Single Molecule Assay for Ultrasensitive Detection of Cathepsin B in Human Blood

Bharani Thangavelu* and Angela M. Boutté

ABSTRACT: Cathepsin B (catB) is a lysosomal cysteine protease expressed in several cells and organs, where it plays a role in protein degradation and turnover. Extracellular, secreted catB has utility as a biomarker for a host of pathological or physiological states, including a myriad of cancers or neurological diseases and injuries. Analytical or diagnostic assessment may be limited by biological sample volume availability. Pathologically relevant changes in catB levels may occur at low-moderate concentrations that require accurate measurement to differentiate from basal levels. Furthermore, biological samples like plasma and serum, often obscure accurate catB measurements because of background and high variance, vastly limiting the ability to detect catB as a peripheral biomarker. Techniques for ultrasensitive protein detection that require low volumes of sample are necessary. To overcome these challenges, a digital enzyme-linked immunosorbent assay (ELISA) was developed for differential detection of catB within less than 5 μL of serum and plasma using the single molecule array (SiMoA) platform, which offers 1000-times more sensitivity and vastly reduced variance compared to colorimetric tests. In buffer, curve-fitting estimated the limit of detection (LoD) to be ~1.56 and ~8.47 pg/mL using two-step or three-step assay configurations, respectively. After correcting for endogenous levels, the estimated LoD was ~4.7 pg/mL in the serum or plasma with the two-step assay. The lower limit of quantitation was ~2.3 pg/mL in the buffer and ~9.4 pg/mL in the serum or plasma, indicating the ability to measure small changes above endogenous levels within blood samples.

1. INTRODUCTION

Cathepsin B (catB) belongs to a family of papain-like, cysteine proteases which are a class of proteolytic enzymes expressed in most cell types. It is primarily localized within subcellular endosomal and lysosomal compartments. While, intra- and peri-cellular catB is involved in metabolic homeostasis, antigen processing and presentation during immune responses, degradation of misfolded proteins, as well as cellular migration, it is also secreted as a myokine, involved in muscle–brain cross talk and neurogenesis. Under pathological conditions, catB expression becomes upregulated in a variety of diseases including metastatic cancers, infections, traumatic brain injury, and neurological diseases. This upregulation is often associated with increased extracellular secretion via active or passive mechanisms. As such, the catB protein content is elevated above basal levels where it may have utility as a biofluid-based marker of diseases, injury, or trauma.

High levels of catB are readily measurable in tissues, liquid biopsies, and cerebral spinal fluid (CSF) using standard, colorimetric assays. For example, catB was found to be elevated in CSF, biopsied tissue lysates, or tumor aspirates using western blotting or colorimetric enzyme-linked immunoassorbent assays (ELISAs). Detection of catB in blood is even more desirable because of the ease of access with little risk to the patient during sampling collection. Elevated catB has been demonstrated in serum or plasma. These assays typically require a fairly large volume (~100 μL) of serum or plasma, offer limited dynamic range, are less sensitive because of the inherent background found in blood samples, and tend to offer broad variance. Thus, changes in catB content that occur as a consequence of disease or injury may not be detectable.

In order to obtain increased accuracy and vastly reduce background noise while utilizing limited biological sample volumes, an ultrasensitive assay was developed for blood using digital ELISAs. Adaptation of catB measurement to the single molecule array (SiMoA) platform improved the estimated limit of detection (LoD) and lower limit of quantitation.
(LLoQ) in human serum and plasma. SiMoA technology has been widely used for analytical quantitation of blood biomarkers, which is inclusive of customized tests. Detailed methodology for measurement of catB derived from low volumes of serum and plasma compared to buffer is provided.

2. RESULTS AND DISCUSSION

2.1. Accuracy and Selectivity of CatB Detection in Assay Buffer. The workflow for ultrasensitive detection of catB in serum and plasma using the SiMoA HD-1 platform is shown (Figure 1). To determine the detection range and quantitation limit of customized digital ELISAs, recombinant human catB protein was spiked into Q-buffer and measured using manufacturer-defined two-step or three-step homebrew configurations. When developing new assays for this platform, it is essential to test both assay configurations in order to determine optimal procedures and interpret data. The two- and three-step SiMoA methods are analogous to sandwich ELISA and indirect ELISA procedures, respectively. In the two-step assay configuration, the sample is incubated with the capture beads and detector antibody in the first step and SβG is added in the second step. In the three-step assay configuration, the sample is incubated with capture beads in the first step and then with detector antibody in the second step; then, SβG is added in the third step. Based on the 4 PL curve fitting extrapolation, the computed LoD, pg/mL, as defined by the SiMoA for two-step and three-step assays are shown.

