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Highly selective and rapidly activatable fluorogenic Thrombin sensors and application in human lung tissue†

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A library of FRET-based peptides were prepared and studied as Thrombin substrates. This identified probes that showed selective activation by Thrombin, low fluorescent background signals, stability to Factor Xa, matrix metalloproteases, and primary human inflammatory cell lysates and supernatant. These were selected for further optimization, creating a second generation of fluorogenic probes with improved solubility and Plasmin resistance. The optimised probe allowed the detection of Thrombin activity in *ex vivo* fibrotic human tissue.

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Introduction

Optical molecular imaging (OMI) offers a powerful approach to the real-time measurement of dynamic molecular processes in diseased tissues. Optical imaging probes¹ that only become fluorescent upon target engagement^{2–4} or undergo signal amplification^{5–7} (e.g. *via* protease turnover) have many advantages compared to “always-on” probes with respect to signal-to-noise and limits of detection making them ideal for rapid point-of-care biochemical measurements.

Thrombin is a serine protease that cleaves soluble fibrinogen, with the products subsequently forming insoluble strands of fibrin, as well as catalysing other coagulation-related reactions, and as such can be considered to be the central orchestrator of the coagulation cascade.⁸ Dysregulated Thrombin activity is implicated in the pathogenesis of multiple diseases^{9–11} ranging from thromboembolism, cancer and coronary heart disease and there is strong evidence that Thrombin plays a critical role in early fibroproliferation,^{12–15} especially in fibroproliferative adult respiratory distress syndrome (ARDS).¹⁶ Indeed, Thrombin activity has been found to be significantly increased in models of acute lung injury and in patients with pulmonary fibrosis.¹⁷

Measurement of Thrombin activity typically takes advantage of the enzyme’s proteolytic properties with the use of Förster Resonance Energy Transfer (FRET) substrates, a well-known and broadly applied strategy in the study of proteases,^{18–20} although aptamers have also been used widely for Thrombin detection.^{21,22} A key issue with such FRET substrates and their potential *in vivo* applications is the lack of selectivity of the probes to the proteases Thrombin and Plasmin. Typical cleavage sites have been identified by a Positional Scanning Combinatorial Library approach for Thrombin and Plasmin, with both displaying a preference for basic amino acids at P1, with P2-Pro, P3-Xaa and P4-Nle/Leu/Ile/Phe/Val for Thrombin, and P2-Tyr/Phe/Trp, P3-Xaa and P4-Lys/Nle/Val/Ile/Phe for Plasmin.²³ More recently a consensus recognition sequence for Thrombin identified by phage display was determined as P2-Pro, P1-Arg, P1'-Ser/Ala/Gly/Thr, P2' (not acidic) and P3'-Arg.²⁴

Several activatable cell penetrating peptides with the sequence DPRSFL derived from the Thrombin substrate protease-activated receptor-1 (PAR1) and variations (e.g. PPRSFL and NleTPRSFL) have been used to image Thrombin activity in atherosclerotic plaques in arteries^{25,26} with mice and after brain injury in rats.²⁷ The extended sequence from PAR1 (P2–P14') has also been incorporated into larger constructs (30-mer peptide) to allow imaging of pulmonary emboli by PET and fluorescence.²⁸

However in the case of Thrombin, due to its pleiotropic roles^{29–32} (including amongst others, platelet aggregation, cellular migration, fibrinolysis and peripheral blood cell activation) and interactions with many target proteins, achieving substrate selectivity is inherently challenging. In addition Thrombin and the ubiquitous protease Plasmin found in extracellular matrix and plasma both belong to the family of serine

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proteases that have a requirement for basic amino acids at the P1 position.

Thus the use of Thrombin activatable imaging probes in complex biological environments, such as the lung would be thwarted due to interfering enzymes such as Plasmin and Factor Xa (possibly at higher concentrations or with far higher specific activities than Thrombin). The development of tools for the *in vivo* analysis of Thrombin thus required the development of highly Thrombin specific substrates/probes and hence, the aim of our work, was to develop a first-in-class Plasmin resistant and highly selective Thrombin optical probe for measuring activity in fibrotic human lung tissue.

Results and discussion

Design and synthesis of Thrombin substrates (FRET peptides)

The first set of peptides synthesised were based on a classical Fluorescein/Methyl Red quenched pair (Fig. 1) with the substrate under investigation located between the fluorophore and quencher which upon enzymatic cleavage releases the fluorophore with a resultant increase in fluorescence. The peptides **1**, **2** and **3** were selected/designed based on recent phage-display technology,²⁴ and were in good agreement with the preference for basic amino acids (preferably Arginine) at P1, a strong preference for Proline at the P2 position, lack of specificity at P3, and the need for an aliphatic amino acid as P4.²³ Other variants (sequences **4**–**10**) were also synthesised and evaluated, with exchange of Leu, Ile and Nle at P4 envisaged as a minor change that could affect the interaction of the ali-

phatic amino acid with its hydrophobic binding pocket. All the peptides were synthesised by manual standard solid-phase Fmoc chemistry, using aminomethyl-Chemmatrix resin and DIC/Oxyma as the coupling combination (Scheme 1). Fmoc-Lys (Dde)-OH was used to allow introduction of Methyl Red *via* selective Dde deprotection.³³ Following cleavage from the resin all the compounds were purified by RP-HPLC and characterized by mass spectrometry (MALDI-TOF; see Experimental and ESI†).

