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Protein Labelling and Albumin Binding Characteristics Of The Near-IR Cy7 Fluorophore, QuatCy

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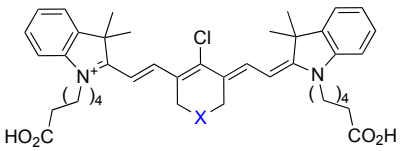
Free cysteine residues react with QuatCy 1, by simply mixing the protein and dye in aqueous buffer at 37 °C. Another dye, MHI-148, can be used for a similar labelling protocol, but QuatCy reacts faster with all proteins studied, except albumin; it emerges here that this is because MHI-148 instantly forms of a non-covalent complex with albumin, but QuatCy does not. Labelling with QuatCy has advantages insofar as it is over five times brighter, and much more photostable, than MHI-148, and combination labelling with this dye pair will allow multiplexing in the near-IR region.

Fluorophores that can conveniently label proteins are valuable if they react with high selectivity between amino acids, and if the attachment procedures can be performed by researchers with no organic chemistry experience or equipment for activation and coupling reactions. If the labelling agents are also *near-IR* fluorescent dyes, their applications can be expanded to *in vivo* and clinical imaging, since light of >750 nm wavelength penetrates tissue most effectively.[1]



A: X = CH_2, MHI-148
1: X = $N^+(CH_3)_2$, QuatCy

Recently our group proved the heptamethine cyanine (Cy7) dye MHI-148 **A** can selectively label proteins with free cysteine side chains,[2] simply by mixing the fluorophore with the protein in aqueous buffer at 37 °C.[3] Under these conditions, only Cys-thiol groups react with **A**, even when free amine, alcohol, and phenol functionalities are exposed on the protein surface. Displacement of *meso*-chloride functionality for dyes like **A** may also be related to their "tumour seeking" properties.

Meso-chloride functionalized Cy7 dyes like **A**, accumulate in solid tumours *in vivo*, persist there for several days, but do not tend to localize in normal tissue.[4, 5] We hypothesized that displacement of the *meso*-Cl on these dyes is related to their tumour seeking properties, and studied the stability of **A** in aqueous environments containing high concentrations of albumin. Albumins have one free cysteine side chain (Cys34 in human serum albumin, HSA), and it transpired that they forms covalent adducts with **A** in aqueous media at 37 °C.[6] The covalent adduct, **A**•HSA, was imported into cancer cells in culture medium. Thus, it seems likely that persistence of the fluorophore in tissue is associated with the exceptionally long *in vivo* half-life of serum albumins. Our data in this area is similar to that obtained independently, and simultaneously, by Goncalves *et al*..[7]

Recently we reported an analog of **A** with a centrally placed quaternary ammonium functionality, *ie* QuatCy **1**.[8] At the onset of the current work we predicted **1** would react with nucleophiles faster than **A** due to inductive enhancement of electrophilicity, but selectivity for *S*- over *O*- and *N*-based nucleophiles might be an issue. Consequently, this communication is about protein-labelling reactions of **1**, especially with albumin; the outcome is not as we predicted.

Two "*meso*-blocked" cyanine dyes were required as controls in this study. Derivative **2** was chosen to be illustrative of displacement of *meso*-Cl by Cys, in peptides and proteins; synthesis of this material was achieved via a nucleophilic displacement (Scheme 1a). Secondly, Suzuki coupling of phenylboronic acid was used to prepare a control compound that cannot undergo nucleophilic displacement at the *meso*-position,[9, 10] *ie meso*-phenyl **3** (Scheme 1b). The photophysical properties of these dyes are included in Table S1 and S2.