Table 1. Quantitation, Dilution Linearity, and Recovery of CatB in Q-Buffera

| C Spike (pg/mL) | C Det. (pg/mL) | SD (pg/mL) | Error (%) | Recovery (%) | C Det. (pg/mL) | SD (pg/mL) | Error (%) | Recovery (%) |
|----------------|---------------|------------|-----------|--------------|---------------|------------|-----------|--------------|
| 1200.00        | 1190          | 14.03      | 0.83      | 99.17        | 1167          | 20.92      | 2.75      | 97.25        |
| 600.00         | 614           | 35.16      | 2.33      | 102.33       | 587           | 11.57      | 2.17      | 97.83        |
| 300.00         | 311           | 9.85       | 3.67      | 103.67       | 289           | 8.65       | 3.67      | 96.33        |
| 150.00         | 149           | 12.57      | 0.67      | 99.33        | 144           | 3.24       | 4.00      | 96.00        |
| 75.00          | 80            | 6.01       | 6.67      | 106.67       | 68            | 1.26       | 9.33      | 90.67        |
| 37.50          | 39            | 3.90       | 4.00      | 104.00       | 35            | 0.59       | 6.67      | 93.33        |
| 18.75          | 18            | 1.02       | 4.00      | 96.00        | 17            | 0.36       | 9.33      | 90.67        |
| 9.38           | 10            | 0.72       | 6.67      | 106.67       | 8.9           | 0.36       | 5.07      | 94.93        |
| 4.69           | 5.2           | 0.68       | 10.93     | 110.93       | 0             | 0.00       | 100.00    | 0.00         |
| 2.34           | 2.64          | 0.29       | 12.64     | 112.64       | 0             | 0.00       | 100.00    | 0.00         |
| 1.17           | 1.61          | 0.35       | 37.39     | 137.39       | 0             | 0.00       | 100.00    | 0.00         |
| 0.00           | 0.00          | 0.00       | N/A       | N/A          | 0             | 0.00       | N/A       | N/A          |

Extrapolated LOD 1.56 Extrapolated LoD 8.47

“CatB (0–1200 pg/mL) was spiked into the Q-buffer. Samples were serially diluted (1/2) in the Q-buffer. The expected (C Spike) and observed (C Det.) concentrations (pg/mL) with standard deviation (SD), percent (%) of error, and recovery are displayed, as well as the extrapolated limit of detection (LoD, pg/mL) as defined by the SiMoA for two-step and three-step assays are shown.”

Cross-reactivity can occur with other proteins and lead to either false-positive or -negative signals. Therefore, selectivity of this assay was determined using related lysosomal cysteine...
protease proteins, cathepsin S (catS) and cathepsin L (catL), as interference proteins. CatB was added to the Q-buffer at 100 pg/mL. CatS and catL were added at concentrations of 2.5, 5, and 10 ng/mL, which were equivalent to 25-, 75-, and 100-fold excess compared to catB. Corresponding AEB values are shown (Figure 3). Additionally, catB was titrated (0−1200 pg/mL) in the presence of 10 ng/mL of catS or catL, such that the ratio of catS or L, each was 25-400 fold in excess of catB (data not shown). No analytical interference was observed and selectivity for catB was observed.

2.2. Measurement of CatB in Human Serum and Plasma in Excess of Endogenous Levels. Feasibility of detection of endogenous catB detection in human blood was determined. Serum or plasma was diluted by 1/256 (e.g. ∼1.17 μL in 298.83 μL Q-buffer) in Q-buffer to achieve an ideal background level of 0.01 AEB (F_{0} = 0.01). Next, the ability to detect known catB concentrations was conducted by adding serially diluted catB (0−1200 pg/mL) to prediluted (with Q-buffer) serum and plasma. Quantitation of catB in serum and plasma was shown (Table 2A,B). The percent of error and recovery values for catB ranges are displayed for serum and plasma and recovery ≥80% for samples spiked with catB for the two-step or the three-step, respectively. The % recovery values are in the analytically acceptable range, 70−130%. The computed serum LoD was ∼4.7 pg/mL for the two-step or three-step assay configurations, respectively. For plasma, the LoD was ∼4.7 pg/mL for the two-step and ∼9.4 pg/mL for the three-step configuration.

