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Full length histone H3 conjugated electrochemical biosensor for extracellular proteolytic Cathepsin L activity detection

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ABSTRACT

Timely and accurate detection of proteolytic extracellular Cathepsin L (CTSL) activity is vital for assessing the metastatic potential of cancer. In this article, extracellular CTSL activity was detected using biotinylated full length histone H3 (BFLH3) treated extended gate (EG) field effect transistors (FETs) with 10^4 times the sensitivity of fluorometric CTSL activity assays. Upon active reaction by CTSL, the treated BFLH3 is cleaved after amino acid 21, inducing a significant charge change on the EG surface due to the large size and charge of the cleaved BFLH3. This change in EG surface charge can be detected by FET, enabling the quantification of proteolytic extracellular CTSL activity. Moreover, self-normalized quantification of CTSL activity was achieved in heterogeneous LNCaP and PANC-1 cultured cell media, which accurately represented higher CTSL levels in LNCaP derived from metastatic lesion of human prostatic adenocarcinoma. CTSL activity detection with EG FET allows multiple measurements of CTSL activity with one FET and opens up the possibility of detecting other proteolytic enzymes for cancer diagnosis and prognosis.

1. Introduction

Cathepsins are highly expressed in various human cancers, associated with tumor metastasis. Among a superfamily of Cathepsins consisting of A, B, C, D, E, F, G, H, L, K, O, S, V, and W family members, Cathepsin L (CTSL) belongs to the cysteine protease of the papain family, which is the largest and best known class of Cathepsins. It is ubiquitously expressed within the cancer cell and specializes in the extracellular matrix degradation and invasion process of the metastatic process [1]. CTSL is overexpressed in a variety of cancer types including ovarian, epithelial ovarian, non-small cell lung, hepatocellular, melanoma, and nasopharyngeal cancer [2–7]. Although CTSL is not a lone cause of cancer, accurate detection of CTSL activity can lead to assessment of the metastatic potential, chemoresistance, and patient survival rate.

To detect CTSL activity, antibody-based, fluorophore-based, and photoaffinity-based detection methods have been used [8–10]. Antibody-based methods are most widely used as it can be employed in a wide variety of detection platforms including western blotting, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence. However, antibody-based detection methods can only quantify the amount of CTSL not the activity of CTSL itself. The activity level of proteolytic CTSL varies widely depending on the locality of CTSL. Of the two types of CTSL, extracellular CTSL is 10 times more active than the lysosomal CTSL and directly involved in the metastatic process [11]. Thus, it is important to detect proteolytic extracellular CTSL activity for diagnosis and prognosis of cancer. Fluorometric detection of CTSL overcomes the limitations of the antibody-based method by enabling the detection of proteolytic forms of CTSL activity. Detection of proteolytic CTSL was made possible by attaching fluorophores such as cresyl violet and rhodamine 110 after the substrate peptide [9]. However, research regarding fluorometric CTSL detection have not established a limit of detection and CTSL detection was made possible only with very high cell densities [12]. Photoaffinity-based detection methods of CTSL can specifically detect CTSL among a set of five cathepsins (B, K, L, S and V) with a limit of detection as low as a 500 pM [10]. However, the low specificity of the probe enabled CTSL detection from crube extracts only when recombinant CTSL is introduced into the cells. In addition, both fluorophore and photoaffinity-based methods of detection are prone to photodegradation, which may reduce the sensitivity of the detector [13].

Electrochemical biosensors such as Field Effect Transistors (FETs), Electrochemical Impedance Spectroscopy (EIS), and potentiometric sensors offer a very attractive alternative to traditional methods of biomarker detection due to its high sensitivity, multiplexing capability, and integrability with computational circuitry [14–16]. Immuno FETs have been the most widely used type of

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FET based biosensors due to its highly specific antibody-antigen interaction. A variety of antigens including avian influenza virus, prostate cancer antigen (PSA), and annexin A3 (ANXA3) have been detected with high sensitivity [17–19]. Utilization of Immuno FETs for CTSL detection is possible but cannot detect CTSL activity itself as do other antibody-based detection methods. Detection of enzymatic activity using FET biosensors have been conducted to detect protein kinase activity [20]. Change in surface charge of FET induced by the addition of phosphate groups by protein kinase was detectable. However, this method offers limited number of measurements per FET as bioconjugation of enzyme substrates occur on the FET itself.

In this paper, we have conjugated a cleavable peptide, biotinylated full length histone H3 (BFLH3), consisting of 136 amino acids onto the surface of the EG FET to detect extracellular CTSL activity. Bioconjugation of BFLH3 occurs on the EG allowing mutliple measurements of CTSL activity using one FET. CTSL cleaves BFLH3 after amino acid 21 which detaches a significant portion (12.97 kDa) and charge (+13.2) at pH7 of BFLH3 from the EG surface. The detachment of the cleaved BFLH3 induces a significant change in surface charge of EG which can be detected by the FET device. We have proved that greater change in EG surface charge induces greater response by FET by comparing the voltage shift induced by the larger BFLH3 peptide and the smaller Methyl red-phenylalanine-arginine-phenylalanine-arginine (MR-FRFR) peptide. We also show that CTSL activity detection is possible in heterogenous media such as the cancer cultured cell media from LNCaP and PANC-1 cell lines with self-normalized detection, which accurately represented higher CTSL levels in LNCaP derived from metastatic lesion of human prostatic adenocarcinoma.