The FRET efficiency of the probes **1**–**10** was initially tested by fluorescence measurements. High quenching of FAM by MR in the FRET pairing is imperative to ensure low background fluorescence of the intact probe.

Evaluation of selectivity and specificity of Thrombin substrates

Peptides **1**–**10** were screened against Thrombin, Plasmin, Factor Xa and MMP-9 to evaluate the specificity and selectivity of the peptide substrates (Fig. 2 and Table S1†). Despite high fold-changes for probes **5**, **6**, **8** and **9** when incubated with Thrombin (15, 10, 16 and 10-fold respectively), unacceptable levels of non-specific probe activation in the presence of Plasmin were observed for each of these probes (4 to 6-fold increase in fluorescence signal); rendering them unsuitable. Probes **1**–**4**, **7** and **10** were each cleaved by Thrombin and showed moderate increases in fluorescence (2.3 to 7-fold increase) with lower levels of fluorescence increase in the presence of Plasmin (<2-fold). Probe **1** was deemed not to be a suitable candidate for further investigation due to the high fluorescence background (Fig. S2†).

Compound	Sequence
P4 - P3 - P2 - P1 - P1' - P2' - P3' - P4'	
1	Leu-Thr-Pro- <i>Arg</i> -Gly-Val-Arg-Leu
2	Leu-Trp-Pro- <i>Arg</i> -Gly-Val-Arg-Leu
3	Leu-Thr-Pro- <i>Arg</i> -Gly-Trp-Arg-Leu
4	Leu-Trp-Pro- <i>Arg</i> -Gly-Trp-Arg-Leu
5	Ile-Thr-Pro- <i>Arg</i> -Gly-Val-Arg-Leu
6	Ile-Thr-Pro- <i>Arg</i> -Gly-Trp-Arg-Leu
7	Ile-Trp-Pro- <i>Arg</i> -Gly-Trp-Arg-Leu
8	Nle-Thr-Pro- <i>Arg</i> -Gly-Val-Arg-Leu
9	Nle-Thr-Pro- <i>Arg</i> -Gly-Trp-Arg-Leu
10	Nle-Trp-Pro- <i>Arg</i> -Gly-Trp-Arg-Leu

Table 1

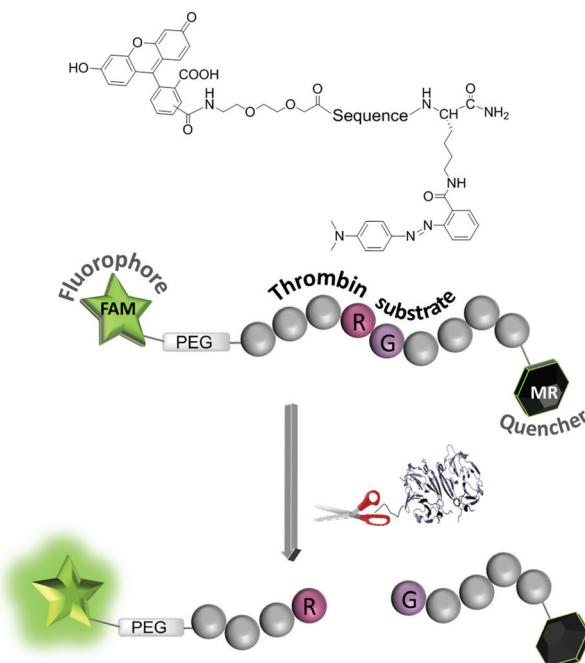
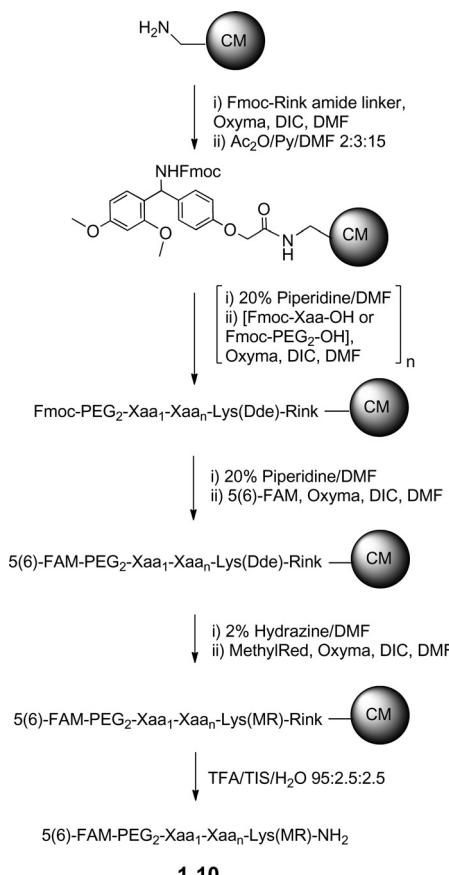