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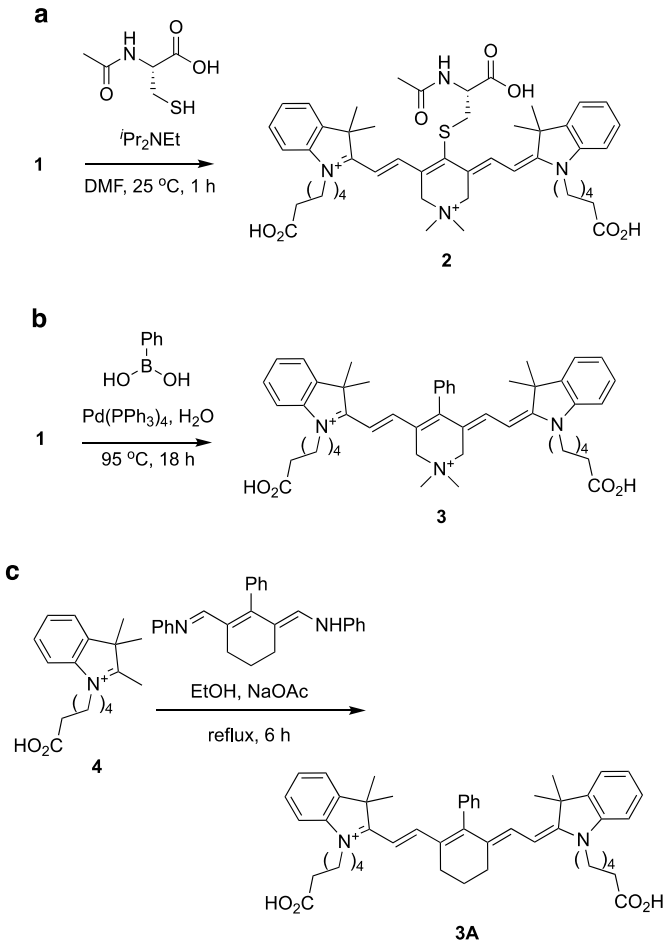
Electronic Supplementary Information (ESI) available: [Synthesis scheme, characterization (NMR, HRMS), photophysical properties, UV-Vis and fluorescence analyses, HPLC data, protein gel assays. This material is available free of charge online.]. See DOI: 10.1039/x0xx00000x



Scheme 1. Syntheses of products from: **a** nucleophilic displacement of chloride from **1** with Ac-Cys-OH. **b** Suzuki coupling of **1** with phenyl boronic acid; and, **c** condensation of phenyl Vilsmeier-Haack reagent with indole **4**.

In a competition experiment (Fig 1b), QuatCy **1** was reacted with amino acid derivatives (Fig 1a) containing most of the common nucleophiles found in peptides and proteins, *ie* representing N_α-amino groups Pro and Lys, and side-chains of Lys, Tyr, Cys. Only the displacement product **2**, from Ac-Cys-OH was detected by analytical HPLC, indicating **1** reacts only with the free thiol group of Cys. Thus, our original concern that QuatCy might be too reactive to combine selectively was unfounded.

Reaction rates for Ac-Cys-OH combined with MHI-148 **A** and with QuatCy **1** were compared. Equimolar MHI-148 **A** and QuatCy **1** were reacted with excess of Ac-Cys-OH, and the results are shown in Fig 1c (Fig S3 shows calibration of HPLC retention times). Displacement from **1** gave **2** rapidly ($t_{1/2}$ ~30 min), and nearly all the QuatCy starting material was consumed within 6 h. Conversely, MHI-148 **A** reacted relatively *slowly* under the same conditions, to give less than 20 % conversion to **2A** after 6 h.

