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A matrix metalloproteinase activation probe for painting human tumours†

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A probe that allows specific ‘painting’ of human tumours is described. Probe activation was mediated by specific matrix metalloproteinases, resulting not only in disruption of a FRET pair, but in the generation of a fragment that “fluorescently paints” human tumours. This probe demonstrated rapid and effective human tumour labelling with the potential to allow margin detection during surgical resection.

The concept of tissue-based fluorescent labelling has gained attention as a method for identification of diseased tissue margins during intra-operative cancer surgery,^{1–6} made possible with key advances in imaging instrumentation.^{7,8} Pivotal to this has been the development of fluorescent probes which provide disease-mediated contrast.⁹ Approved fluorescent contrast agents in this area include compounds such as 5-aminolevulinic acid which is selectively up-taken and metabolized by cancerous tissues to generate protoporphyrin IX.¹⁰ Other optical agents in clinical studies¹¹ include labelled nanobodies,¹² peptides, such as chlorotoxin (tozuleristide)¹³ which is currently in phase II/III studies for pediatric CNS tumours, and the bis-cyclic peptide GE-137, which targets the human hepatocyte growth factor receptor (c-MET).¹⁴ There are also a wide variety of protease based probes,^{15–21} with cathepsins being a key target where signals are generated by either covalent modification of the enzyme²² or by FRET dequenching.^{23,24} Such probes have been able to detect margins in nonmelanoma skin cancer.²⁵ Other examples of probes explored for cancer imaging include those targeting DPP-IV for esophageal cancer,^{26,27} folate receptor targeted probes,²⁸ PSMA for prostate cancer²⁹ or those developed by Tsien,³⁰ whereby MMP-2 activation liberated a cell penetrating peptide that locally tagged proximal tissue. Matrix metalloproteinase 13 (MMP-13 or

collagenase 3) is an endopeptidase overexpressed in the micro-environment around both lung tumours and fibrotic tissue, and has been shown to play a role in early invasive pulmonary adenocarcinoma.³¹ MMP-2 and -9 (gelatinase A and B) are cancer-associated endopeptidases overexpressed in a variety of malignant tumors and often associated with aggressiveness and poor prognosis.^{32,33} Here we report on the rational design, synthesis and evaluation on human cancerous tissue of a novel MMP-imaging agent (3) which allowed MMP-mediated ‘painting’ of resected human tumour tissue.

The designed probe (3) contained both a FRET pair between 5-carboxyfluorescein (FAM) and the quencher methyl red reporting on enzyme activity, as well as the incorporation of an ‘always-on’ far-red fluorophore (an in-house synthesized Cy5.5, ex/em 670/693 nm) with a spectral window distinct from FAM and away from tissue autofluorescence. The Cy5.5-fragment released enzymatically was responsible for the “tissue-painting” ability of the activated probe, which is attributable to the hydrophobicity of this fragment compared to the parent compound (Fig. 1a).

The peptide sequence (Pro-Phe-Gly-Nle-Lys-βAla, previously reported as MMP-2,9,13 substrate^{34,35}) was synthesized by Fmoc solid-phase peptide synthesis on ChemMatrix resin using Oxyma/DIC as the coupling combination (Scheme 1). At the carboxy-terminus of the peptide, three replicates of bis-ethyleneglycol and D-lysine were added to ensure both solubility and stability against proteases.³⁴ As part of this strategy we developed a novel Cy5.5 red dye, that was readily prepared on large scale in 4 steps, and contained a (5-carboxypyridin-2-yl) group to allow ready incorporation *via* an amide bond to the peptide by solid phase methods (ESI† for details). The Cy5.5, 5-carboxyfluorescein and methyl red were sequentially incorporated at the amino terminus of the peptide, on the Lys side chain (after Dde deprotection) and conjugated to the Lys(N₃) residue *via* azide/alkyne cycloaddition respectively (Scheme 1 and ESI†). Probe 3 was purified and characterized by RP-HPLC and MALDI TOF MS and was fully aqueous soluble (log *P* –1.5) (ESI† for details).

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Fig. 1 Cleavage of compound **3** by target MMPs and generation of the tissue painting fragment. (a) Mode of action of **3** showing the quenched and non-quenched fluorophores and resultant tissue staining with the Cy5.5 fragment released following MMP cleavage; (b) cleavage of **3** (5 μ M) measured as fluorescence increase (compared to enzyme-free control) at 15 min; ex/em 485/528 nm (FAM, green bars) and 680/710 nm (Cy5.5, red bars) (M: marimastat, MMP inhibitor). Data is the mean of three independent replicates performed in duplicate. Error bars represent s.e.m. Statistical analysis was performed with a one-way ANOVA test compared to an enzyme-free control. * $P = 0.0147$; **** $P < 0.0001$; (c) RP-HPLC analysis revealed the decreased polarity of the Cy5.5-fragment after cleavage compared to the parent peak; (d) visual change in the octanol–water distribution before and after enzymatic cleavage of **3** showing the generation of the hydrophobic Cy5.5 fragment; (e) structure of **3** showing the fragments generated following cleavage at the Gly–Nle site by MMPs.

Target and off-target proteases were incubated with **3**. Within 15 min, as anticipated, an increase in green fluorescence (FAM signal) was measurable from the probe incubated with the active domains of MMP-2, -9 and -13 (Fig. 1b and Fig. S1, ESI[†]), which was blockable in the presence of pan-MMP inhibitor marimastat.

Probe **3** was stable against the off-target proteases (Fig. 1b), with cleavage by MMPs specifically at Gly \uparrow Nle, as confirmed by MALDI TOF MS (Fig. S2, ESI[†]). Importantly the Cy5.5 residue did not interfere with fluorescence in the green channel following MMP activation, with data similar to the control compound **4** that contained FAM & methyl red only (see Fig. S3 and S4, ESI[†]). In solution the Cy5.5 fluorescence intensity remained constant before and after cleavage (Fig. 1b), demonstrating that its

intensity was independent of the FRET pairing. HPLC analysis confirmed that the Cy5.5-fragment obtained after cleavage was much more hydrophobic than the other two components, with its retention factor shifting from $k = 4.2$ for parent compound **3**, to $k = 8.0$ for the Cy5.5 fragment (Fig. 1c and e), while MMP-13 treatment of the peptide **3** in biphasic buffer/octanol led to migration of the cleaved Cy5.5-labelled peptide into the octanol phase (Fig. 1d and Fig. S5, ESI[†]) demonstrating the hydrophobicity “switch-on” upon cleavage.

3 (5 μ M) was applied onto human lung tumour tissue, from three individual patients: two with squamous cell carcinoma and one with adenocarcinoma (MMPs presence within these tissues was confirmed by gelatin zymography,³⁶ Fig. S6, ESI[†]) and imaged over 30 min (Fig. 2 and ESI[†] videos) with a fibre-based imaging





Fig. 3 Macroscopic imaging of **3** delineating tumour margins. (a) Bright field microscopy image and fluorescence image of freshly excised lung slice, with pathologically identified adenocarcinoma (**Ad**), transition zone (**Tr**) and normal (**N**) tissue, following incubation with compound **3**. (b) Control tissue from the same patient sample without the addition of compound **3** was used as a measure of tissue autofluorescence within this spectral window. Scale bar is 1 mm.

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Conflicts of interest

There are no conflicts to declare.

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