Fig. 1 Design of Thrombin probes and mode of action. Table 1. Library of peptide probes synthesised for the *in vitro* analysis of Thrombin (the cleavage site is indicated in italics (Nle: Norleucine)). Right: Enzymatic activation of the peptide releases a 5(6)-carboxyfluorescein (FAM) peptide fragment from its proximity to its quencher MethylRed (MR).





Scheme 1 Synthesis of Thrombin probes **1–10** by solid-phase synthesis. CM = Chemmatrix resin; DIC = *N,N'*-diisopropylcarbodiimide; Fmoc-PEG₂-OH = [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid; TFA = trifluoroacetic acid; TIS = triisopropylsilane.

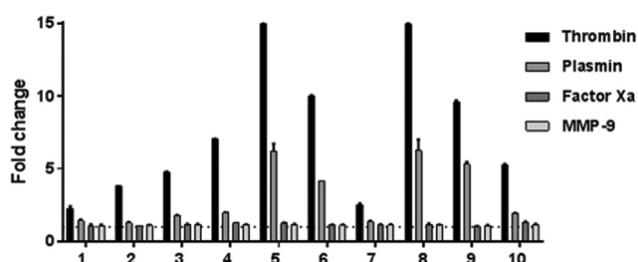


Fig. 2 Relative increase (fold change) in fluorescence of probes **1–10** (10 μ M) after 10 min incubation with either Thrombin (5 U ml⁻¹), Plasmin (30 nM), Factor Xa (0.5 μ M) or MMP-9 (83 kDa, 30 nM), compared to control (probe in buffer alone (ex/em 485/528 nm)).

MALDI-TOF MS analysis following enzymatic treatment showed that Thrombin cleavage was site specific (X-X-P-R \uparrow G-X-R-L) for all the sequences tested (see ESI[†]), while no cleavage by MMP-9 and Factor Xa was observed for any probe.

As expected some differences in the performance of probes **4**, **7** and **10** were observed (Fig. 2, 3 and S1[†]) following the exchange of Leu for Ile or Nle at P4, and could be attributed to the small differences in the aliphatic amino acid side chain and its interaction with the binding site.

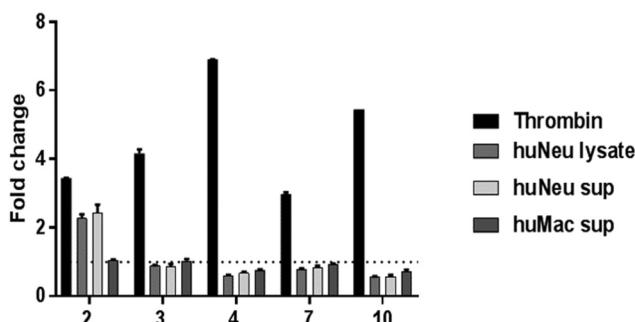


Fig. 3 Evaluation of the activation of FRET peptides **2**, **3**, **4**, **7**, **10** (10 μ M) by Thrombin (5 U ml⁻¹), human neutrophil (huNeu) lysates and supernatant (sup) and macrophage supernatant (huMac sup). Fold-changes in fluorescence (ex/em 485/528 nm) compared to probe in buffer only.

Lung related selectivity studies for the Thrombin probes

Since probes **2**, **3**, **4**, **7** and **10** were shown to be relatively stable in the presence of Plasmin and had low fluorescence backgrounds, they were evaluated further in complex biological environments to assess nonspecific cleavage. The probes were evaluated with supernatants and lysates of freshly isolated human neutrophils and human macrophages (Fig. 3) with relative increases in fluorescence compared for each probe. All probes (with the exception of **2**) were stable under these conditions and demonstrated large increase in fluorescence signal with Thrombin (3–4-fold; **4** 7-fold; **7** 3-fold; **10** 5.5-fold). Specific cleavage by Thrombin was confirmed by MALDI-TOF MS as P-R \uparrow G-V-R-L, although probe **2** demonstrated an additional cleavage site P-R-G-V \uparrow R-L (Fig. 4), an effect also observed in other probes containing the motif Val-Arg, such as probe **8** (Fig. S3[†]). Plasmin cleavage at the carboxyl side of Lys and Arg is well recognised³⁴ and was observed with our first generation probes. The main Plasmin cleavage site was determined as -P-R-G-W-R \uparrow L- by MALDI-TOF MS analysis of probe **10** following incubation with Plasmin (Fig. 5).

