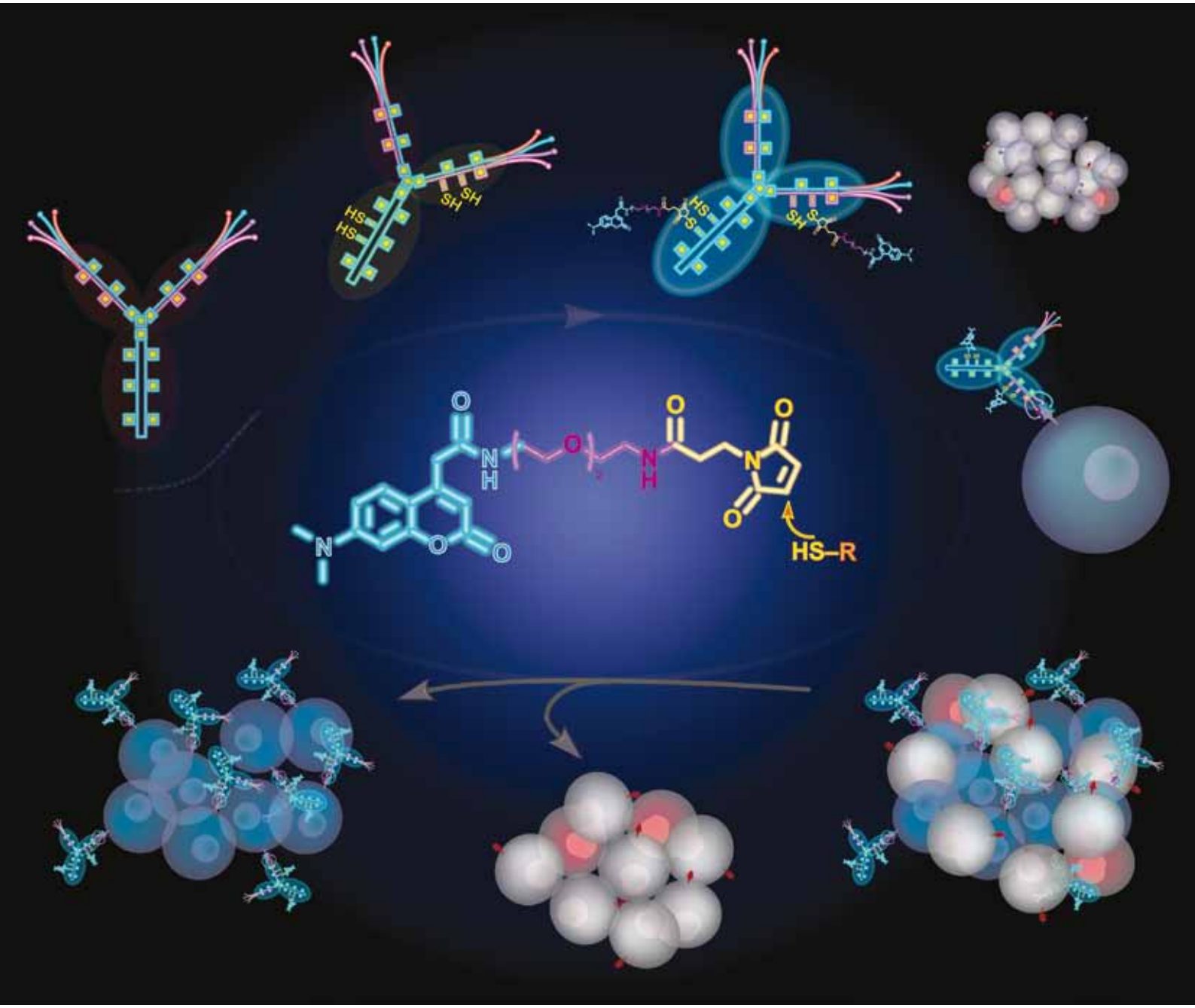
Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 7 | Number 17 | 7 September 2009 | Pages 3345–3612



ISSN 1477-0520

RSC Publishing

FULL PAPER
Hong Y. Song *et al.*
Practical synthesis of maleimides and coumarin-linked probes for protein and antibody labelling *via* reduction of native disulfides

PERSPECTIVE
Murat Sunbul and Jun Yin
Site specific protein labeling by enzymatic posttranslational modification