The estimated lower limit of quantification (LLoQ) (signal > zero, recovery ≥80%, error ≤20%) for catB spiked in serum and plasma was 9.4 pg/mL for the two-step and ∼9.4 pg/mL for the three-step configuration.

Next, assay performance was further determined by comparing the LLoQ values to endogenous protein levels estimated from the samples measured without addition of recombinant catB (Table 3B). The estimated discriminant values for the two step assay for serum and plasma were 5.3 and 7.5%, respectively; values for the three-step assay were 23.5 and 39.2%, correspondingly. Lastly, data derived from commercially available colorimetric ELISAs are shown for comparison (Table 4). The LoD of the proposed SiMoA assay for catB detection was lower than that of traditional ELISAs.

3. CONCLUSIONS

This work shows development of an ultrasensitive assay for the detection of catB with >80% selectivity and recovery. Although this assay may be used with any biological sample within the buffer systems described, substantially lower LoD/LLoQ, the...
ability to differentiate small concentration changes, and utilization of very small sample volumes (∼2 μL) compared with commercially available assays for serum or plasma is shown. Due to improved detection ranges and differential quantification compared to baseline measurements, this customized assay is a promising method for accurate measurement of incremental catB biomarker changes within the broad range of physiological states, diseases, or trauma. CatB detection with this method has utility within a wide range of research applications, and potentially, clinical settings that may require early detection as well as prolonged care monitoring.

4. EXPERIMENTAL SECTION

4.1. Reagents and Instrumentation. SiMoA homebrew kits included carboxylated paramagnetic beads, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), barcoded labels, sample diluent, detector diluent, biotinylation reaction buffer (BBR), bead wash buffer (BWB), bead conjugation buffer (BCB), and a bead diluent. The streptavidin-β-galactosidase (SβG), resorufin-β-o-galactopyranoside (RGP), system buffer (wash buffer one and wash buffer two), sealing oil, 96-well plates, conductive tips, array discs, and cuvettes and the HD-1 instrument were purchased from Quanterix, Inc. (Billerica, MA). Fetal bovine serum (FBS) was substituted according to the instructions provided by the manufacturer. Briefly, capture catB antibodies were subjected to buffer exchange by one freeze–thaw cycle prior to use.

4.2. Preparation of Capture Antibody-Coated Magnetic Beads. The catB capture antibodies (Catalog# MAB2177, R&D Systems, Minneapolis, MN) were reconstituted according to the instructions provided by the manufacturer. Briefly, capture catB antibodies were subjected to buffer exchange by first adding 0.08 mg of antibody solution that was diluted into BCB (Quanterix, Inc.; final volume of 500 μL) to an Amicon 50K MWCO filter (Catalog# C82301, Millipore Sigma, Burlington, MA) and then centrifuging at 4 °C at 14,000 g for 5 minutes (min). The eluate was discarded

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Table 2. Quantitation and Recovery of CatB in Human Blood

| C Spike (pg/mL) | A: Quantitation of CatB in Serum | Three-Step Protocol |
|----------------|---------------------------------|---------------------|
|                | C Det. (pg/mL) | SD (pg/mL) | C Det. (pg/mL), Adjusted | Error (%) | Recovery (%) | C Det. (pg/mL) | SD (pg/mL) | C Det. (pg/mL), Adjusted | Error (%) | Recovery (%) |
| 1200.00        | 1501           | 15.92      | 1325                     | 10.42     | 110.42       | 1240           | 11.75      | 1160                     | 3.33      | 90.00          |
| 600.00         | 845            | 18.24      | 699                      | 11.50     | 111.50       | 645            | 13.92      | 565                      | 5.83      | 94.17          |
| 300.00         | 512            | 11.07      | 336                      | 12.00     | 112.00       | 364            | 9.55       | 284                      | 5.33      | 94.67          |
| 150.00         | 349            | 15.67      | 173                      | 15.33     | 115.33       | 210            | 5.93       | 130                      | 13.33     | 86.67          |
| 75.00          | 259            | 39.76      | 83                       | 10.67     | 110.67       | 146            | 4.53       | 66                       | 12.00     | 88.00          |
| 37.50          | 213            | 10.06      | 37                       | 1.33      | 98.67        | 110            | 2.59       | 30                       | 20.00     | 80.00          |
| 18.75          | 196            | 17.41      | 20                       | 6.67      | 106.67       | 96             | 3.61       | 16                       | 14.67     | 85.33          |
| 9.38           | 186            | 17.97      | 10                       | 6.67      | 106.67       | 82             | 2.65       | 2                        | 78.67     | 21.33          |
| 4.69           | 178            | 10.09      | 2                        | 57.33     | 42.67        | 82             | 4.20       | 2                        | 57.33     | 42.67          |
| 2.34           | 176            | 6.62       | 0                        | 100       | 0            | 80             | 3.22       | 0                        | 100       | 0              |
| 1.17           | 176            | 8.88       | 0                        | 100       | 0            | 80             | 2.57       | 0                        | 100       | 0              |
| 0.00           | 176            | 1.16       | 0                        | N/A       | N/A           | 80             | 2.51       | 0                        | N/A       | N/A            |
| Extrapolated LoD |                |            |                          | 4.7       |               |                |            |                          | 4.7       |               |