2nd generation of Thrombin probes with Plasmin stability

As some of the probes demonstrated very fast, yet selective, responses even under exposure to highly oxidative and proteolytic environments such as lysates and supernatants of primary human leucocytes, increasing aqueous solubility and gaining greater selectivity over Plasmin was the main goal for the synthesis of the next generation of Thrombin probes. Solubility was enhanced by adding polyethyleneglycol (PEG₂) units to the peptide **10** which gave good performance in all previous assays. D-Lysine residues were also incorporated as a non-natural amino acid to introduce charged/solubilising groups to improve solubility and at the same time prevent non-specific protease cleavage. Improvements of selectivity for Thrombin over Plasmin were envisioned by modifying the key amino acids involved at the Plasmin cleavage site to their D-enantiomers. This thus gave probes **11–13** (Fig. 6A).

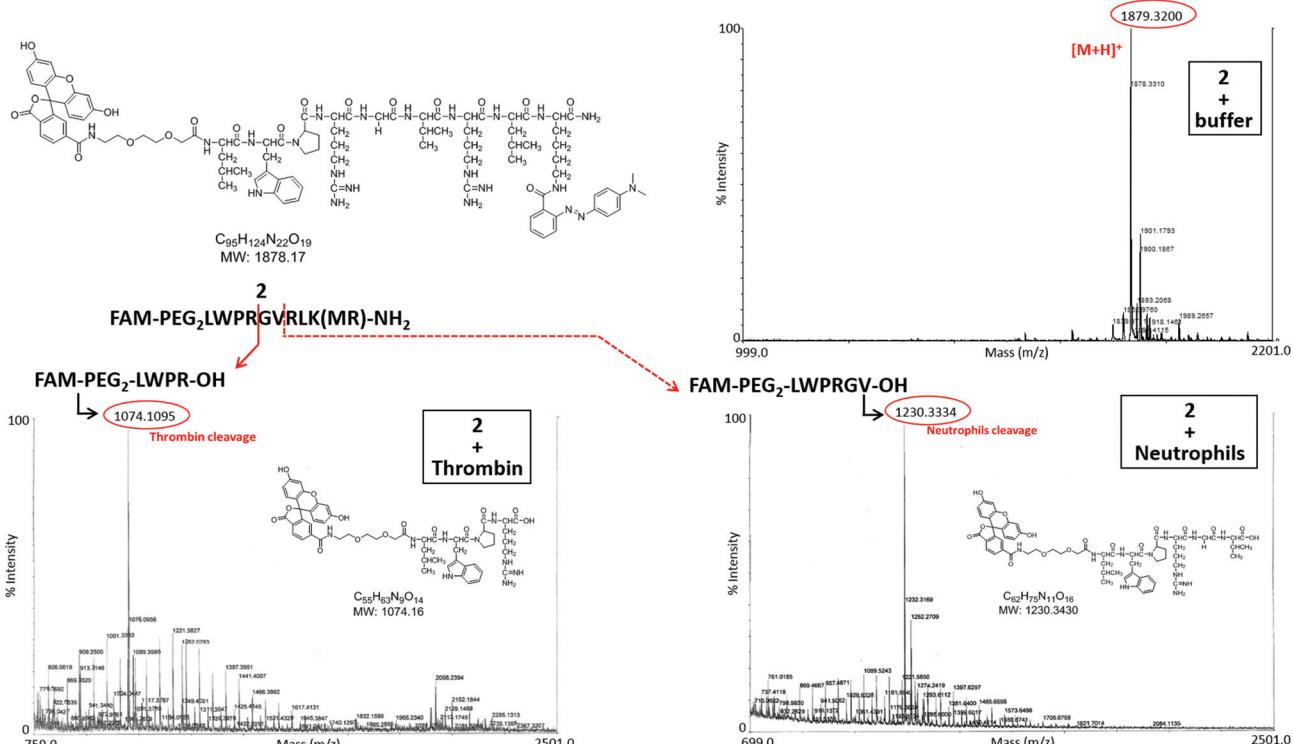


Fig. 4 MALDI-TOF MS analysis of peptide 2, demonstrating specific vs. non-specific cleavage by Thrombin and Neutrophils respectively.