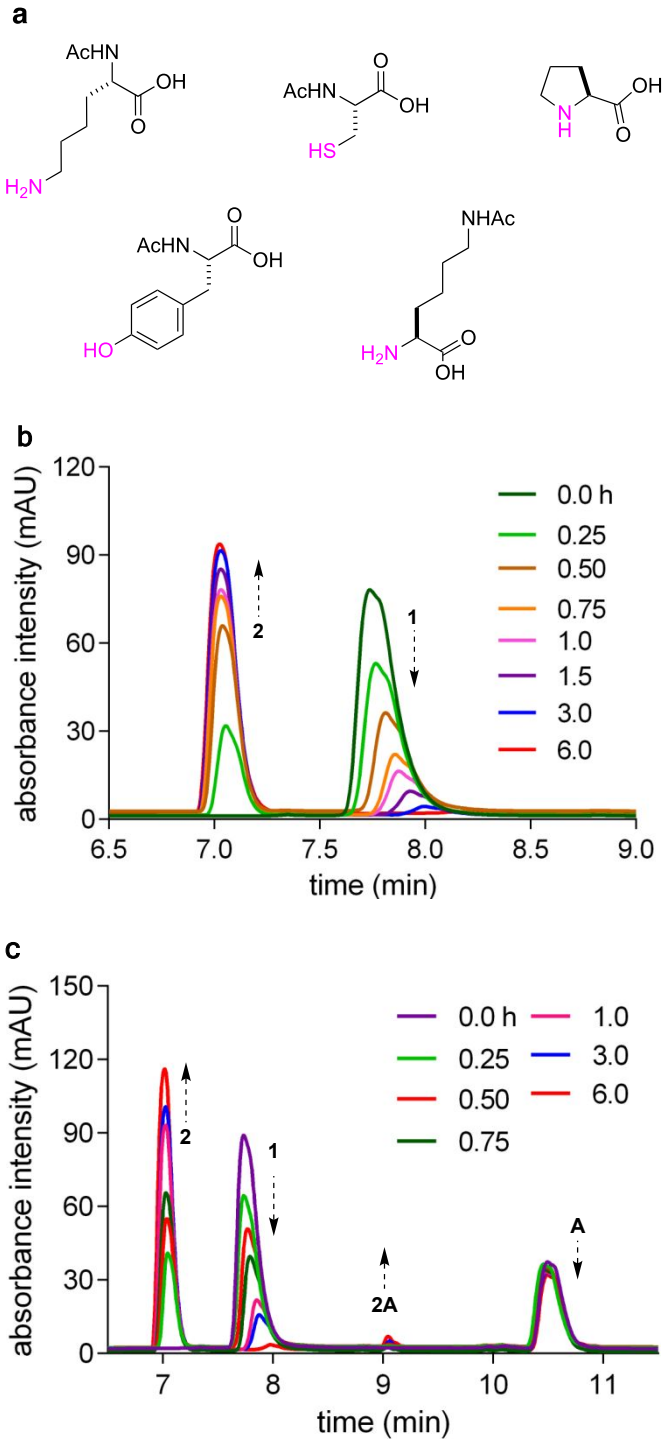


Fig 1. **a** Competitor nucleophiles: *N*-acetyl-L-Cys, *N*-acetyl-L-Tyr, N^α-acetyl-L-Lys, N^ε-acetyl-L-Lys, and L-proline. Zoomed in region of HPLC chromatograms of: **b 1** (200 µM) mixed with 300 µM of each the competitor amino acid derivatives, in 1 M HEPES buffer pH 8.0 at 37 °C (only Cys-adduct **2** formed); and, **c** 200 µM of **A** and **1** mixed with 300 µM of *N*-acetyl-L-Cys in 1 M HEPES buffer pH 8.0 incubating at 37 °C (QuatCy **1** is consumed much more quickly). The complete chromatogram is in supporting Fig S4.

Vimentin is an abundant intracellular protein involved in spindle formation with no apparent binding pockets; it comprises two intertwined, non-identical helical chains, wherein Cys328 is the only free thiol.[11] Reactions of vimentin with **A** and **1** were monitored by gel electrophoresis and visualized by excitation in the near infra

