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Highly specific, multi-branched fluorescent reporters for analysis of human neutrophil elastase†

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Human neutrophil elastase (HNE) is a serine protease implicated in the pathogenesis of acute and chronic inflammatory disease. Here a series of, internally quenched, single fluorophore fluorescent reporters were synthesised that allowed the rapid, highly specific and sensitive analysis of HNE activity over closely related proteases.

Introduction

Human neutrophil elastase (HNE) is a proteolytic enzyme stored within the primary azurophilic granules of polymorphonuclear cells (PMN).[1] It is a protease whose major function is the proteolytic destruction of bacteria, but when uncontrolled is involved in the pathogenesis of acute and chronic inflammatory diseases. Excessive HNE degrades matrix, cleaves cellular receptors, activates profibrogenic mediators and contributes to epithelial and endothelial damage,[2–5] and thus tight control and monitoring of HNE activity is important therapeutically.

Assessment of HNE activity requires probes with subtle molecular specificity that permit specific detection of HNE over other closely related serine proteases such as proteinase 3 and cathepsin G.[6,7] Gauthier *et al.*[8] has previously described FRET (Förster resonance energy transfer) labelled peptides such as Abz-APEEIMRRQ-EDDnp (Abz: *ortho*-aminobenzoyl, EDDnp: *N*-(2,4-dinitrophenyl)ethylenediamine) as substrates for the analysis of neutrophil serine proteases on the surfaces of neutrophils, but these have limitations, notably because of their short emission wavelengths.

Previously we reported an assay for proteases based on multi-branched fluorescently-labelled scaffolds which display the phenomenon of internal or self-quenching.[9,10] This strategy offers a single fluorophore-based method for the analysis of proteolytic activity, where amplification of signal upon substrate cleavage is possible, without the need of having to rely on two dyes as required in conventional FRET systems. Here this approach was applied to the synthesis of substrates for purified HNE, as a fore-runner for the analysis of HNE activity in or on cells. The multi-valent fluorescent peptides were readily synthesised by solid-phase techniques,[11,12] allowing the generation of mono-disperse products,[13] in addition, they demonstrated high biocompatibilities, low toxicities and an ability to access intracellular compartments.[14,15]

Results and discussion

The three multi-branched peptides **S1**, **S2** and **S3** shown in Fig. 1, were based on the peptide sequences optimised for HNE cleavage by Gauthier and co-workers[6] (APEEIMDRQ and APEEIMRRQ) but utilised a single fluorophore fluorescein (λ_{ex} = 494 nm and λ_{em} = 518 nm), with **S1** and **S2** based on a trivalent scaffold, whilst **S3** was hexa-valent.

Synthesis required the preparation of the monomer (6) which was synthesised in six steps[11] as shown in Scheme 1 and monomer (12) which was synthesised in five steps as shown in Scheme 2. Monomer (6) was prepared by the 1,4 addition of the hydroxy groups of 1,1,1-tris(hydroxymethyl)-amino-methane onto acrylonitrile, followed by amino protection (Boc). Reduction of the nitrile groups with borane–THF complex gave (3) which was treated with Dde-OH to give the tris-Dde (2-acetyl-dimedone) protected amine (4). Following removal of the Boc protecting group, the isocyanate (6) was prepared following the procedure of Knölker.[16] Monomer (12)[17] was synthesised from α-resorcylic acid by esterification followed by alkylation with 2-(Boc-amino)ethyl bromide in the presence of potassium carbonate in DMF to yield (9). Saponification (NaOH in MeOH/dioxane), followed by removal of the Boc protecting groups and reprotection with Fmoc-*N*-hydroxysuccinimide gave the branched monomer (12).

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†Electronic supplementary information (ESI) available: The experimental details of the synthesis of monomers (6), (12) and probes **S1–S3** are described in details. Biological experimental also described. See DOI: 10.1039/c3ob40212f
‡The authors NA and MD contributed equally to this work.