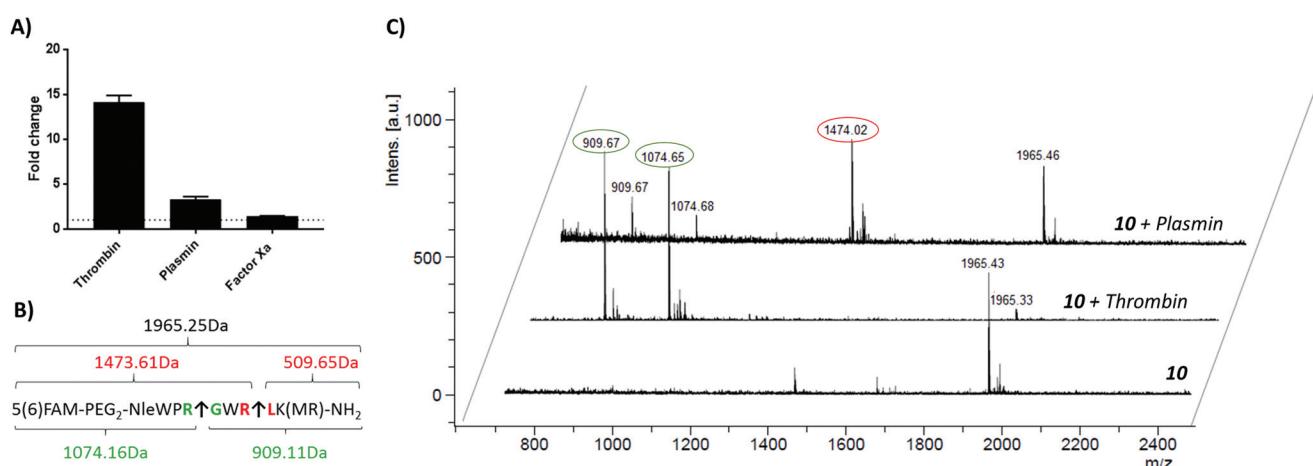


Fig. 5 (A) Compound 10 ($10 \mu\text{M}$) showing Thrombin (5 U ml^{-1}) and Plasmin (30 nM) cleavage while Factor Xa ($0.5 \mu\text{M}$) did not affect the probe (ex/em $485/528 \text{ nm}$). (B) Molecular weight of probe 10 and its fragments obtained after enzymatic hydrolysis of shown cleavage sites. (C) MALDI-TOF MS spectra of probe 10 before and after incubation with the different enzymes (Thrombin cleavage site in green with two fragments observed at 1074 and 909 Da; Plasmin main cleavage site in red with fragment observed at 1474 Da).

Evaluation with recombinant Thrombin and Plasmin demonstrated that these modifications afforded probes that were highly selective, with increases in fluorescence in response to Thrombin measured at 15, 15 and 10-fold for probes 11, 12, and 13 respectively within 12 minutes (Fig. 6B), with no increase in fluorescence detected for any probe in the presence of Plasmin.

Due to its high aqueous solubility and Plasmin resistance, probe 11 was selected as the lead candidate for further investigation. The stability of probe 11 was assessed against a larger

panel of enzymes, known to be highly abundant within fibrotic tissue (Fig. S4†). None of these, apart from Thrombin, were able to activate the probe. Moreover, the K_m value of $5.4 \mu\text{M}$ demonstrated good binding affinity while the turn-over number of 10.8 s^{-1} showed good catalysis, with an impressive k_{cat}/K_m value of $1.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ obtained for thrombin with probe 11 *in vitro*. This is in contrast to k_{cat}/K_m values in the order of $10^5 \text{ M}^{-1} \text{ s}^{-1}$ previously reported for thrombin with alternative thrombin activated FRET probes.²⁵