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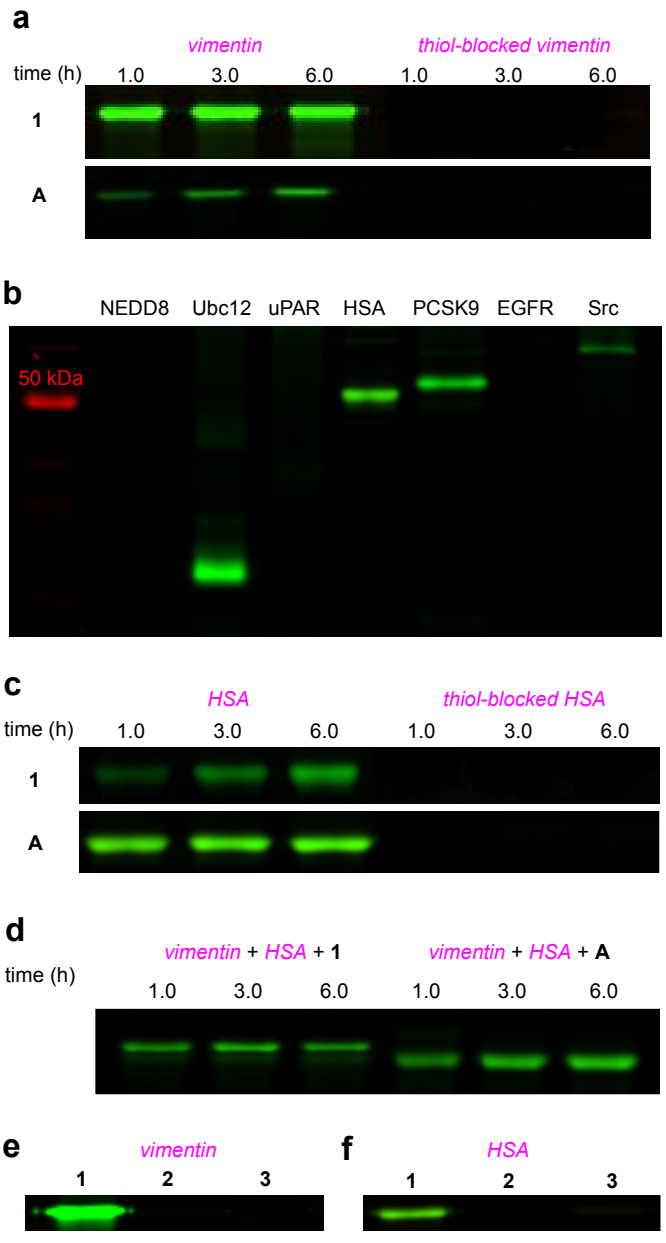
red-region (Odyssey® CLx LI-COR). Peak intensities in these gels are not indicative of concentrations because of the usual problems encountered when matching excitation lasers and observation filters. However, the data collected (Fig 2a) shows product from **A** forms slowly over 6 h, whereas the analogous vimentin adduct from **1** appears to reach maximum within 1 h. Pre-treatment of vimentin with excess 6-maleimidohexanoic acid to cap the free thiol gave a blocked vimentin that did not combine with compound **1**. Thus, vimentin reacted with **1** *faster* than **A**, and the reaction occurred exclusively on the Cys.



