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A dual optical and nuclear imaging reagent for peptide labelling *via* disulfide bridging†

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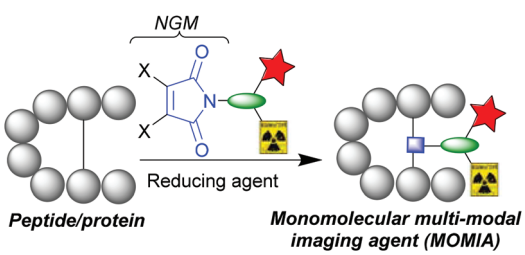
We report a concise approach to a multimodal imaging reagent for peptide labelling *via* disulfide bridging. The reagent is constructed using a one pot, three component, [3 + 2] cycloaddition of a fluorescent azide with a dithiomaleimide-alkyne, with concomitant incorporation of ^{125}I. The dithiomaleimide handle then enables site selective conjugation to a disulfide bond of a peptide whilst retaining the key structural bridging functionality, as exemplified on the therapeutic peptide octreotide.

Employing two complimentary imaging techniques synergistically can allow for more accurate staging and localisation of tumours and is a concept being explored in cancer diagnosis. Administering two separate imaging agents to patients carries problems however, as both will have differing pharmacokinetic properties to consider and the toxic effects of each component may well be additive. To circumvent this, strategies have been explored to combine multiple imaging agents into one construct, such as in micelles and nanoparticles.[1] However, these carry their own drawbacks, as such large molecules have inherently slow diffusion rates into the target tissue and rapid uptake and excretion by the liver.[2]

A recent concept is the incorporation of desired reporter groups into one molecule, to create a monomolecular multimodal imaging agent (MOMIA). MOMIAs contain two or more imaging agents in a 1 : 1 signalling ratio, with each agent providing different but complimentary information.[3] Coupling optical imaging with nuclear techniques such as positron emission tomography (PET) or single photon emission computed tomography (SPECT) allows for the strengths of the individual methods to be combined. For instance, PET and SPECT allow whole body imaging and detection of tumours within deep tissue, but are limited by low resolution. Once the diseased region has been located, high resolution imaging of the tumour can be carried out by highly sensitive optical methods.[3,4]

Peptides present themselves as an attractive targeting moiety in clinical applications due to their small size, good stability and ease of synthesis and characterisation.[5] This combined with their high specificity and affinity for their targets has led to their successful application in clinical diagnostics and therapeutics.[5] Octreotide, a long-acting analogue of the hormone somatostatin, is a cyclic octapeptide with high affinity for the somatostatin receptor.[6] Many neuroendocrine tumours are reported to over-express one subtype of this family of receptor, predominantly subtype 2 (SSTR2).[7] With its high potency and specificity for the SSTR2, radiolabelled octreotide has been employed for imaging of neuroendocrine tumours in patients for many years.[8] Recently, Edwards *et al.* reported on a derivative of octreotide which incorporates a chelating group at the N-terminus for binding either ^{177}Lu or ^{64}Cu (for imaging by PET or SPECT) and the fluorescent dye cypate (for optical imaging) conjugated at the C-terminus.[4] Excellent correlation in the biodistribution of the MOMIA by optical and nuclear imaging techniques was observed.

We have previously reported on the application of a new class of maleimide reagent, referred to as next generation maleimides (NGMs, see Scheme 1), for the rapid and cysteine-selective modification of peptides and proteins.[9] Most recently, the use of these reagents in the effective re-bridging of the two cysteine residues of a reduced disulfide bond has been



Scheme 1 A platform for MOMIA construction by disulfide bridging.

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exploited; in this way, cystines can become tenable as sites for conjugation as the structural integrity conferred by the disulfide bond is retained.[10] This disulfide re-bridging strategy has proved effective in the functionalisation of peptides, proteins, antibody fragments and full antibodies.[10,11] By careful design of the maleimide reagents for a desired application, a range of variants can be synthesised that differ in activity or cargo.[10–12]

We envisaged that multi-functionalised NGMs could provide a versatile platform for the construction of peptide and protein-derived MOMIAs (Scheme 1), as well as theranostic agents, by allowing a variety of imaging reporter groups and drug constructs to be combined. Such reagents would offer a one-step approach to the dual functionalisation of disulfide bonds, which would complement the elegant double click strategy described by Chudasama, Caddick and co-workers.[13] Thus we report herein the synthesis of a multi-modal NGM, carrying a fluorophore to enable optical imaging, and radioiodine for nuclear imaging techniques. We tested the ability of this novel reagent to insert into the disulfide bond of a model targeting peptide, octreotide.

