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# A Fluorogenic Peptide-Based Smartprobe for the Detection of Human Neutrophil Elastase in Inflammation

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## Introduction

Neutrophils are one of the most abundant immune cells and are first to be recruited to sites of inflammation where they can fight invading pathogens very effectively [1]. Activation of neutrophils results in the release of proteases, with human neutrophil elastase (hNE) being the most abundant. This protease is involved and implicated in a variety of inflammatory diseases and includes association with so-called Neutrophil Extracellular Traps (NETs). NETs are networks of extracellular fibers, produced by dying neutrophiles, that are primarily composed of extruded DNA, embedded with proteases that effectively kill pathogens. These structures have been associated with a variety of chronic inflammatory diseases such as idiopathic pulmonary fibrosis [2] while the fibrillar nature and excessive development of NETs in SARS-CoV-2 infection has been linked to the development of acute respiratory distress syndrome and blood vessel blockage.

Peptide based "dendritic" or multi-branched fluorogenic probes [3] can provide significantly higher signal amplification and lower background noise when compared to their linear counterparts as well as offering high sensitivity (multiple copies of the fluorophore are released upon cleavage). In this area our group previously reported a self-quenching probe for the detection of hNE [5]. However, this probe, the so-called "Neutrophil Activation Probe" (NAP) suffered from high background signals and showed poor levels of signal amplification, making it unsuitable for *in vivo* application and was also unsuccessful in the visualization of NETs *in vitro* [5].

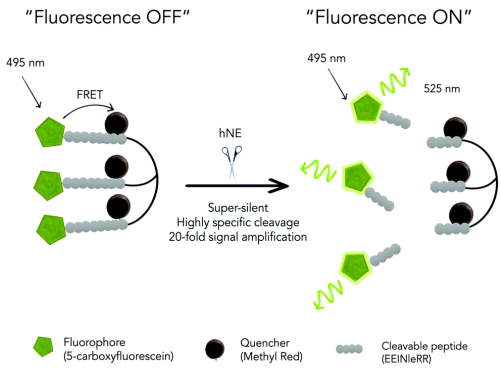


*Fig. 1. A triple-quenched, super-silent tri-branched probe (**HNE-FQ**), which upon specific cleavage by human neutrophil elastase (hNE) liberates three fluorescent molecules resulting in an increase in fluorescence.*

Herein, we report a highly sensitive fluorogenic probe for the detection of hNE in activated neutrophils and Neutrophil Extracellular Traps (NETs). It was based on the previously reported triple self-quenched, tri-branched probe (NAP), but with amplification of signal dramatically enhanced by the addition of three copies of a traditional FRET quencher (methyl red), and generated a >20 fold increase in fluorescence upon specific cleavage by hNE (Figure 1) [6].

## Results and Discussion

The probe contained the highly specific hNE cleavable peptide (Glu-Glu-Ile¦Nle-Arg-Arg) and was synthesized on an aminomethyl ChemMatrix resin functionalized with the Fmoc-Rink Amide linker (Figure 2). The starting trivalent isocyanate (**1**) was synthesized following a previously reported procedure [7] and immobilized onto the solid support. Removal of the Dde protecting groups exposed the three terminal amino groups (**2**) that served for the synthesis of the FRET-peptides using Fmoc solid-phase peptide synthesis with DIC/Oxyma as the coupling combination. The quencher was incorporated as an intact Fmoc-Lys(MR)-OH building block to simplify the synthesis and avoid the need for orthogonal deprotection on the resin. A bis-ethylene glycol unit, between the fluorophore and the peptide, was introduced to increase water solubility.