| B: Quantitation of CatB in Plasma | Three-Step Protocol |
|----------------------------------|---------------------|
| C Spike (pg/mL) | C Det. (pg/mL) | SD (pg/mL) | C Det. (pg/mL), Adjusted | Error (%) | Recovery (%) | C Det. (pg/mL) | SD (pg/mL) | C Det. (pg/mL), Adjusted | Error (%) | Recovery (%) |
| 1200.00        | 1376           | 60.38      | 1251                     | 4.25      | 104.25       | 1214           | 13.20      | 1166                     | 2.83      | 97.17          |
| 600.00         | 759            | 22.55      | 634                      | 5.67      | 105.67       | 608            | 9.01       | 560                      | 6.67      | 93.33          |
| 300.00         | 455            | 31.08      | 330                      | 10.00     | 110.00       | 330            | 6.31       | 282                      | 6.00      | 94.00          |
| 150.00         | 284            | 9.38       | 159                      | 6.00      | 106.00       | 187            | 5.92       | 139                      | 7.33      | 92.67          |
| 75.00          | 208            | 12.77      | 83                       | 10.67     | 110.67       | 110            | 3.59       | 62                       | 17.33     | 82.67          |
| 37.50          | 165            | 11.64      | 40                       | 6.67      | 106.67       | 80             | 2.78       | 32                       | 14.67     | 85.33          |
| 18.75          | 145            | 5.39       | 20                       | 6.67      | 106.67       | 66             | 1.48       | 18                       | 4.00      | 96.00          |
| 9.38           | 135            | 6.09       | 10                       | 6.67      | 106.67       | 50             | 2.85       | 2                        | 78.67     | 21.33          |
| 4.69           | 126            | 4.15       | 1                        | 78.67     | 21.33        | 48             | 2.46       | 0                        | 100       | 0              |
| 2.34           | 125            | 2.36       | 0                        | 100       | 0            | 48             | 1.15       | 0                        | 100       | 0              |
| 1.17           | 125            | 6.63       | 0                        | 100       | 0            | 48             | 3.14       | 0                        | 100       | 0              |
| 0.00           | 125            | 3.48       | 0                        | N/A       | N/A           | 48             | 1.06       | 0                        | N/A       | N/A            |
| Extrapolated LoD |                |            |                          | 4.7       |               |                |            |                          | 9.4       |               |

“Serum or plasma was prepared in Q-buffer (1/256) and then spiked with recombinant catB at 1200 ng/mL. Samples were then serially diluted (1/2), such that the content of serum or plasma remained constant. For (A) serum and (B) plasma, the expected (C Spike) and observed (C Det.) concentrations (pg/mL) measured before and after subtraction of baseline levels, respectively are shown. The standard deviation (SD), percent (%) of error, and recovery are displayed. The extrapolated limit of detection (LoD, pg/mL) as defined by the SiMoA for two-step and three-step assays is indicated for each assay configuration.
before washing the membrane twice with 450μL of ice-cold BCB. The beads were then washed two times with 300μL of BCB and resuspended in 290μL of BCB. 10μg of fresh EDC was reconstituted in 1mL of BCB just before use. To activate the beads, 10μL of EDC was added to the bead suspension (final concentration 0.3mg/mL, final volume 300μL) and mixed on an HulaMixer orbital shaker (ThermoFisher Scientific) for 30 min at 4°C. The supernatant was aspirated and then washed two times with 300μL of ice-cold BCB.

