(k_{cat}/K_M) \cdot [E_0]$ was calculated as $2.81 \times 10^{-3} \text{ s}^{-1}$, about twice of the statistical value $(1.25 \pm 0.30) \times 10^{-3} \text{ s}^{-1}$ on the random VACNF NEA at 30.7 nM cathepsin B concentration. Accordingly, the specificity constant k_{cat}/K_M was 9.2×10^4 , about twice of $(4.11 \pm 0.67) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ measured with random VACNF NEAs. Clearly, the regular VACNF NEAs worked much better than the random VACNF NEAs. The smaller diameter (~100 nm vs. 150 nm) may help to reduce the steric hindrance for enzymes to interact with the immobilized peptide. The uniform size and exposed length as well as the regular spacing of the CNFs made the regular VACNF NEAs more reproducible and perform better than the random VACNF NEAs.

4. Conclusions

We have demonstrated electrochemical detection and kinetic analysis of cathepsin B activity in proteolysis of Fc-appended tetrapeptides that are immobilized on VACNF NEAs using enhanced AC voltammetry. A normalization algorithm was used to extract the proteolysis signal from the largely varied raw data, which enabled consistent comparison and quantitative analyses of cathepsin B activity. This algorithm was first validated with purified cathepsin B solutions and then demonstrated in complex lysates of human breast tissue, the lysates spiked with purified cathepsin B, and the immunoprecipitated lysate. The cathepsin B concentration in normal tissue lysate can be quantitatively assessed by comparing these results. The regular VACNF NEAs fabricated with precise e-beam lithography showed faster kinetics of cathepsin B proteolysis, illustrating the potential for further improving detection sensitivity. This electrochemical method has great potential as a portable multiplex system for simple and rapid protease profiling of lysate, serum, or blood samples, which may be used in cancer diagnosis and treatment monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A) A representative AC voltammogram (ACV) of a random VACNF NEA immobilized with $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CO}-\text{Leu}-\text{Arg}-\text{Phe}-\text{Gly}-\text{NH}-\text{CH}_2-\text{Fc}$ measured at AC frequency $f = 800$ Hz and AC voltage amplitude $V_0 = 150$ mV. Insert: Schematic of cathepsin B induced proteolytic cleavage of the tetrapeptide substrate. (B–D) Four representative background-subtracted ACVs from the continuously repeated ACV measurements after 25 μL of cathepsin B was added into the electrochemical cell. The final concentrations of cathepsin B were (B): 15.5 nM ($0.5 \text{ ng} \cdot \mu\text{L}^{-1}$); (C): 30.7 nM ($0.9 \text{ ng} \cdot \mu\text{L}^{-1}$); and (D): 62.1 nM ($1.8 \text{ ng} \cdot \mu\text{L}^{-1}$), respectively. (E–G) The change of ACV peak current $i_{p,acv}$ versus the reaction time while the cathepsin B solution at the concentration corresponding to (B–D) was added to the electrochemical cell. The continuous lines are fitting curves to the proteolysis using Equation 1.

Fig. 2.

The proteolysis kinetics represented by the extracted proteolysis signal $S = (i_t - i_b)/i_0$ vs. the reaction time after adding purified cathepsin B into the electrochemical cell. The final concentrations of cathepsin B are 15.5, 30.7, and 62.1 nM, respectively. Each set of data was fitted with a simple exponential decay.

Fig. 3.

(A) Plot of the reaction rate $-dS/dt$ versus extracted proteolysis signal $S = (i_t - i_b)/i_0$ at different cathepsin B concentrations. (B) Plot of $(k_{\text{cat}}/K_M)[E_0]$ with respect to cathepsin B concentration $[E_0]$ in the proteolytic reactions. The error bars are the standard deviation of the results from at least triplicated experiments.

Fig. 4.

The proteolysis curves of (A) 20.0 $\mu\text{g}\cdot\text{mL}^{-1}$ (pink diamond) and 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$ (black square) of the whole breast tissue lysate (normal adult human); (B) 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$ whole tissue lysate (black square) and this tissue lysate spiked with 0.9 $\mu\text{g}\cdot\text{mL}^{-1}$ (30.7 nM) (red dot) and 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ (15.5 nM) (blue triangle) cathepsin B, respectively; and (C) 29.1 $\mu\text{g}\cdot\text{mL}^{-1}$ tissue lysate before (black square) and after four rounds of immunoprecipitation (4IP) in order to deplete the cathepsin B (green dot). All ACV measurements were carried out at $f = 800$ Hz and AC voltage amplitude $V_0 = 150$ mV. (D) Western blot analysis using cathepsin B antibody: Lysate lane – 25.0 μg of the original whole tissue lysate; Pellet lane – the immunoprecipitated fraction of 0.5 mg of tissue lysate using cathepsin B antibody; Supernatant lane – the supernatant fraction after four rounds of immunoprecipitation. The proenzyme cathepsin B is ~ 37 kDa and the active cathepsin B is ~ 25 kDa.

Fig. 5. SEM images at 45° perspective view of (A) a 3 × 3 VACNF NEA chip, and (B) the e-beam patterned regular VACNF NEA partially embedded in SiO₂ matrix at each microelectrode pad. (C) The extracted proteolysis signal $S = (i_t - i_b)/i_0$ was derived from continuously repeated ACV measurements while 25 μL of purified cathepsin B was added into the electrochemical cell to a final concentration of 30.7 nM (0.9 ng·μL⁻¹). All ACV measurements were carried out at $f = 800$ Hz and AC voltage amplitude $V_0 = 150$ mV.