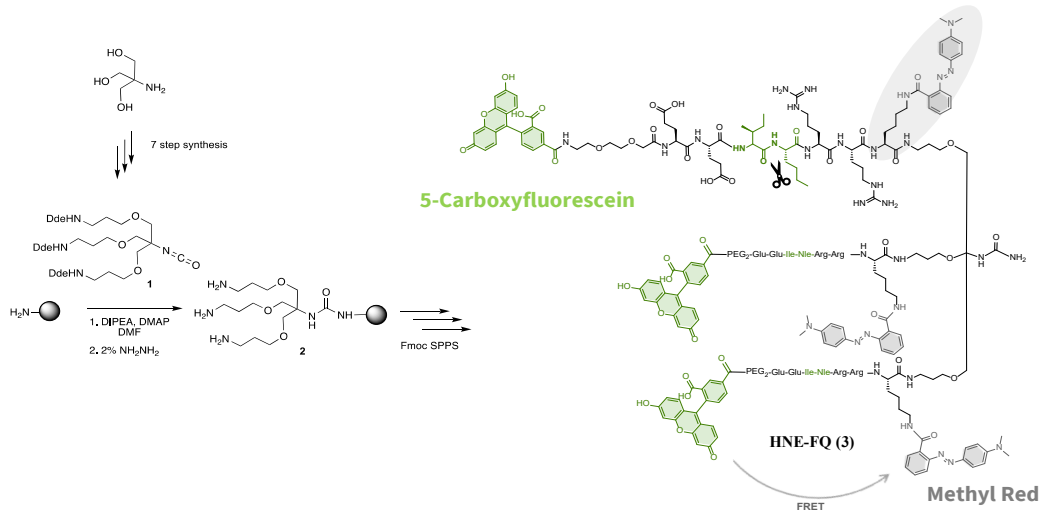


*Fig. 2. Probe synthesis started by attachment of the tribranched scaffold **1** onto a Rink-amide-ChemMatrix resin-linker combination [6]. This was followed by deprotection of the Dde protecting groups, and synthesis of the FRET-peptide using an Fmoc/tBu solid-phase peptide synthesis strategy. The FRET pair consisted of 5-carboxyfluorescein as the fluorophore and methyl red as the dark quencher. Fmoc-Lys(MR)-OH was incorporated as the first amino acid residue (shadowed).*

The probe's maximum excitation was at 490 nm with a maximum emission at 520 nm [6] with activation of **HNE-FQ** in presence of the hNE proving to be rapid, with a 20-fold increase in fluorescence within minutes while *Sivelestat* fully inhibited probe activation (Figure 3a). Specificity of the probe was confirmed by exposure to two closely related neutrophil serine proteases: Cathepsin G and Proteinase 3, with negligible cleavage observed in both cases (Figure 3b).

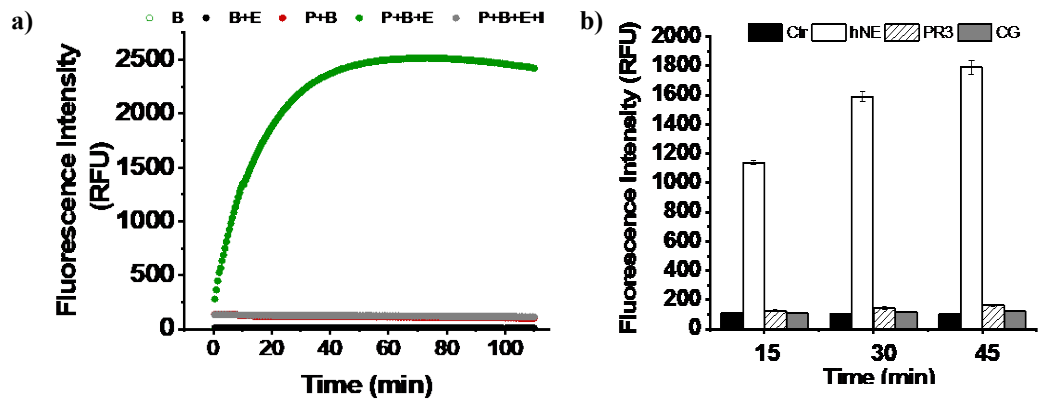


*Fig. 3. a) The time-course dequenching of probe (5 µM) following incubation with hNE (100 nM) in the presence or absence of the inhibitor Sivelestat (100 µM) n = 3. B = buffer; E = enzyme; P = probe; I = inhibitor. b) Specificity assessment of probe (5 µM) incubated in the presence of hNE (100 nM) and related serprocidins PR3 (100 nM) and Cathepsin G (300 nM) at different time points at 37°C, n = 3.*

The activity of **HNE-FQ** was compared to the previous probe **NAP (4)** a tribranched compound containing three copies of a hNE cleavable sequence, capped with 5-carboxyfluorescein on its *N*- terminus (Figure 4a). In **NAP** the fluorophores are in close proximity to each other leading to self-quenching. However, **NAP (4)** suffers from high background fluorescence levels and limited signal amplification (3.6-fold) [5]. In addition, **HNE-FQ** activation was compared to another tribranched probe that targets a broader range of serprocidins, the probe **NES** (**5,** Figure 4a). **NES** uses a different peptide sequence but shares structural similarities, since it also consists of a tribranched scaffold and contains a FRET quencher (Methyl Red) to enhance quenching of carboxyfluorescein. However, in NES, the Methyl Red groups are attached at the *N*-terminus and the carboxyfluorescein fluorophores on the *C*-terminus [4]. Thus, although **NES (5)** provided an improvement in amplification of signal upon activation when compared to **NAP (4),** this was still limited (approximately 7-fold). This can be explained by the effect of self-quenching following cleavage, since, in NES, upon activation, the Methyl Red containing fragments are released, but the three molecules of 5-carboxyfluorescein remain attached to the tribranched "core", leading to self-quenching. The optimised design in **HNE-FQ** tackles the limitations of the two previous designs by reorienting the FRET pair labels on the peptide sequence (5-carboxyfluorescein on the *N*-terminus and Methyl Red on the *C*-terminus), allowing full release of three carboxfluorescein fluorophores. This rearrangement resulted in a significant improvement in the signal amplification upon probe activation, (22-fold). Taken together, optimisation of the first- and second-generation elastase probes lead to probe **HNE-FQ**, which showed a significant improvement in fluorescence signal amplification and a major reduction in background fluorescent levels.