The capture antibody solution (300μL) was then added to the beads, vortexed, and placed on the rotator for 45 min at room temperate for blocking. The membrane was inverted atop an Eppendorf tube and centrifuged at 4°C at 1000g for 2 min. The filter was rinsed with 50μL of BCB before repeating centrifugation. The antibody concentration was measured using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific), then diluted to 0.2mg/mL in BCB, and stored on ice until use.

Carboxylated paramagnetic beads (1.4×10^9) were transferred into a 1.5mL microtube and washed three times with 300μL of BWB. The beads were then washed two times with 300μL of BCB and resuspended in 290μL of BCB. 10μg of fresh EDC was reconstituted in 1mL of BCB just before use. To activate the beads, 10μL of EDC was added to the bead suspension (final concentration 0.3mg/mL, final volume 300μL) and mixed on an HulaMixer orbital shaker (ThermoFisher Scientific) for 30 min at 4°C. The supernatant was aspirated and then washed two times with 300μL of ice-cold BCB.

The SiMoA assay LoD is 100-fold lower in serum or plasma compared to commercially available kits. N/A, Not applicable.

A: Quantitation of CatB at Ultralow and Moderate Concentrations

B: Estimated Discriminant Values of CatB

Table 4. Comparative Summary of CatB Assays

| Manufacturer or Developer | Assay Name          | Catalog # | Concentration Format | Detection Range (pg/mL) | Approximate LoD (pg/mL) | Recovery in Serum or Plasma |
|--------------------------|---------------------|-----------|----------------------|-------------------------|-------------------------|-----------------------------|
| R&D Systems              | Total Cathepsin B   | DY2176    | Solid Phase Sandwich | 62.5-4000               | ~62μL                   | ≤20%                        |
| Abcam                    | Cathepsin B ELISA   | ab119584  | Solid Phase Sandwich | 156-10,000              | ~156μL                  | ≥99% (Range 106–115%)       |
| WRAIR                    | CatB-2 Step         | N/A       | Single Molecule, Digital ELISA | 1.56-10,000             | 8.47μL                  | ≥80% (Range 80-97%)         |
| WRAIR                    | CatB-3 Step         | N/A       | Single Molecule, Digital ELISA | 8.5-10,000              |                        |                             |

The SiMoA assay LoD is 100-fold lower in serum or plasma compared to commercially available kits. N/A, Not applicable. aReported by manufacturer/vendor. bVariance based on data provided herein after subtracting for endogenous levels in biological samples.
(3.36 × 10^6 beads/mL) was used to determine the concentration and fraction that is monomeric (e.g., non-aggregated) using the “bead aggregation test” (Quanterix, Inc). The average fraction of monomeric beads was ≥0.85, in excess of ≥0.75 which is the minimum suggested by the manufacturer.

4.3. Preparation of the Detection Antibody. The biotinylated CatB detection antibody (Catalog# BAF953, R&D Systems) was reconstituted according to the instructions provided by the manufacturer. Briefly, the detection CatB antibody was subjected to buffer exchange by adding 0.2 mg of detection antibody solution diluted into BRB (final volume of 500 μL) to an Amicon 50K MWCO filter (Catalog# C82301, Millipore Sigma) and then centrifuged at 4 °C at 14,000g for 5 min. The eluate was discarded before washing the membrane twice with 450 μL with BRB. To collect the detection antibody solution, the filter was inverted upon an Eppendorf tube and centrifuged at 4 °C at 1000g for 2 min. The filter was rinsed with 50 μL of BRB before repeating centrifugation. The concentration was measured using a NanoDrop 2000 spectrophotometer as previously stated and then stored at 4 °C until use.