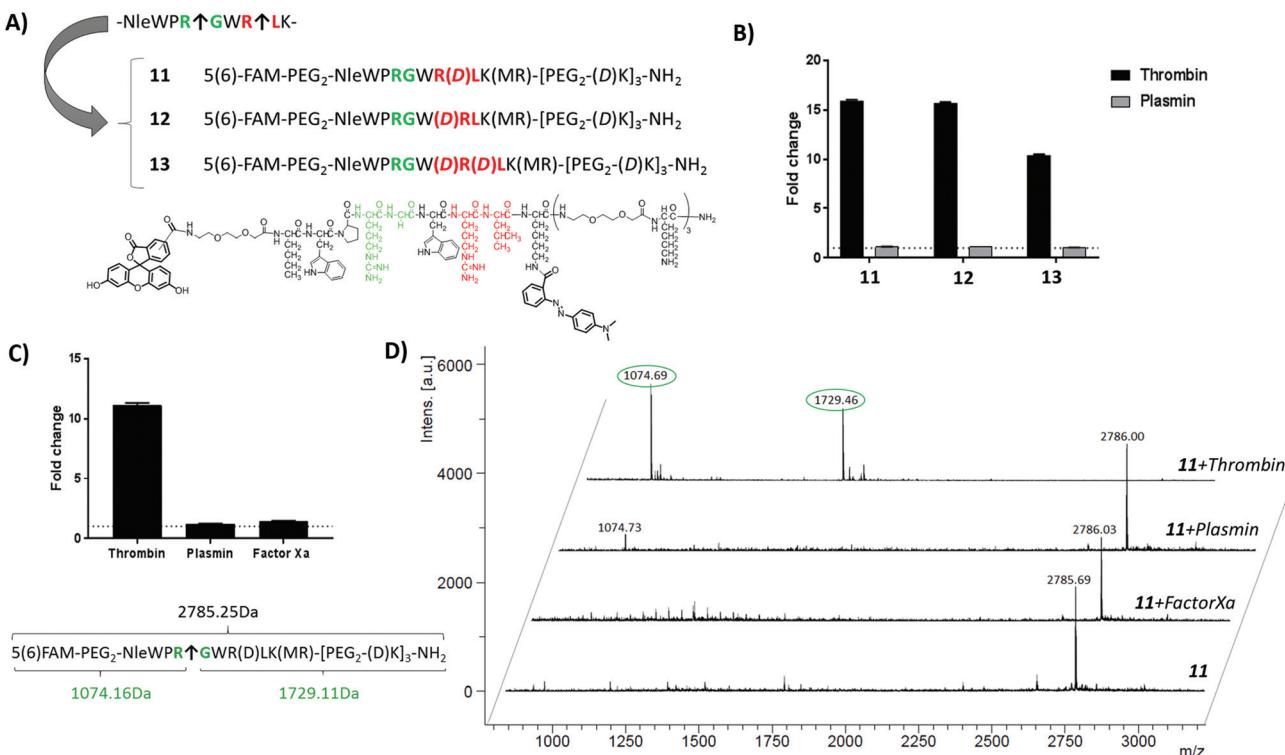


Fig. 6 (A) Structure of the second generation Thrombin probes **11–13** with the Thrombin cleavage site in green and the modified Plasmin cleavage site in red; (B) data showing the relative fold increase of each probe after incubation with Thrombin or Plasmin (ex/em 485/528 nm); (C) the change in fluorescence provided by probe **11** (10 μ M) (excitation/emission 485/528 nm) with recombinant human Thrombin, Plasmin and Factor Xa (at 5 μ M, 30 nM and 0.5 μ M respectively); (D) MALDI-TOF MS spectra of **11** showing Thrombin cleavage while Plasmin and Factor Xa did not significantly affect the probe.

Validation of Thrombin probes in *ex vivo* fibrotic lung tissue

As Thrombin is centrally implicated in the fibroproliferative cascade, the lead optimized probe **11** was evaluated on *ex vivo* human lung tissue homogenates from patients with idiopathic pulmonary fibrosis (IPF) and control non-fibrotic tissue from

the same patients. A significant increase in fluorescence was detected with probe **11** (1 μ M) incubated with fibrotic tissue homogenates compared to the ‘healthy control’ equivalents $P = 0.0003$ (Mann–Whitney test) (Fig. 7A) with specific cleavage due to Thrombin present within the tissue samples confirmed by MALDI-TOF MS analysis (Fig. 7B).

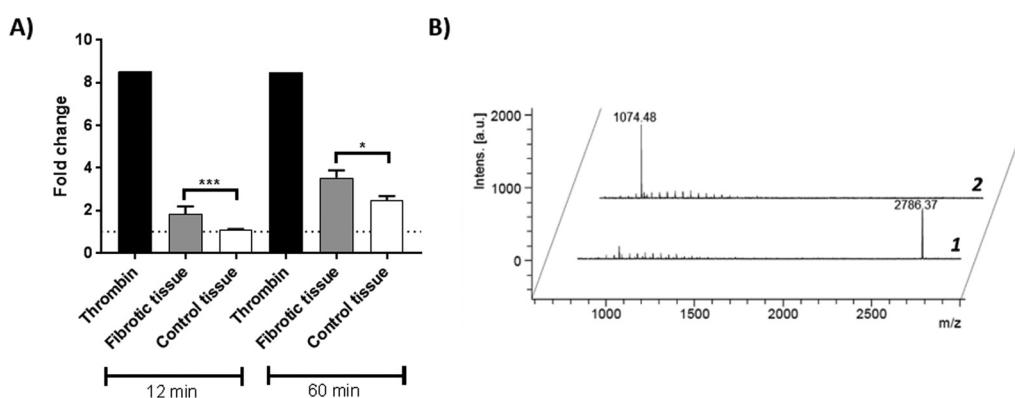


Fig. 7 Addition of probe **11** onto *ex vivo* human tissue: (A) fluorescence signal generated by the cleavage of probe **11** (1 μ M) by human fibrotic tissue homogenate, control tissue homogenate or recombinant Thrombin (ex/em 485/528 nm); (B) MALDI-TOF MS analysis of probe **11** following treatment with control human tissue (trace 1) and human fibrotic tissue (trace 2) showing the fragment obtained after specific cleavage by Thrombin.



Conclusions

A number of peptide sequences were evaluated as potential Thrombin substrates with the best sequences selected based upon activation by Thrombin, stability to Factor Xa, human inflammatory cell lysates and supernatants. In the second generation of probes, the inclusion of unnatural D-amino acids at certain positions within the enzyme cleavable peptide sequence resulted in the sequence being resilient to cleavage by Plasmin, whilst still being selectively cleavable by Thrombin.