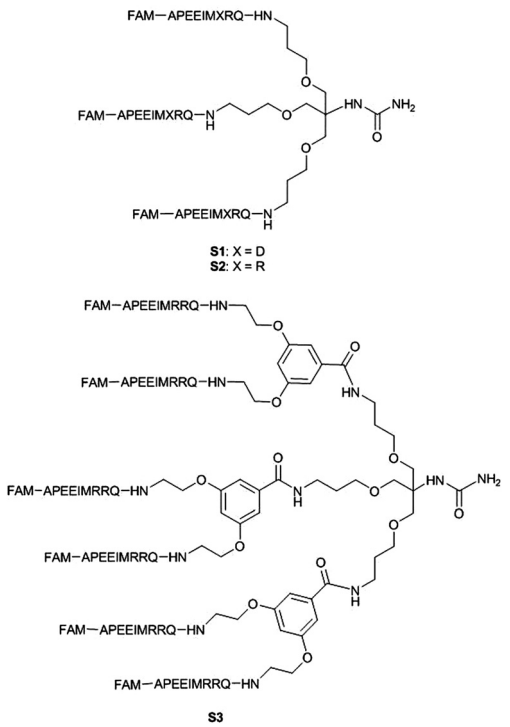
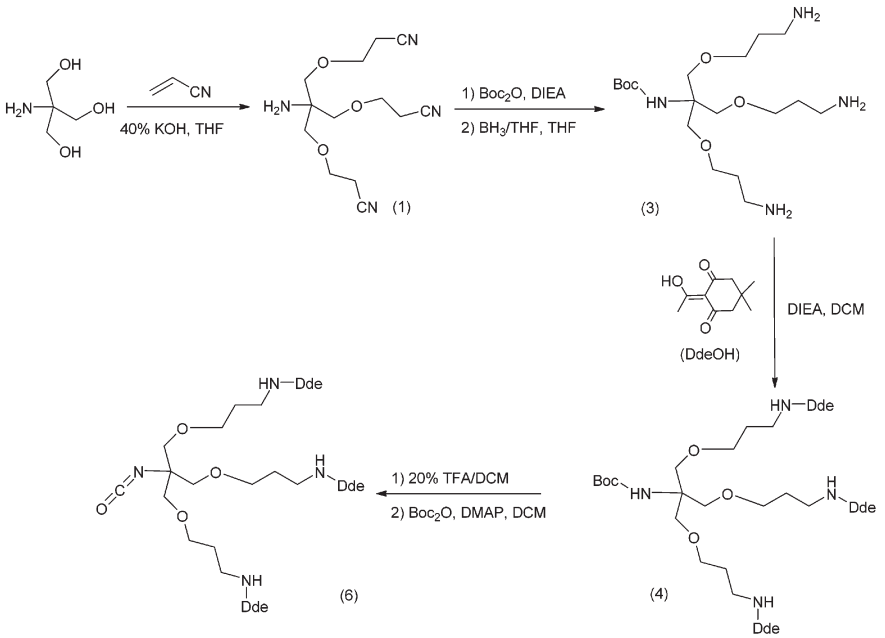


Fig. 1 Structures of the three multivalent probes **S1**, **S2** and **S3** (FAM: 5(6)-carboxyfluorescein amide).

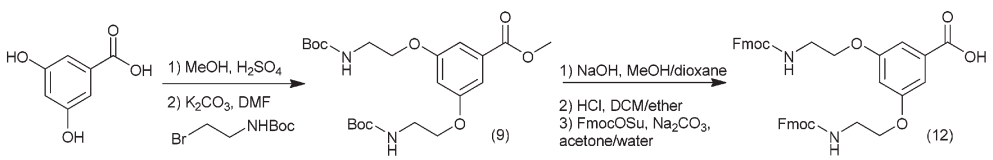
The probes were synthesised on an amino methyl polystyrene resin (1.6 mmol g^{-1}, 1% DVB, 100–200 mesh) derivatized with an Fmoc-Rink Amide type linker (Scheme 3). The linkers were loaded with monomer (6) or (12) to give the tri or hexa-branched scaffolds (14 and 15). Following removal of the Dde protecting groups (2% hydrazine in DMF) or the Fmoc groups the appropriate Fmoc-protected amino acids were coupled followed by the attachment of 5(6)-carboxyfluorescein and cleaved from the resin using TFA/phenol/water/TIS (88/5/5/2).

Probes **S1**, **S2** and **S3** were evaluated *in vitro* for their selectivity for recombinant HNE, in comparison with the closely related serine proteinases, cathepsin G and proteinase 3 (also found in PMN) (see ESI-S9†). **S1** displayed poor selectivity for HNE over Pr3 and cathepsin G, **S2** was better, while **S3** showed excellent specificity (see Table 1).