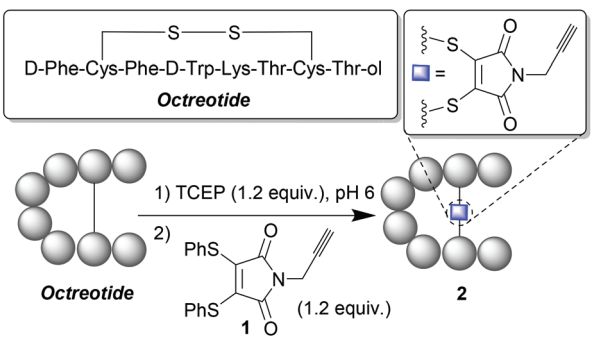
The chemistry of the NGM motif allows for the facile functionalisation of the ring nitrogen. The incorporation of an alkyne at this position has been reported and creates a reactive chemical handle available for further derivatisation.[11e,14] We envisaged exploiting this reactivity to install our desired functionalities, and thus *N*-alkyne dithiophenolmaleimide **1** was synthesised.[15] This reagent was employed to test the efficiency of the bridging protocol for octreotide, and to investigate the effect of the bridging on the biological activity.

Octreotide was first reduced using 1.2 equivalents of tris-(2-carboxyethyl)phosphine (TCEP) for 1 h before addition of 1.2 equivalents of maleimide **1** (Scheme 2). Complete conversion to the modified octreotide **2** was observed within 10 min (see ESI Fig. 1†).

To examine whether the bridging modification had an effect on the biological activity of octreotide, a signalling assay was established to test its activity. Somatostatin and its analogues such as octreotide are known to exert their pharmacological effects through the hyperpolarisation of neuroendocrine and neuronal cell membranes.[16] This hyperpolarisation is facilitated by the activation of the G protein-coupled inwardly rectifying K^+ (GIRK) channel family. The somatostatin receptor is known to couple to GIRK channels; activation of the receptor leads to subsequent activation of the GIRK channel by means of G protein signalling.[16] To model this system, a Human Embryonic Kidney (HEK) 293 cell line stably expressing the GIRK1/2a channel subtype was transiently transfected with the SSTR2. The GIRK1/2a subtype was chosen as it is known to predominate in neuroendocrine cells, providing the most physiologically relevant system.[17] Current activation by inwards flux of K^+ through GIRK1/2a channels upon application of modified octreotide **2** was monitored by whole-cell patch clamp experiments. This was carried out over a range of concentrations (from 1 nM to 10 μM) to allow construction of a dose–response curve. These experiments were repeated using native octreotide (at concentrations from 0.01 nM to 100 nM) to serve as a control (Fig. 1).

From the dose–response analysis, an EC_{50} of 118 nM was calculated for the modified octreotide **2**. In comparison to the native peptide (EC_{50} calculated as 0.66 nM), there is an attenuation of biological activity as a result of disulfide bridging using the maleimide reagent **1**. This is likely due to the small, rigid nature of octreotide, leading a 2 carbon-bridge to be sufficient to perturb the pharmacophore. This attenuation of activity revealed that a MOMIA based on labelling the disulfide bridge of octreotide would not be ideal for *in vivo* imaging. However this peptide still represented a suitable model system for testing the dual labelling strategy, in the knowledge that future applications on larger, structured proteins would be unaffected by disulfide bridging.[11a,c,e,f,13,18] Furthermore, *in vitro* experiments could still be explored to reveal information about the cellular distribution of the peptide.

The free alkyne moiety of maleimide **1** lends itself to a click cycloaddition reaction as a route to functionalising the maleimide with the imaging agents of choice. Rhodamine B was chosen as the fluorophore as it has good photostability, quantum yield and water solubility,[19] as well as a carboxylic acid group which can be readily functionalised with an azide. The targeted rhodamine B-azide was synthesised in 4 steps (Scheme 3). The carboxylic acid of rhodamine B was first con-



Scheme 2 Octreotide disulfide bridging using maleimide **1**.

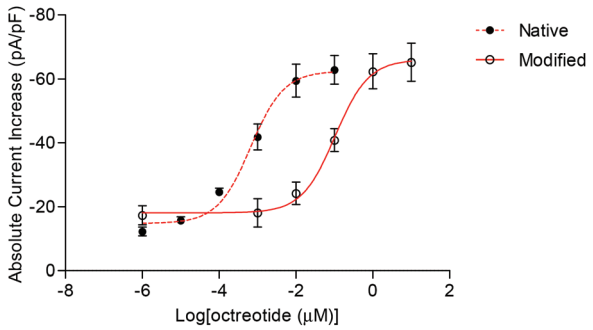
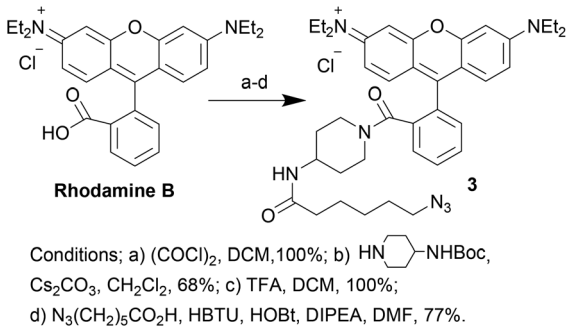


Fig. 1 Mean absolute current increase (pA/pF) with increasing concentration of modified octreotide **2** (solid line) and native octreotide (dashed line).