4.4. SiMoA Assay Implementation and Data Analysis. Antibody-coated capture beads were diluted in a bead diluent to a concentration of 2.0 × 10^7 beads/mL. Biotinylated detector antibodies were diluted in a detector diluent (final one μg/mL). Streptavadin-β-galactosidase (S/βG) concentrate was diluted to 100 pM in a S/βG diluent. Recombinant human CatB protein (Catalog# CY953, R&D Systems) was serially diluted (0–2400 pg/mL) in Q-buffer [25% FBS (Gibco/ThermoFisher Scientific), 75% Sample Diluent (Quanterix, Inc.)] and used as a calibrator to generate standard curves. Capture antibody-coated beads, biotinylated detection antibody, and S/βG were placed in barcoded plastic bottles (Quanterix, Inc.), and the calibrators were loaded onto a 96-well plate (Quanterix, Inc.). Both two-step and three-step assay configurations were tested based on the manufacturer’s instructions (Quanterix, Inc). In the two-step assay configuration, 25 μL of bead solution (5.0 × 10^6 beads), 100 μL of the sample, and 20 μL of the detector antibody (final conc. 1.0 μg/mL) were pipetted into a reaction cuvette and incubated for 35.25 min. The beads were then pelleted with a magnet, and the supernatant was removed. Following several washes, 100 μL of S/βG (final conc. 100 pM) was added and incubated for 5.25 min. The beads were washed, resuspended in RGP solution, and loaded onto the array disc. In a three-step assay configuration, 25 μL of bead solution (5.0 × 10^6 beads), and 100 μL of sample were incubated for 30 min.

Next, beads were then pelleted with a magnet and the supernatant was discarded. Following automated washes, 100 μL of detection antibody (final conc. 1.0 μg/mL) was pipetted into a reaction cuvette and incubated for 5.25 min. The beads were then pelleted with a magnet, and the supernatant was removed. Following a series of washes, 100 μL of S/βG (final conc. 100 pM) was added and incubated for 5.25 min, washed, resuspended in RGP solution, and loaded onto the array disc. Each disc contains 24 arrays with 216,000 microwells/array; one sample is tested/array. The array disc was then sealed with oil and imaged with a high-resolution charge-coupled device camera. Images of the arrays were analyzed, and the AEB values were calculated by the SiMoA HD-1. The calibration curves were fit using a four-parameter logistic regression (4 PL) fit with a 1/y2 weighting factor using a preset program designed by the manufacturer. The calibration curves generated in Q-buffer were used to determine concentrations of the unknown human serum and plasma samples. All measurements were performed in duplicate. The limit of detection (LoD) of the assay was calculated as three standard deviations (SDs) above the background.

Protein recovery was determined by comparing the calculated concentrations of the spiked samples and subtracting endogenous levels against the known spiked concentration. The percent (%) error or recovery was each determined based on the following equations:

\[
\% \text{ error} = \frac{C_{\text{Det}} - C_{\text{Spike}}}{C_{\text{Spike}}} \times 100
\]

\[
\% \text{ recovery} = \frac{C_{\text{Det}} - C_0}{C_{\text{Spike}}} \times 100
\]

\(C_{\text{Det}}\) is the measured concentration of the spiked sample, \(C_0\) is the concentration of the original sample without spiked recombinant protein, and \(C_{\text{Spike}}\) is the spiked concentration in the sample.

The assay performance was determined based on following equation:

\[
\text{assay performance} = \frac{\text{LLoQ value}}{\text{endogenous value}} \times 100
\]

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**Author Contributions**

The manuscript was written through contributions of all the authors, who have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest. Angela M. Bouté is the founder of Aries Biotechnologies, Consulting (Oakland, CA), which had no role in this work. Angela M. Bouté: angela.m.boute.civ@mail.mil