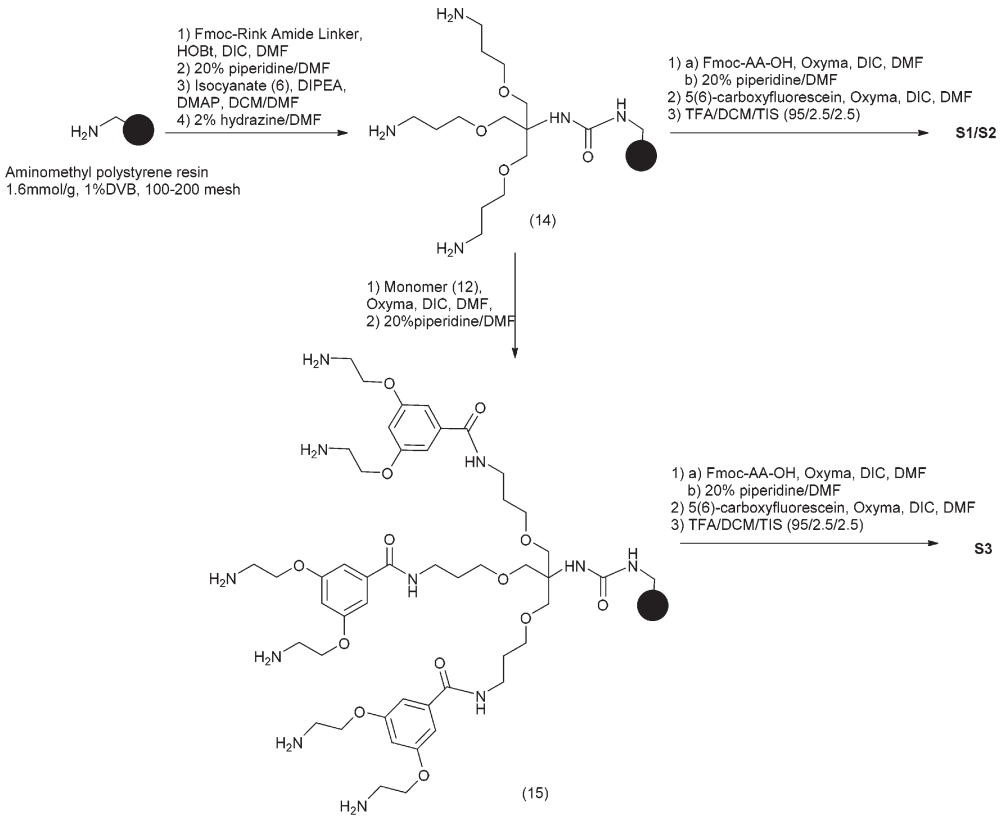
Probes **S1** and **S2** were evaluated on activated primary human neutrophils (PMN), known to secrete HNE, and on primary human mononuclear cells (peripheral blood mononuclear cells, PBMC). Sivelestat, a specific HNE inhibitor, was added to both cell types to confirm selective neutrophil-dependent dequenching of both **S1** and **S2** (Fig. 2A). To validate that the cleavage of the probe was due to cellular HNE activity, whole neutrophils and primary human neutrophil lysates were pre-incubated with selective inhibitors. This included secretory



Scheme 1 Synthesis of monomer (6).



Scheme 2 Synthesis of monomer (12).



Scheme 3 Synthesis of the multivalent probes **S1**, **S2** and **S3**.

Table 1 Relative cleavage rates for probes **S1**, **S2** and **S3** with HNE, Pr3 and CatG. (Values obtained from three independent experiments each in duplicate. Data represent average initial velocity expressed as arbitrary fluorescent units per minute)

Probe	HNE	Pr3	CatG
S1	77.8 ± 33.8	43.1 ± 11.5	16.3 ± 21.3
S2	109.0 ± 25.5	12.5 ± 9.5	11.9 ± 8.3
S3	351.0 ± 190.0	8.3 ± 11.0	10.6 ± 0.6

leukocyte protease inhibitor (SLPI) which inhibits HNE but not Pr3, and alpha-1-proteinase inhibitor (αPI) that inhibits both HNE and Pr3 and alpha 1 antichymotrypsin (α1ACT) which inhibits cathepsin G but neither HNE nor Pr3.[6] The selective inhibitory profiles of these inhibitors validated the specificities of compounds **S1** and **S2** for HNE (Fig. 2B). In the presence of SLPI, residual Pr3 activity was observed with **S1**, whereas it is not visible with **S2**, confirming the specificity of the peptide sequence used in **S2** for HNE over Pr3. The difference observed between intact and lysed PMN can be explained by the difference between the amount of HNE secreted and the quantity that remains within the cell.[18] Since HNE is one the most destructive enzymes within a neutrophil, its release is tightly regulated and as such it is stored in azurophilic granules.

