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Electrochemical sensing of human neutrophil elastase and polymorphonuclear neutrophil activity

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ABSTRACT

Human neutrophil elastase (HNE) is a serine protease, produced by polymorphonuclear neutrophils (PMNs), whose uncontrolled production has been associated with various inflammatory disease states as well as tumour proliferation and metastasis. Here we report the development and characterisation of an electrochemical peptide-based biosensor, which enables the detection of clinically relevant levels of HNE. The sensing platform was characterised in terms of its analytical performance, enzymatic cleavage kinetics and cross-reactivity and applied to the quantitative detection of protease activity from PMNs from human blood.

1. Introduction

Human neutrophil elastase (HNE), a serine protease secreted by polymorphonuclear neutrophils (PMNs), plays an important role in many physiological and pathological processes (Korkmaz et al., 2008). The proteolytic activity of this enzyme contributes to the body's defence against infectious agents by promoting the destruction of pathogenic bacteria (Meyer-Hoffert and Wiedow, 2011; Nathan, 2006). High levels of unregulated HNE have also been associated with the inflammatory state found in a wide range of acute and chronic diseases (Kolaczkowska and Kubes, 2013; Pham, 2006) with excess HNE causing extracellular matrix degradation, cellular receptor cleavage and healthy tissue disruption (Abe et al., 2009; Chua and Laurent, 2006; Shapiro, 2002). Furthermore, recent studies have suggested a direct role for HNE in promoting tumour proliferation and metastasis (Galdiero et al., 2013; Sato et al., 2006). HNE is therefore a potential diagnostic marker for a number of disease states and detecting it with high sensitivity is clinically important (Henriksen and Sallenave, 2008; Ho et al., 2014; Korkmaz et al., 2010). Common methods to monitor HNE levels rely on immunoassays (de la Rebière de Pouyade et al., 2010; Dunn et al., 1985) and aptamer or peptide-based sensors typically labelled with fluorescent reporters (Avlonitis et al., 2013; Bai et al., 2017; Ferreira et al., 2017; He et al., 2010). In this context, it is interesting to note that electrochemical peptide-based biosensors have proven to be valuable tools for the detection of protease activity (Anne et al., 2012; Liu et al., 2006; Shin et al., 2013; Swisher et al., 2015, 2014, 2013) – as well as a wider range of applications (Huang et al., 2016; Li et al., 2015, 2014; Puiu et al., 2014). Recently, our research group reported an electrochemical peptide-based biosensor which used self-assembled monolayers (SAMs) on gold electrodes for the detection of the model protease trypsin (González-Fernández et al., 2016). This constituted a short peptide sequence, which acts as substrate for the target enzyme, methylene blue as redox reporter and a polyethylene glycol (PEG) spacer that was shown to be important in tuning both the anti-fouling properties and the probe's flexibility (González-Fernández et al., 2018). Upon enzymatic cleavage, a redox-labelled probe fragment is released, leading to a measurable decrease in the electrochemical signal. This system provided a sensitive platform with a clinically relevant limit of detection (LOD) of 250 pM. Building on this approach, here we target the translation of this model system to the detection of HNE in biological (human blood) samples through the development of a novel methylene blue-tagged peptide-based biosensor with an HNE-specific cleavage sequence employing ternary SAMs on a gold surface. This is the first reported example of HNE detection based on a reagent-free labelled electrochemical strategy.

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2. Materials and methods

2.1. Instrumentation

Electrochemical measurements were performed using a conventional three-electrode electrochemical cell driven by a computer-controlled AutoLab PGstat-30 potentiostat running the GPES 4.9 software (EcoChemie, The Netherlands). A platinum wire and a 2 mm diameter polycrystalline gold electrode (IJ Cambria, UK) were used as auxiliary and working electrodes, respectively. All the potentials were referenced to a Ag | AgCl | KCl (3 M) reference electrode (Bioanalytical Systems, Inc., USA). MALDI TOF MS were run on a Bruker Ultraflextreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in H_2O/CH_3CN/TFA (50/50/0.1).

2.2. Reagents

Human neutrophil elastase, HNE (MW 29.5 kDa) was obtained from Athens Research and Technology Inc. (USA). Cathepsin G, bovine serum albumin (BSA), casein, 6-mercaptohexanol (MCH), 2,2′-(ethylenedioxy) diethanethiol (PDT), HEPES buffer, sodium chloride, sodium acetate were all obtained from Sigma. All reagents were of analytical grade and solutions were prepared using protease-free deionised water. HNE was reconstituted in 50 mM acetate, pH 5.5, with 150 mM NaCl and stored at −80 °C until use.

2.3. Experimental methods

2.3.1. Synthesis

The detailed synthetic experimental procedures are described in Appendix A: Supplementary Data.