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**References**

1. Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; Turk, D. Cysteine cathepsins: from structure, function and regulation to new frontiers. Biochim. Biophys. Acta 2012, 1824, 68–88.
(2) Cavallo-Medved, D.; Moin, K.; Sloane, B. Cathepsin B: Basis Sequence: Mouse. ACF Soc Nat Pol Pages. 2011, 2011, A000508.
(3) Reiser, J.; Adair, B.; Reinheckel, T. Specialized roles for cysteine cathepsins in health and disease. Clin. Invest. 2010, 120, 3421–3431.
(4) Yan, S.; Sloane, B. F. Molecular regulation of human cathepsin B: implication in pathologies. Biol. Chem. 2003, 384, 845–854.
(5) Cavallo-Medved, D.; Rudy, D.; Blum, G.; Boggo, M.; Caglic, D.; Sloane, B. F. Live-cell imaging demonstrates extracellular matrix degradation in association with active cathepsin B in caveolae of endothelial cells during tube formation. Exp. Cell Res. 2009, 315, 1234–1246.
(6) Murphy, N.; Lynch, M. A. Activation of the P2X7 receptor induces migration of glial cells by inducing cathepsin B degradation of tissue inhibitor of metalloproteinase 1. J. Neurochem. 2012, 123, 761–770.
(7) Pedersen, B. K. Physical activity and muscle-brain crosstalk. Nat Rev Endocrinol 2019, 15, 383–392.
(8) Boutté, A. M.; Friedman, D. B.; Boggo, M.; Min, Y.; Yang, L.; Lin, P. C. Identification of a myeloid-derived suppressor cell cystatin-like protein that inhibits metastasis. FASEB J. 2011, 25, 2626–2637.
(9) Breznik, B.; Limbach Stekin, C.; Kos, J.; Klurshed, M.; Hira, V. V.; Bošnjak, R.; Lah, T. T.; Van Noorden, C. J. F. Cysteine cathepsins B, X and K expression in peri-arteriolar glioblastoma stem cell niches. J. Mol. Histol. 2018, 49, 481–497.
(10) Vasiljeva, O.; Papazoglou, A.; Krüger, A.; Brodoeff, H.; Korovin, M.; Deussing, J.; Augustin, N.; Nielsen, B. S.; Almholz, K.; Boggo, M.; Peters, C.; Reinheckel, T. Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer. Cancer Res. 2006, 66, 5242–5250.
(11) Coleman, M. D.; Ha, S. D.; Haeryfar, S. M. M.; Barr, S. D.; Kim, S. O. Cathepsin B plays a key role in optimal production of the tissue inhibitor of metalloproteinase 1. J. Virol. Antivir. Res. 2018, 7, 1–20.
(12) Memmert, S.; Damanaki, A.; Nogueira, A. V. B.; Eick, S.; Nokhebsaim, M.; Papadopoulou, A. K.; Till, A.; Rath, B.; Jepsen, S.; Sloane, B. F. Live-cell imaging demonstrates extracellular matrix degradation in association with active cathepsin B in caveolae of endothelial cells during tube formation. Exp. Cell Res. 2009, 315, 1234–1246.
(13) Boutté, A. M.; Hook, V.; Thangavelu, B.; LaValle, C. R.; Nemes, J.; GildorJ, J.; Shear, D. A.; Kamimori, G. H. Brain-related proteins as serum biomarkers of acute, subconcussive blast overpressure exposure: A cohort study of military personnel. PLoS One 2019, 14, No. e0221036.
(14) Hook, V.; Yoon, M.; Mosier, C.; Ito, G.; Podvin, S.; Head, B. P.; Rissman, R.; O’Donoghue, A. J.; Hook, G. Cathepsin B in neurodegeneration of Alzheimer’s disease, traumatic brain injury, and related brain disorders. Biochim. Biophys. Acta Protein Proteonomics 2020, 1868, 140428.
(15) Ruan, H.; Hao, S.; Young, P.; Zhang, H. Targeting Cathepsin B for Cancer Therapies. Horiz. Cancer Res. 2015, 56, 23–40.
(16) Vidak, E.; Javorsek, U.; Vizovicsek, M.; Turk, B. Cysteine Cathepsins and their Extracellular Roles: Shaping the Microenvironment. Cells 2019, 8, 264.
(17) Boutẗe, A. M.; Hook, V.; Thangavelu, B.; Sarks, G.; Abbatiello, B.; Hook, V.; Jacobsen, J. S.; Robertson, C. S.; GildorJ, J.; Yang, Z.; Wang, K. W.; Shear, D. A. Penetrating Traumatic Brain Injury Triggers Subacute Dysregulation of Cathepsin B Protein Levels Independently of Cysteine Protease Activity in Brain and Cerebral Spinal Fluid. J. Neurotrauma 2020, 37, 1574.
Wilson, D. H.; Rissin, D. M.; Kan, C. W.; Fournier, D. R.; Piech, T.; Campbell, T. G.; Meyer, R. E.; Fishburn, M. W.; Cabrera, C.; Patel, P. P.; Frew, E.; Chen, Y.; Chang, L.; Ferrell, E. P.; von Einem, V.; McGuigan, W.; Reinhardt, M.; Sayer, H.; Vielsack, C.; Duffy, D. C. The Simoa HD-1 Analyzer. *J. Lab. Autom.* 2016, 21, 533–547.