Scheme 3 The synthetic route to rhodamine B-azide **3**.

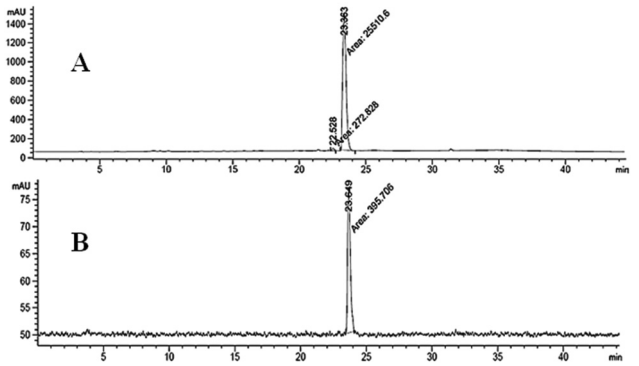
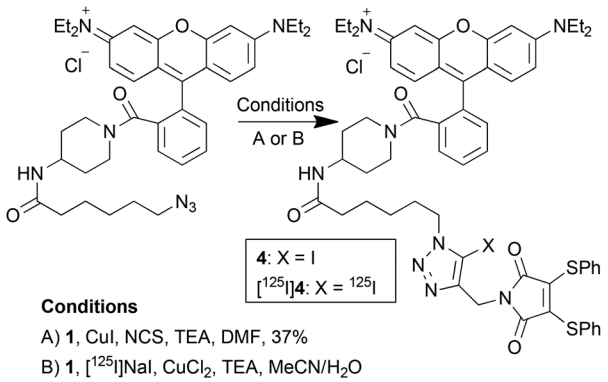


Fig. 2 (A) HPLC trace of the non-radioactive reference compound **4**; (B) radio-chromatogram of the dual optical and nuclear labelling reagent [^{125}I]**4**.

verted to the acid chloride and then reacted with 4-(*N*-Boc-amino)piperidine to form the tertiary amide.[20] Deprotection with TFA led to quantitative formation of the TFA salt, which was then coupled with 6-azidohexanoic acid[21] to afford the desired rhodamine B-azide **3** in good yield.

With the fluorescent azide and dithiophenolmaleimide alkyne in hand, focus turned to the click cycloaddition reaction. Yan *et al.* recently reported on a novel route to incorporation of the radiolabel ^{125}I into the triazole product of a click cycloaddition in a one-pot, three-component, copper(II)-mediated reaction.[22] We envisaged employing this reaction in a highly efficient synthesis affording a NGM bearing both a fluorophore and a radiolabel. To enable structural characterisation the non-radioactive iodinated compound was first synthesised. Maleimide **1**, azide **3**, triethylamine, and *N*-chlorosuccinimide were added to a solution of copper(I) iodide and stirred in an overnight reaction to form the triazole **4** (Scheme 4).[23] With this reference reagent in hand, work progressed on to the synthesis of the radioactive compound. In this case, maleimide **1**, copper(II) chloride and triethylamine were combined prior to the addition of [^{125}I]NaI and azide **3** to form radioactive reagent [^{125}I]**4** (Scheme 4). The structural identity of the radiolabelled product was confirmed by comparison of the radio-chromatogram with the UV trace from the non-radioactive reference compound (Fig. 2). The analytical



Scheme 4 The synthesis of reagents **4** and [^{125}I]**4**.

radiochemical yield was determined to be 58%, while the isolated radiochemical yield was 47%.

To test whether this multi-modal maleimide was efficient in the re-bridging of the disulfide of octreotide, it was trialled in the method previously employed; octreotide was first reduced with 1.2 equivalents of TCEP before addition of 1.2 equivalents of maleimide **4**. Efficient conversion to octreotide-MOMIA was observed, with full conversion reached within 15 min (see ESI Fig. 3†). With all components in place, the octreotide-MOMIA was tested for its efficacy as an imaging agent for somatostatin receptor-positive tumours. To examine whether the modified octreotide still bound to the somatostatin receptor and whether the attached rhodamine moiety maintained its fluorescence, confocal microscopy experiments were conducted. HEK293 cells were transiently transfected with SSTR2 and enhanced green fluorescent protein (eGFP) as a transfection marker. The cells were incubated with the octreotide-MOMIA (1 μM) for 20 min before its removal. The cells were washed and then visualised at two wavelengths: 488 nm to visualise eGFP and confirm SSTR2 expression and 543 nm to visualise rhodamine fluorescence (Fig. 3) and to confirm octreotide-MOMIA binding. Non-transfected HEK293 cells were included as a negative control.