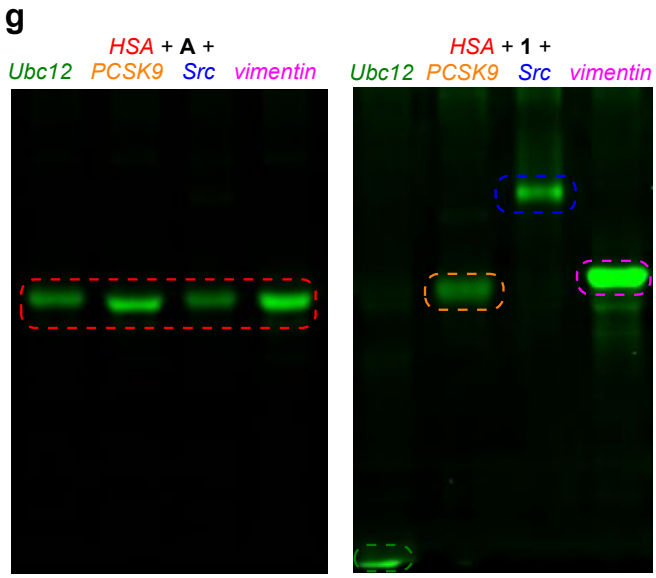


Fig 2. NIR- gel images for equimolar **1** or **A** incubated with: **a** vimentin and thiol-blocked vimentin; **b** dye **1** reacted with proteins having free thiols (Ubc12, HSA, PCSK9, Src), and not combining with ones without (NEDD8, uPAR, EGFR); **c** HSA and thiol-blocked HSA at 1, 3 and 6 h; **d** vimentin and HSA (both 4 μM). Incubation of **1**, **2**, and **3** with: **e** vimentin; **f** HSA for 6 h. **g** Thiol-presenting proteins (HSA, Ubc12, PCSK9, Src and vimentin; 4 μM each) were incubated with 1 μM **A** (left) or **1** (right) at 1.5 h. Red dotted box indicates albumin adducts, green is Ubc12m blue is Src, and pink is vimentin. HEPES buffer, pH 8.0 37 °C, throughout.

QuatCy **1** was reacted with proteins to test generality of our labelling procedure (Fig 2b). Four proteins having free Cys-*SH* (Ubc12, HSA, PCSK9, Src) and three without (NEDD8, uPAR, EGFR) were incubated with **1** in 50 mM HEPES for 6 h at 37 °C. Labelling was observed only for the four proteins that contain free thiols. Stability of vimentin labelled with **1** (10 μM) in the presence of glutathione (0.5 mM; 37 °C, PBS pH 7.4; by HPLC; Fig S7) was explored. Approximately 80 % of the dye labelled protein was present after 12 h. Consequently, the *meso-S* bond is not impervious to intracellular thiol nucleophiles, but is stable enough for short-term microscopy experiments.

Experiments similar to those described above were undertaken to investigate reactions of **1** and human serum albumin (HSA); based on the data above, it was expected that **1** would react *faster* than **A**, but, it reacted *slower* (Fig 2c). To check this surprising result (Fig 2d), equimolar solutions (4 μM) vimentin and HSA were incubated with: (i) **1**; and, (ii) **A**. Only QuatCy **1** reacted with vimentin under these conditions, while **A** combined solely (to within the limits of detection, throughout) with HSA; thus **A** and **1** have juxtaposed relative reaction rates with vimentin and HSA.

Experiments were performed to check covalent adducts correlated with *meso*-Cl on the fluorophores. Fig 2e shows data for vimentin incubated with QuatCy **1**, Ac-Cys-QuatCy **2**, and Ph-QuatCy **3**; only the dye with a *meso*-Cl reacted, *ie* **1**. Similarly, only **1** reacted with HSA (Fig 2f) in a similar set of experiments (but much slower than **A**).

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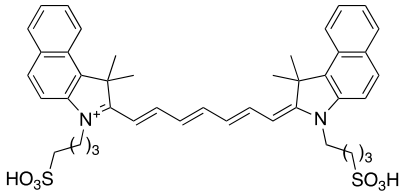
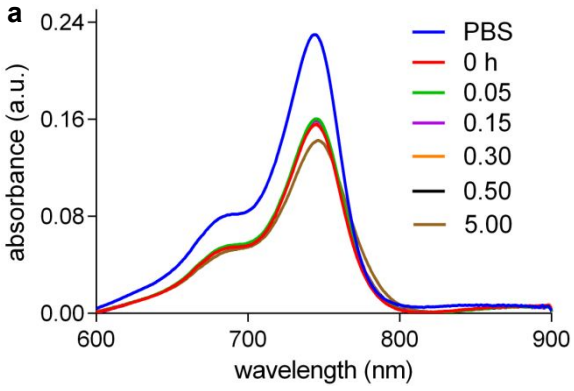
The following experiment was performed to check the selectivity of albumin for **A** relative to the other proteins that tend to react with **1** first. Thus, four-fold excesses of a mixture of five Cys-thiol presenting proteins (HSA, Ubc12, PCSK9, Src and vimentin) were reacted with **A** (Fig 2g, left) and **1** (right). MHI-148 **A** preferentially labelled HSA whereas **1** preferentially labelled the other proteins demonstrating that the reaction of QuatCy with albumin is relatively slow, but relatively fast with the four other Cys-presenting proteins.