Practical synthesis of maleimides and coumarin-linked probes for protein and antibody labelling *via* reduction of native disulfides†

Hong Y. Song,[a,b] Mun H. Ngai,[a] Zhen Y. Song,[c] Paul A. MacAry,[c] Jonathan Hobley[b] and Martin J. Lear*[a]

Received 26th February 2009, Accepted 9th June 2009
First published as an Advance Article on the web 3rd July 2009
DOI: 10.1039/b904060a

The cellular tracking, detection and sensing of protein or antibody movement are important aspects to advance our understanding of biomolecular interactions and activity. Antibodies modified with fluorescent dyes are also valuable tools, especially in immunology research. We describe here a proof-of-principle study of a new water-soluble coumarin probe with a maleimide thiol-reacting unit to fluorescently tag biomolecules. Highlights include: (1) a convenient water-based preparation of *N*-substituted maleimides, (2) a one-pot preparation of activated maleimido-esters, and (3) a bio-conjugation protocol for the selenol-promoted reduction of native disulfide bonds and the 'site-specific' labelling of antibodies with no significant loss of activity.

Introduction

Due to the rapid development of fluorescent probes and digital fluorescence microscopy, the design of new and versatile fluorescent water-soluble molecular probes is of high demand in the biotechnological, environmental, and biomedical disciplines, especially for studying the dynamic movement and interactions of proteins.[1] In contemporary biomolecular research, 'site-specific' protein labelling plays an important role as a method to manipulate or visualize individual proteins. Tagged proteins are typically generated to study protein function, movement, isolation, interaction, and cellular localization to their targeted biomolecular entities.[2] The green fluorescent protein (GFP), for example, has been used most prominently as a marker or tag to observe the localization and fate of endogenous proteins in cells. Its large size (238 amino acids) and environmental dependability can, however, limit its use, especially for laboratories lacking the necessary biochemical machinery or techniques.[3] Therefore, new small-molecule fluorescent probes, which can be conveniently, covalently and chemoselectively attached to proteins, that combine all priorities of minimal invasiveness, wide photophysical varieties, 'site-specificity', ease-of-use and no dissociation concerns,[4] are still highly desirable.

Indeed, the chemically-controlled or 'specific' labelling of a particular biomolecule within a complex biological environment without disrupting proper functioning is an ongoing challenge. In 2001, Sharpless and co-workers identified a number of heterocycle-forming reactions called 'click chemistry', which can respond to this challenge.[5] Amongst these reactions, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), also known as Huisgen 1,3-dipolar cycloaddition, has become a prime example that is widely used in bio-conjugation work.[6] Despite these virtues, the 'site-specific' pre-installation of azido or terminal alkynyl functionality into biomolecules is still a challenge, as well as overcoming the potential cytotoxicity of copper-catalysts. A noteworthy advance by the Bertozzi group is the development of copper-free 'click chemistry' using a highly strained and electron-deficient difluorinated cyclooctyne (DIFO), although the 10-step synthesis or current commercial unavailability may limit its wide use.[7]

In our study towards molecular imaging and ligand targeting,[8] we chose to focus on a direct (one-step) functionalization of a biomolecule, which can be performed under physiological conditions without the requirement of metal promoters. To this end, we selected to advance cysteine–maleimide coupling as a labelling stratagem. We thus report here practical methods to make *N*-substituted maleimides and illustrate the use of a new coumarin-based fluorescent probe that was bioconjugated to an antibody #130 (specific for the human leukocyte antigen-A2, HLA-A2)[9] *via* sulfhydryl groups (cysteine) generated by the selenol-promoted reduction of native disulfide bonds. These methods are chemoselective, robust and rapid, and provide a practical entry into the 'site-specific' labelling of thiol-bearing biomolecules.