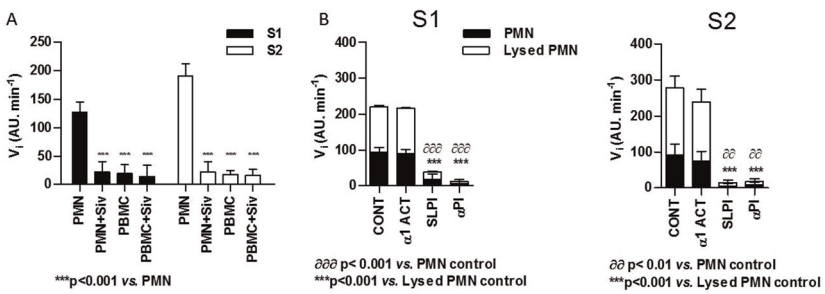


Fig. 2 (A) Cellular specificity of cleavage of probes **S1** and **S2**. Neutrophils (PMN) show cleavage of **S1** and **S2** unlike mononuclear cells (PBMCs) while activation is blocked by sivelestat (Siv: a specific HNE inhibitor); (B) selective inhibition of **S1** and **S2** in whole and lysed PMNs by antiproteinases (α1ACT: alpha one antichymotrypsin, inhibits CatG but not Pr3 and HNE; SLPI: secretory leukocyte protease inhibitor, inhibits of HNE but not Pr3, and αPI: α1 protease inhibitor, inhibits both HNE and Pr3).

Probe **S3** was evaluated on activated PMNs and PBMCs and showed specific activation on human neutrophils (Fig. 3A). The cleavage specificity of the probe due to the cellular activity of HNE was determined with pre-incubation of primary human neutrophils and primary human neutrophil lysates with serine proteinase inhibitors (Fig. 3B). As expected, the specificity of **S3** for HNE was very high compared to Pr3. However, in a cellular environment the initial cleavage of **S3** was observed to be very similar to **S2**, nevertheless the low fluorescence background observed in **S3** confers a real advantage compared to **S2**. The six-branched reporter **S3** improved the signal to noise ratio of the probes with the identical peptide sequence found in **S2** (Fig. 1). The six branched scaffold **S3** displayed more efficient quenching than **S2**, and led to a 2-fold higher turn-on fluorescence signal upon enzymatic cleavage by HNE (Fig. 4). In addition, the probes did not impair cellular integrity or metabolism as confirmed by cytotoxicity assays, while cells remained unaffected by the presence of 100 μM **S1**, **S2** or **S3** (see ESI S13-Fig. 2 and S12-Fig. 1†) showing their biocompatibilities.[19]

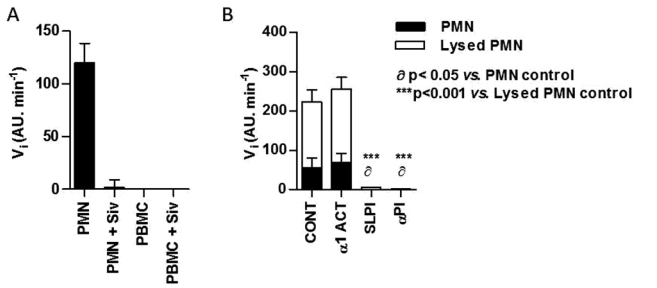


Fig. 3 (A) Cellular specificity of cleavage of probe **S3**. Neutrophils (PMN) cleavage **S3** unlike mononuclear cells (PBMC) while activation is blocked by sivelestat (Siv: a specific HNE inhibitor); (B) selective inhibition of **S3** in whole and lysed PMNs by antiproteinases (SLPI: secretory leukocyte protease inhibitor and αPI: α1 protease inhibitor).

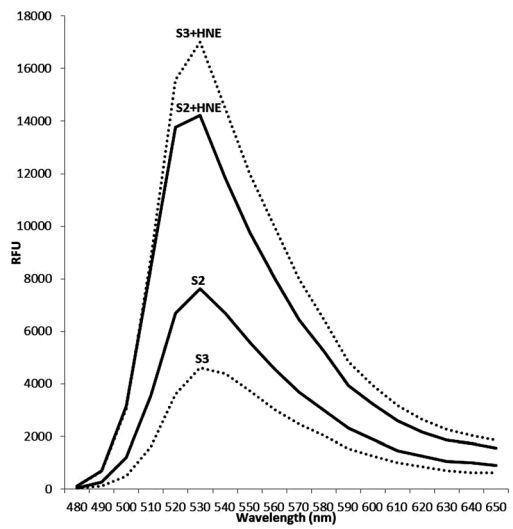


Fig. 4 Fluorescence emission spectra of **S2** and **S3** (10 μM in PBS) before and after incubation with HNE (0.19 μM in HEPES) at 37 °C (excitation wavelength 490 nm).

Conclusions

In summary incorporating HNE specific peptide sequences into multivalent scaffolds allowed the development of HNE specific reporters. They are rapid to synthesise and display multiple advantages for the use in biological assays over conventional FRET systems. These compounds are adaptable, with the number of branches, peptide sequence and fluorophores readily changed to improve their efficacy. These compounds are non-toxic and are suitable for the use in a variety of assays, notably on cells where their ability to access intracellular compartments allows analysis of intracellular proteases.

Acknowledgements

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