Good fluorescence correlation was observed; octreotide-MOMIA bound to cells expressing the SSTR2. Octreotide has maintained its ability to bind to SSTR2 despite chemical modification. Control cells with no SSTR2 expression revealed no non-specific binding of octreotide-MOMIA. A high concentration of rhodamine fluorescence was observed on the cell membrane while intracellular fluorescence was less concentrated.

The confocal imaging experiments were repeated on a second model system with endogenous expression of the SSTR2. Human pancreatic BON-1 cells derived from a neuroendocrine tumour are reported to display considerable SSTR2 expression and served as an ideal cell line to test the efficacy of octreotide-MOMIA.[24] Confocal experiments were conducted as previously described, with visualisation at 543 nm (Fig. 4).

Cell membrane localisation of octreotide-MOMIA was observed, confirming SSTR2 binding of the peptide. This

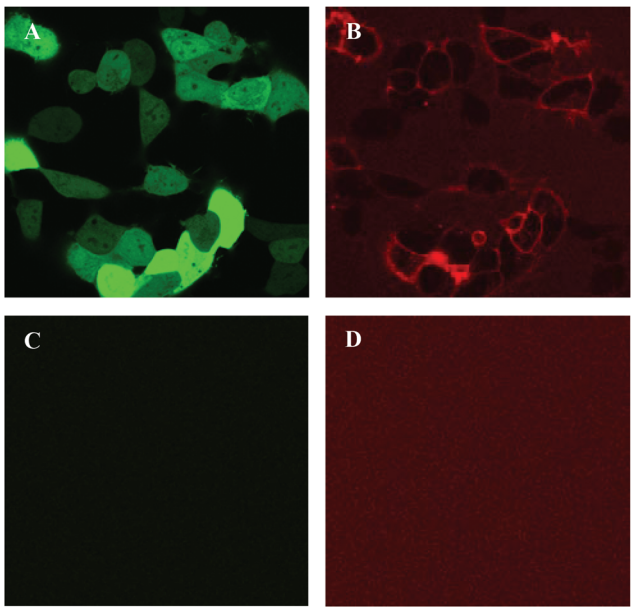


Fig. 3 Confocal images of HEK293 cells after incubation with octreotide-MOMIA (1 μM) for 20 min at 37 °C prior to washing. (A) Cells transfected with SSTR2 and eGFP and visualised at 488 nm. (B) Cells transfected with SSTR2 and eGFP and visualised at 543 nm. (C) Non-transfected cells visualised at 488 nm. (D) Non-transfected cells visualised at 543 nm.

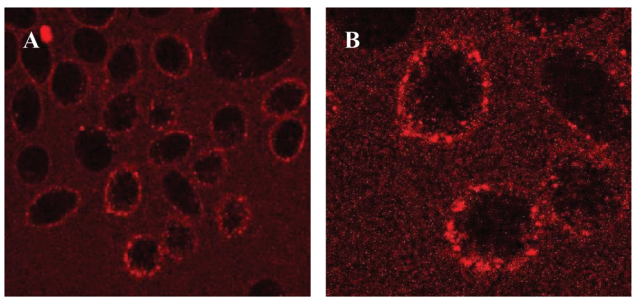


Fig. 4 (A) Confocal image of BON-1 cells visualised at 543 nm after incubation with octreotide-MOMIA (1 μM) for 20 min at 37 °C prior to washing. (B) A zoomed image of a group of cells from image (A).

demonstrates that octreotide-MOMIA allows effective fluorescence imaging of somatostatin receptor-positive cells, common in neuroendocrine and some forms of breast cancer.

Conclusions

In conclusion we have developed a concise synthetic route to a multi-modal disulfide bridging reagent. This approach enables direct incorporation of radioiodine concomitantly with a fluorophore into a peptide by the functional re-bridging of a reduced, native cystine. Such MOMIAs offer opportunities in the next generation of diagnostics, helping to improve the accuracy of the staging and localisation of cancers. The reagent described could also be readily applied to other receptor-specific peptides and proteins, to access a range of dual modality imaging agents. Furthermore, this strategy represents a general approach to the efficient construction of dual-functionalised protein conjugates. For example the use of a drug in the place of the fluorophore would enable efficient access to protein–drug conjugates detectable by nuclear imaging techniques, with prospective application in theranostics.

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