The lead optimized probe enables the reliable and accurate detection of Thrombin in tissue samples where other ubiquitous and highly similar proteinases that orchestrate the coagulation cascade are present such as Plasmin and Factor Xa. The development of a fast and selective probe for Thrombin detection has utility in a number of fibroproliferative and fibrotic disorders where Thrombin levels are increased, and has important implications in the improved diagnosis and therapeutic stratification of early stages of diseases characterised by dysregulated Thrombin activity.

Experimental

Synthesis of the probes

The FRET-peptides were synthesized by standard Fmoc solid-phase peptide chemistry, with dyes and quenchers coupled while the probe was on the solid support. After cleavage and purification by RP-HPLC the compounds 1–13 were characterized by MALDI-TOF MS and analytical HPLC. Detailed synthetic procedures and characterization are described in the ESI.†

MALDI-TOF MS analysis. MALDI spectra were acquired on a Brüker Ultraflextreme MALDI-TOF MS with a matrix solution of sinapic acid (10 mg mL⁻¹) in H₂O/CH₃CN/TFA (50/50/0.1).

Enzyme assays

Enzymatic assays were run in a 384-well format in a PCR opaque microplate (Thermo Scientific). All experiments were performed at least in duplicate. Dilutions and reactions were prepared in MMP buffer (50 mM Tris, 10 mM CaCl₂, 0.15 M NaCl, 0.05% Brij-35, pH 7). Proteolytic activity was determined by calculating the fold change in fluorescence over background signal provided by the corresponding dilution of the probe with exogenous enzymes using a multiwell plate fluorimeter (Synergy H1 Hybrid Reader, BioTek instruments Ltd) at excitation/emission 485/528 nm. Unless otherwise stated, all probes were investigated at 10 μM. Recombinant human MMPs (Catalytic domain MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13 (Enzo Life Sciences) and full-length MMP-2, -9, -12 and -13 (Merck/Millipore)) were used at 30 nM. Pro-MMP-13 (R & D Systems) was activated by incubating with 1 mM 4-amino-phenylmercuric acetate (APMA) for 2 h at 37 °C. Recombinant human Thrombin (Sigma-Aldrich, used at 5 U mL⁻¹), Plasmin (Sigma-Aldrich, used at 30 nM) and Factor Xa (Sigma-Aldrich, used at 0.5 μM) were used to identify the lead molecular probe

sequences. Human neutrophils and monocytes were isolated from human blood from healthy volunteers (following approval of the appropriate regional ethics committee (REC) and with informed consent of the patients), as previously described.³⁵ Neutrophil and Macrophage lysate and supernatant preparation is described within ESI.†

Human tissue supernatant

Human lung tissue was obtained from surgical biopsies for the investigation of interstitial lung disease (*n* = 5). Informed consent was obtained and the study was approved by the Regional Ethics Committee. Under sterile condition, the tissue was dissected and stored at -80 °C for further analysis. For the preparation of tissue supernatant, frozen tissue was suspended in PBS and homogenised (Bio-Gen PRO200 homogeniser, Pro-Scientific) on ice. Samples were centrifuged at 13 000 rpm for 15 min at 4 °C and the debris-free supernatant collected. Total protein concentrations were determined using at PierceTM BCA kit (Thermo Scientific). The samples were aliquoted and stored at -20 °C or -70 °C until further analysis. All tissue homogenates were investigated at a protein concentration of 333 μg mL⁻¹.

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References

- 1 S. Lee, J. Xie and X. Chen, *Biochemistry*, 2010, **49**, 1364–1376.
- 2 T. Aslam, A. Miele, S. V. Chankeshwara, A. Megia-Fernandez, C. Michels, A. R. Akram, N. McDonald, N. Hirani, C. Haslett, M. Bradley and K. Dhaliwal, *Chem. Sci.*, 2015, **6**, 4946–4953.
- 3 M. Eun Jun, B. Roy and K. Han Ahn, *Chem. Commun.*, 2011, **47**, 7583–7601.
- 4 C. R. Drake, D. C. Miller and E. F. Jones, *Curr. Org. Synth.*, 2011, **8**, 498–520.
- 5 N. Avlonitis, M. Debonne, T. Aslam, N. McDonald, C. Haslett, K. Dhaliwal and M. Bradley, *Org. Biomol. Chem.*, 2013, **11**, 4414–4418.
- 6 A. R. Akram, N. Avlonitis, A. Lilienkampf, A. M. Perez-Lopez, N. McDonald, S. V. Chankeshwara, E. Scholefield, C. Haslett, M. Bradley and K. Dhaliwal, *Chem. Sci.*, 2015, **6**, 6971–6979.



7 S. Lee, K. Park, K. Kim, K. Choi and I. C. Kwon, *Chem. Commun.*, 2008, 4250–4260.

8 E. Di Cera, *Mol. Aspects Med.*, 2008, **29**, 203–254.

9 J. M. Siller-Matula, M. Schwameis, A. Blann, C. Mannhalter and B. Jilma, *Thromb. Haemostasis*, 2011, **106**, 1020–1033.