Reactions of MHI-148 and QuatCy with HSA were explored further by UV spectroscopy. Absorbance maxima for QuatCy **1** and *meso*-Ph-QuatCy **3** were *not* red-shifted by HSA (Fig 3a and b, respectively), indicating **1** does not enter into non-covalent interactions with HSA. The alternative, non-covalent interactions without shifting the UV maxima, is unlikely because it would have to apply to both QuatCy **1** *and meso*-Ph-QuatCy **3**. The decrease in absorbance of QuatCy is directly related concentration of HSA in the solution, presumably via an environment effect. Indocyanine green (ICG **B**) and HSA are known to give a red-shift due to non-covalent interactions,[12] underlining the assertion that non-covalent interactions of Cy7 dyes with albumin give red-shifted fluorescence.



indocyanine green (ICG), **B**

Assertions regarding lack of non-covalent albumin interactions for **1** and **3** are further supported by the behaviour of **A** in a similar experiment (Fig 3c). The absorbance maximum of MHI-148 **A** is red-shifted to ~805 nm immediately upon addition of HSA, presumably due to non-covalent complex formation. However, after 5 h the absorption maximum moves to ~791 nm corresponding to the covalent adduct; a similar shift was *not* observed for the *meso*-blocked compound **3A** (Fig 3d).



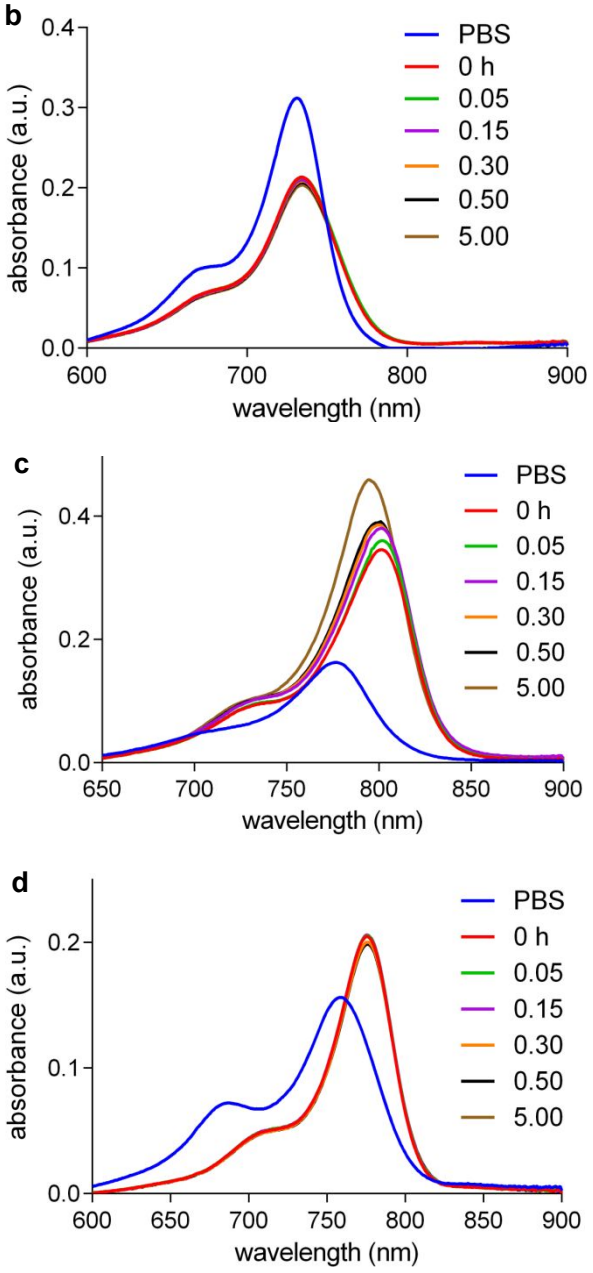


Fig 3. Absorption spectra of fluorophores incubated at 37 °C with HSA (50 μM) in 10 mM pH 7.4 phosphate buffer saline (PBS). **a** QuatCy **1**; **b** the *meso*-Ph substituted control for QuatCy, **3**; **c** MHI-148 **A**; and, **d** the *meso*-blocked control for MHI-148, **3A**.