In order to visualize NETs using **HNE-FQ**, neutrophils were differentiated from HL-60 cells by exposure to with all-*trans*-retinoic acid (ATRA) as previously reported [6] and stained with probe (5 μM), 4′,6-Diamidine-2′-phenylindole dihydrochlorideI (DAPI; 0.2 mM) and the extracellular DNA intercalating dye (SYTOX Orange; 5 μM). Morhologically, ATRA/PMA treated cells exhibited four different stages of NETosis, based on nuclear morphology and hNE distribution (Figure 5). Early stages of neutrophil activation exhibited a small and round nucleus with hNE colocalized with condensed chromatin (stage 1; yellow circles). At later stages, chromatin decondensation led to spherical (stage 2; green circles) or more "cloud-like spread" shapes (stage 3; red circles). In the final stage, neutrophils formed extracellular chromatin filaments composed by cytoplasmic granules and hNE (stage 4; Figure 5e).

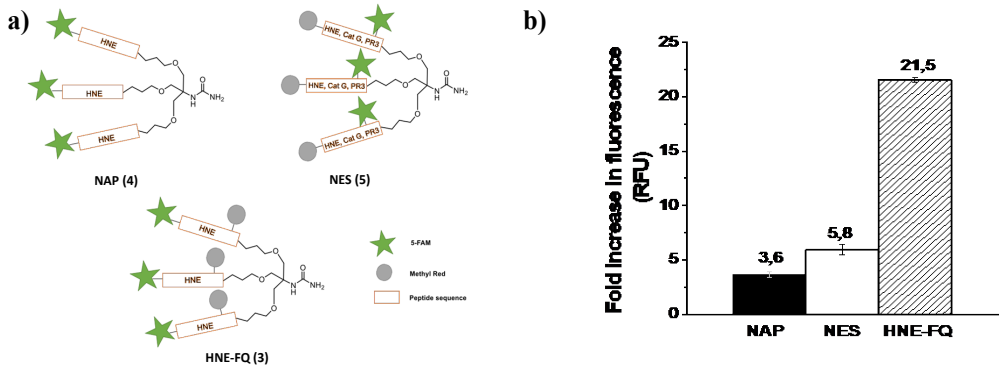


*Fig. 4. a) Structures of **NAP (4)**, **NES (5)** or **HNE-FQ (3)**; b) Probes (5 μM) were exposed to elastase (100 nM) with activation monitored over time. The bar plot shows the maximum fold increase upon activation of all three probes.*

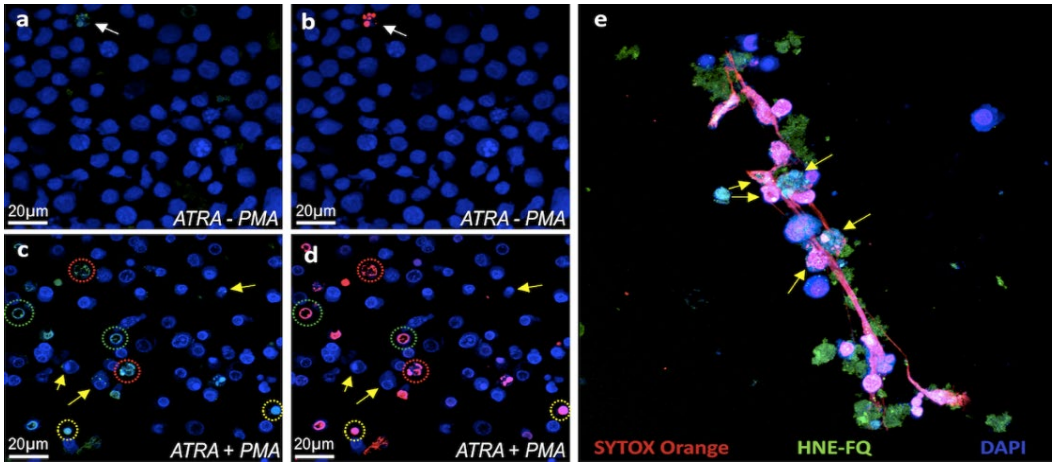


*Fig. 5. Fluorescence microscopy images of HL-60 cells stained with DAPI (blue), **HNE-FQ** (green) and SYTOX Orange (red). Top row left: Control HL-60 neutrophils in absence of PMA a) Blue/Green channels b) Blue/Red channels. Bottom row left: HL-60 neutrophils stimulated with PMA c) Blue/Green channels and d) Blue/Red channels. Yellow arrows indicate cells expressing low hNE (likely at the beginning of NETosis process) while those encircled in green, red and yellow are cells at different stages of NETosis (see text for description). e) 3D image slice of a typical NET stained as in the panels on the left. Arrows indicate chromatin studded with **HNE-FQ**, indicative of chromatin release by activated cells.*

In conclusion, a tri-branched human Neutrophil Elastase activatable fluorescent probe (**HNE-FQ)** was designed and synthesized and allows the rapid, specific, and sensitive detection of hNE in activated neutrophils and NETs. The data shows that **HNE-FQ** was highly specific for the staining of human neutrophil activation and highlights its potential as a diagnostic tool to detect and quantify NETosis and activated neutrophils, with current efforts focused on the synthesis of a near infrared (NIR) variant of the sensor.

## Acknowledgments

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