10 K. M. Snyder and C. M. Kessler, *Semin. Thromb. Hemostasis*, 2008, **34**, 734–741.

11 M. E. Maragoudakis, N. E. Tsopanoglou and P. Andriopoulou, *Biochem. Soc. Trans.*, 2002, **30**, 173–177.

12 N. A. Hernandez-Rodriguez, A. D. Cambrey, N. K. Harrison, R. C. Chambers, A. J. Gray, A. M. Southcott, R. M. duBois, C. M. Black, M. F. Scully and R. J. McAnulty, *Lancet*, 1995, **346**, 1071–1073.

13 R. C. Chambers, *Biomed. Health Res.*, 2000, **34**, 115–128.

14 G. Verma, M. Binnie and C. K. N. Chan, *Curr. Respir. Med. Rev.*, 2007, **3**, 188–192.

15 R. C. Chambers and C. J. Scotton, *Proc. Am. Thorac. Soc.*, 2012, **9**, 96–101.

16 R. C. Chambers, P. Leoni, O. P. Blanc-Brude, D. E. Wembridge and G. J. Laurent, *J. Biol. Chem.*, 2000, **275**, 35584–35591.

17 T. Ohba, J. K. McDonald, R. M. Silver, C. Strange, E. C. LeRoy and A. Ludwicka, *Am. J. Respir. Cell Mol. Biol.*, 1994, **10**, 405–412.

18 H.-Y. Hu, S. Gehrig, G. Reither, D. Subramanian, M. A. Mall, O. Plettenburg and C. Schultz, *Biotechnol. J.*, 2014, **9**, 266–281.

19 S. S. van Berkel, B. van der Lee, F. L. van Delft, R. Wagenvoord, H. C. Hemker and F. P. J. T. Rutjes, *ChemMedChem*, 2012, **7**, 606–617.

20 C.-H. Tung, R. E. Gerszten, F. A. Jaffer and R. Weissleder, *ChemBioChem*, 2002, **3**, 207–211.

21 A. Sassolas, L. J. Blum and B. D. Leca-Bouvier, *Biosens. Bioelectron.*, 2011, **26**, 3725–3736.

22 B. Deng, Y. Lin, C. Wang, F. Li, Z. Wang, H. Zhang, X.-F. Li and X. C. Le, *Anal. Chim. Acta*, 2014, **837**, 1–15.

23 B. J. Backes, J. L. Harris, F. Leonetti, C. S. Craik and J. A. Ellman, *Nat. Biotechnol.*, 2000, **18**, 187–193.

24 M. Gallwitz, M. Enoksson, M. Thorpe and L. Hellman, *PLoS One*, 2012, **7**, e31756.

25 M. Whitney, E. N. Savariar, B. Friedman, R. A. Levin, J. L. Crisp, H. L. Glasgow, R. Lefkowitz, S. R. Adams, P. Steinbach, N. Nashi, Q. T. Nguyen and R. Y. Tsien, *Angew. Chem., Int. Ed.*, 2013, **52**, 325–330.

26 E. S. Olson, M. A. Whitney, B. Friedman, T. A. Aguilera, J. L. Crisp, F. M. Baik, T. Jiang, S. M. Baird, S. Tsimikas, R. Y. Tsien and Q. T. Nguyen, *Integr. Biol.*, 2012, **4**, 595–605.

27 B. Chen, B. Friedman, M. A. Whitney, J. A. V. Winkle, I.-F. Lei, E. S. Olson, Q. Cheng, B. Pereira, L. Zhao, R. Y. Tsien and P. D. Lyden, *J. Neurosci.*, 2012, **32**, 7622–7631.

28 M. J. Page, A. L. Loureco, T. David, A. M. LeBeau, F. Cattaruzza, H. C. Castro, H. F. VanBrocklin, S. R. Coughlin and C. S. Craik, *Nat. Commun.*, 2015, **6**, 8448.

29 S. Narayanan, *Ann. Clin. Lab. Sci.*, 1999, **29**, 275–280.

30 C. T. Griffin, Y. Srinivasan, Y.-W. Zheng, W. Huang and S. R. Coughlin, *Science*, 2001, **293**, 1666–1670.

31 K. Croce and P. Libby, *Curr. Opin. Hematol.*, 2007, **14**, 55–61.

32 J. T. B. Crawley, S. Zanardelli, C. K. N. K. Chion and D. A. Lane, *J. Thromb. Haemostasis*, 2007, **5**, 95–101.

33 J. J. Diaz-Mochon, L. Bialy and M. Bradley, *Org. Lett.*, 2004, **6**, 1127–1129.

34 F. J. Bikker, G. Koop, N. B. Leusink, K. Nazmi, W. E. Kaman, H. S. Brand and E. C. I. Veerman, *Vet. Res. Commun.*, 2014, **38**, 271–277.

35 A. G. Rossi, J. C. McCutcheon, N. Roy, E. R. Chilvers, C. Haslett and I. Dransfield, *J. Immunol.*, 1998, **160**, 3562–3568.