Protein labelling with QuatCy **1** differs to that using MHI-148 **A**. That complementarity may be advantageous to potential users for at least two reasons. First, the absorption and emission spectra of the two dyes are sufficiently different to allow multiplexing (abs/em in aqueous PBS buffer: **1**, 745/768; **A**, 779/807 nm; full peak width at half height is 50 nm for **1** and 70 for **A**; Table S1 and S2). Secondly, **1** is more than fivefold brighter and significantly more photostable than **A** in PBS buffer.[8] QuatCy **1** tends to react faster than **A** with most proteins other than albumin. Labelling procedures with both **1** and **A** involve simply mixing the fluorophores and the protein in aqueous buffer at 37 °C without any activation procedures.

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References

1. J. V. Frangioni, *Curr. Opin. Chem. Biol.*, 2003, **7**, 626-634.
2. C.-M. Lin, S. M. Usama and K. Burgess, *Molecules*, 2018, **23**, 2900.
3. S. M. Usama, C.-M. Lin and K. Burgess, *Bioconjugate Chem.*, 2018, **29**, 3886-3895.
4. X. Yang, C. Shi, R. Tong, W. Qian, H. E. Zhau, R. Wang, G. Zhu, J. Cheng, V. W. Yang, T. Cheng, M. Henary, L. Strekowski and L. W. K. Chung, *Clin. Cancer Res.*, 2010, **16**, 2833-2844.
5. C. Zhang, T. Liu, Y. Su, S. Luo, Y. Zhu, X. Tan, S. Fan, L. Zhang, Y. Zhou, T. Cheng and C. Shi, *Biomaterials*, 2010, **31**, 6612-6617.
6. Z. Wang, B. Z. Olenyuk, J. C. Shih and J. Wang, *J. Pharm. Anal.*, 2018, **8**, 153-159.
7. C. Canovas, P.-S. Bellaye, M. Moreau, A. Romieu, F. Denat and V. Goncalves, *Org. Biomol. Chem.*, 2018, **16**, 8831-8836.
8. S. Thavornpradit, S. M. Usama, J. Shrestha, G. K. Park, H. S. Choi and K. Burgess, *Theranostics*, 2019, **9**, 2856-2867.
9. H. Lee, J. C. Mason and S. Achilefu, *J. Org. Chem.*, 2006, **71**, 7862-7865.
10. D. Su, C. L. Teoh, A. Samanta, N.-Y. Kang, S.-J. Park and Y.-T. Chang, *Chem. Commun.*, 2015, **51**, 3989-3992.
11. D. Perez-Sala, C. L. Oeste, A. E. Martinez, M. J. Carrasco, B. Garzon and F. J. Canada, *Nat. Commun.*, 2015, **6**, 7287.
12. X. Li, Y. Fu, L. Ma, Z. Wang and H. Zhang, *Chem. Res. Chin. Univ.*, 2016, **32**, 343-347.

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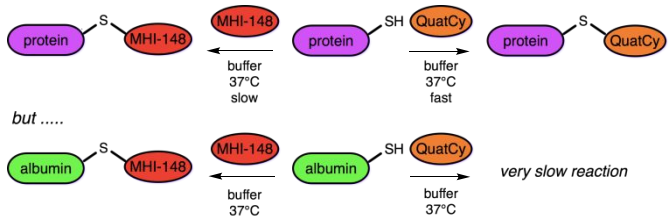

"QuatCy" selectively labels Cys side-chains, and proteins with exposed Cys residues over albumin.

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