2.3.2. Electrode cleaning and pre-treatment

After immersing in the minimum volume of piranha solution (3:1-H_2SO_4 (95%): H_2O_2 (33%)) (CAUTION piranha solution is strongly oxidising and must be handled with care!) for 10 min in order to eliminate any organic matter from the gold surface, the working electrode was successively polished on a polishing cloth using alumina slurries of 1, 0.3 and 0.05 μm particle size (Buehler, Germany). Afterwards, this electrode was further cleaned by immersion in H_2SO_4 (95%) and then HNO_3 (65%) at room temperature for 10 min. Finally, the working electrode was subjected to cyclic voltammetry, carrying out potential cycles between 0 and + 1.6 V in 0.1 M H_2SO_4 at a scan rate of 100 mV s^{-1} until the characteristic voltammogram of clean polycrystalline gold was obtained.

2.3.3. Sensing phase preparation protocol

The sensing phase was formed as a mixed SAM on the gold electrode surface by immersing the freshly cleaned and pre-treated gold working electrode overnight at 4 °C in a 40 μM ethanolic solution of the appropriate redox-labelled peptide (substrate, composed of L-amino acids; or control, with all amino acids replaced with their respective D-amino acid analogues) and freshly prepared PDT (600 μM). After washing with ethanol, the resulting SAM-modified electrode was immersed in 1 mM MCH in ethanol for 1 h at room temperature. Finally, two washing steps were carried out, firstly in ethanol and then in HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 7.4). The modified electrodes were stored in HEPES buffer at 4 °C until use.

2.3.4. Sensor measurements

The modified electrodes were immersed in HEPES buffer and electrochemically interrogated using square wave voltammetry (SWV, at a frequency of 60 Hz and with an amplitude of 25 mV and a step potential of 5 mV) until a stable signal was obtained. After addition of the target enzyme, the SWV signal was continuously monitored with time, with the resulting signal decrease being expressed as the relative change in SWV peak current with respect to the initial peak current (henceforth shown as a negative % signal change).

2.3.5. Blood sample treatment for activated/non-activated PMNs

Freshly isolated PMNs – obtained from healthy donors following the blood preparation protocol previously described (Avlonitis et al., 2013) – were re-suspended at 2×10^6 cells/mL in PBS containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}. The sample was divided in two tubes, and to one of them A23187 (a calcium ionophore, 10 μM final concentration) was added in order to activate the PMNs. The other one was kept non-activated as a negative control. Both tubes were heated at 37 °C for 10 min in a water bath and then centrifuged for 5 min. The cell pellet was discarded and the supernatant stored at −20 °C until use.

3. Results and discussion

The sensing platform was based on the immobilisation of a redox-labelled peptide sequence, which contained a specific cleavage site for the enzyme, on a gold surface as a SAM. The peptide sequence (APEEIMRRQ) has been reported as a highly specific HNE cleavable sequence in a fluorescent assay for HNE detection (Avlonitis et al., 2013). A novel peptide probe has been designed and synthesised, adding further functionality to this previously reported sequence. In order to generate an HNE electrochemical probe, a methylene blue redox tag was attached to the amino-terminus of the peptide with the addition of a 2-unit ethylene glycol moiety (PEG-2) and a cysteine at the carboxy-terminus, which enables facile SAM immobilisation of the probe through the formation of a S-Au bond (Fig. 1A). A probe containing the equivalent non-cleavable D-amino acid sequence was also synthesised as a negative control probe. The sensing mechanism relies on specific enzyme-catalysed cleavage followed by the release of the labelled peptide fragment from the SAM-modified gold surface into solution (Fig. 1B). This results in a decrease of the electrochemical signal, which is interrogated before and during enzyme exposure by square wave voltammetry (SWV) and presented as a negative % signal change (a decrease in the relative peak SWV current with respect to the initial value recorded before enzyme addition). The cleavage site, immediately after the methionine residue, was confirmed in solution through cleavage fragment mass analyses by MALDI-TOF MS (Fig. S1). The sensing surfaces were generated using a previously optimised ternary-SAM (T-SAM) configuration (González-Fernández et al., 2016) established as showing enhanced SAM biosensing, according to the protocol detailed in Section 2.3.2. This is denoted a ternary-SAM as it is composed of 3 different thiols on the gold surface: the peptide-probe, a co-adsorbed pegylated dithiol and mercaptohexanol.

3.1. Analytical performance against varying HNE concentrations

Substrate-modified gold electrodes were prepared and their performance evaluated upon immersion in solutions containing varying HNE concentrations (10–150 nM). The modified-electrodes were electrochemically interrogated by SWV in real-time when exposed to different HNE concentrations (Fig. 2). As shown in Fig. 2A the presence of HNE caused a decrease of the % signal, and as expected, the higher the HNE concentration the faster the % signal change recorded, resulting from faster proteolytic cleavage of the SAM-modified surface; where the (negative) % signal change $= (I_{SWV}(t) - I_{SWV}(t = 0))/ I_{SWV}(t = 0) \times 100$, with I_{SWV} ($t = 0$) defined as the signal recorded before the addition of the enzyme. As previously observed for other SAM systems, the signal did not decrease by 100%, suggesting that a subset of the immobilised peptides are not available for cleavage, which has been previously attributed to polycrystalline gold roughness leading to site inaccessibility (González-Fernández et al., 2018). Furthermore, additional cyclic voltammetry was performed in order to calculate the change of surface coverage of active methylene blue-peptide upon HNE incubation (100 nM), which showed the expected decrease, consistent