2. Experimental

2.1. Fabrication of FET

FET was fabricated using the previously described methods [21]. In brief, a p-type (100) 6 in. silicon-on-insulator (SOI) wafer was used to form the dual-gate structure. The thicknesses of the buried oxide (BOX) layer, top silicon layer, and top oxide layer were 750, 100, and 10 nm, respectively. The active region was defined by employing photolithography and etching process using an inductively coupled plasma reactive-ion etching (ICP-RIE) system. The top oxide layer was formed using a dry oxidation method. To form the gate electrode structure, a 150 nm thick TiN was deposited using a sputtering system and the TiN metal layer was etched using photolithography and etching process. The source and drain regions were defined by arsenic (As) ion implantation (concentration: 3×10^{15} cm^{-3}). 150 nm thick TiN contact pads on the source and drain were formed by deposition and lift-off processes.

2.2. Preparation of EG attached with detection chamber

To fabricate the Polydimethylsiloxane (PDMS) detection chamber, PDMS was made by mixing sylgard 184 silicone elastomer base (Sigma-Aldrich) and 184 elastomer curing agent (Sigma-Aldrich) with a 10:1 w/w ratio. The mixture was poured onto a glass plate for subsequent degassing for 2 h. Then, the PDMS solution was heated at 60 °C overnight. Blocks of PDMS with 19×19 mm^2 dimension was punctured with four 6 mm diameter holes and attached on top of Indium tin oxide (ITO) glass substrate with 19×27 mm^2 dimension. O_2 plasma was treated using an inductively coupled plasma reactive-ion etching on both PDMS and ITO glass prior to attachment. The fabricated EG attached with PDMS chamber allowed multiple measurements of CTSL activity using one FET since EG can be replaced multiple times for CTSL activity measurement.

2.3. Bioconjugation of BFLH3 and MR-FRFR on EG

After treating the PDMS chamber attached EG with O_2 plasma, 5 v/v% (3-aminopropyl)triethoxysilane (APTES, Sigma-Aldrich) dissolved in ethanol was reacted with the EG surface for 1 h at room temperature (RT). Then, EG was sonicated with ethanol for 1 min followed by pipet washing 3 times. The EG is then dried by blowing compressed nitrogen gas. The surface of EG was further dried by incubating the EG at 120 °C for 5 min. 2.5 v/v% glutaraldehyde (Sigma-Aldrich) dissolved in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5) was subsequently treated for 1 h.

2.3.1. For the MR-FRFR conjugated EG

5 μL of reconstituted MR-FRFR (Immunochemistry Technologies) was diluted with 1 × PBS and incubated on EG for 1 h.

2.3.2. For the BFLH3 conjugated EG

166.7 nM of streptavidin (SA, Sigma-Aldrich) was treated for 1 h, followed by 166.7 nM of BFLH3 (BPS Bioscience) for 1 h.

Both MR-FRFR and BFLH3 conditions were treated with 0.2 M ethanolamine (Sigma-Aldrich) for 1 h and 5% BSA for 1 h for blocking the EG surface.

2.4. FET CTSL measurement setup

A commercial Ag/AgCl reference electrode and Hewlett-Packard 4156B high precision semiconductor parameter analyzer were utilized for FET measurements. The drain source current was measured against a sweep in the back gate voltage, while the reference electrode in the solution was grounded. The top gate was electrically connected to the electrode on the EG [17].

2.5. FET CTSL activity measurement in CTSL buffer and cultured cell media

CTSL from human liver (Sigma-Aldrich) was diluted with CTSL buffer containing 20 mM malonate, 1 mM EDTA, 400 mM NaCl at pH 5.5. Increasing doses of CTSL ranging from 0.1 ng/mL to 10 μg/mL was incubated for 30 min onto the BFLH3 conjugated EG for drain source current measurements against a back gate voltage sweep. Drain source currents were also taken from MR-FRFR treated EG after incubating 100 ng/mL CTSL diluted in CTSL buffer for up to 30 min. Voltage shift was measured and plotted for every 10 min up to 30 min. For measurements from cultured cell media, RPMI and DMEM buffer containing 10% fetal bovine serum (FBS) supplemented with HEPES (50 mM) was measured as control. Buffers were spiked with 0.5 ng/mL and 5 ng/mL CTSL for changes in voltage shift induced by CTSL. Cultured cell media was collected from LNCaP and PANC-1 cell lines that were cultured for 24 h at a density of 10^4 cells/well in 96 wells prior to FET measurement.

2.6. Cell culture and ELISA/western blot for CTSL quantification

Prostate and pancreatic cancer cell lines IPC, PANC-1, Capan-1, LNCaP, DU145, RWPE-1, and 22RV-1 were grown in media conditions recommended by American Type Culture Collection (ATCC). Cells were cultured for 24 h at a density of 10^6 cells/well on 6 well plates prior to cell collection. Cells were then collected and lysed with RIPA buffer and all the collected cell lysates were diluted to 250 ng/mL. Subsequent ELISA quantification of CTSL from each cell lysate was conducted by following the manufacturer's protocol of the CTSL ELISA kit (Abcam). Western blot quantification of CTSL was conducted after adding 4 × Sodium dodecyl sulfate (SDS) buffer (Sigma-Aldrich) and 5 mM Dithiothreitol (Sigma-Aldrich).