Design-wise, a fluorescent probe typically includes a fluorophore, a linker, and a reacting unit that can conjugate chemoselectively to a particular functionality on a biomolecule. Our choice of 7-dimethylaminocoumarin-4-acetic acid[10] as the fluorophore stemmed from several favourable characteristics: it possesses desirable photophysical properties, such as a large Stokes shift and visible excitation and emission wavelengths ($\lambda_{ex} = 370$ nm, $\varepsilon = 22\,000$ M^{-1} cm^{-1}, $\lambda_{em} = 459$ nm, $\Phi = 0.1$–0.4),[11] lacks biological activity[8], is small[12] and is water soluble;[13] it does not localize within cells and can be readily removed by media washing or dialysis. By further possessing immunoaffinity-fluorescent (IAF) properties, one additional facet of this fluorophore is that it allows biomolecule (typically protein) identification and isolation by co-immunoprecipitation with its own specific antibody partner.[8]

[a]*Department of Chemistry, and Medicinal Chemistry Program of the Life Sciences Institute, National University of Singapore, 3 Science Drive 3, Singapore 117543. E-mail: chmlmj@nus.edu.sg; Fax: +65-67791691; Tel: +65-65163998*
[b]*Institute of Materials Research and Engineering, A*STAR, 3 Research Link, Singapore 117602*
[c]*Immunology Programme, Centre for Life Sciences, National University of Singapore, 28 Medical Drive, Singapore 117456*

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C spectra for all compounds. See DOI: 10.1039/b904060a

While a short polyethylene glycol (PEG) chain functions as both a linker and a hydrophilic group, the maleimide functionality was chosen as a thiol reacting unit over iodoacetamides, since it requires smaller molar quantities of reagent, less reaction time and is more effective at alkylating thiols at physiological pHs.[14]

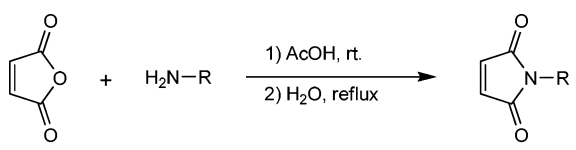
Results and discussion

Practical synthesis of *N*-alkylated maleimides and maleimidocarboxylic NHS-esters

Maleimide-incorporated probes are used extensively in biology and chemistry. Commercially available maleimide derivatives are not cheap (Sigma-Aldrich: **2b** S$600/g; **2c** S$1155/g; **3a** S$223/25mg), while the syntheses reported have shortcomings, such as harsh conditions, the use of large amounts of toxic reagents, and low yields.[15] In our study, we found that *N*-substituted maleimides and their corresponding *N*-hydroxy succinimide (NHS) esters could be readily prepared from maleic anhydride (S$ 49/Kg) in high yield and in a practical fashion. Table 1 summarizes our results to synthesize maleimide derivatives. Typically, maleic anhydride **1** was mixed with an α, β, γ or related amino acid in acetic acid and the mixture was stirred at room temperature for 2 to 8 hours until a mono-coupled amide intermediate precipitated out. Filtration, followed by dissolution at reflux in water, then gave the maleimides **2** as a precipitate in good yields (68% to 87%).

Due to a need to make activated ester versions of **2**, we next developed a one-pot method to sequentially couple maleic anhydride, an amino acid and *N*-hydroxylsuccinamide to generate **3** (Table 2). Here, maleic anhydride and the amino acid were reacted in DMF at room temperature for 2 hours, cooled to 0 °C, DCC and NHS were added, and the mixture was then allowed to warm to room temperature and was stirred overnight. The solid was filtered off, and the filtrate was poured onto ice, producing a white to pale yellow solid. The solid was collected and dried to obtain the desired NHS-ester **3** in good to excellent yield (61% to 90%). Alternatively, after pouring onto ice, the solid could be collected by dissolving in CH_2Cl_2, washed and extracted, dried over Na_2SO_4 and concentrated to provide **3**.

Table 1 Convenient synthesis of *N*-alkylated maleimides[a]

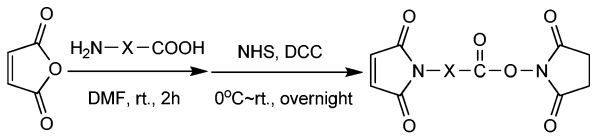


1 2

Entry	-R	Product	Yield (%)[b]
1	-CH_2COOH	**2a**	83
2	-CH_2CH_2COOH	**2b**	87
3	-$CH_2CH_2CH_2COOH$	**2c**	85
4	-$CH_2CONHCH_2COOH$	**2d**	76
5	-Cyclohexane	**2e**	68

[a] Maleic anhydride **1** (0.1 mol), amine (0.1 mol), acetic acid (100 mL). [b] Isolated yield.

Table 2 One-pot preparation of maleimido-carboxylic NHS-esters[a]

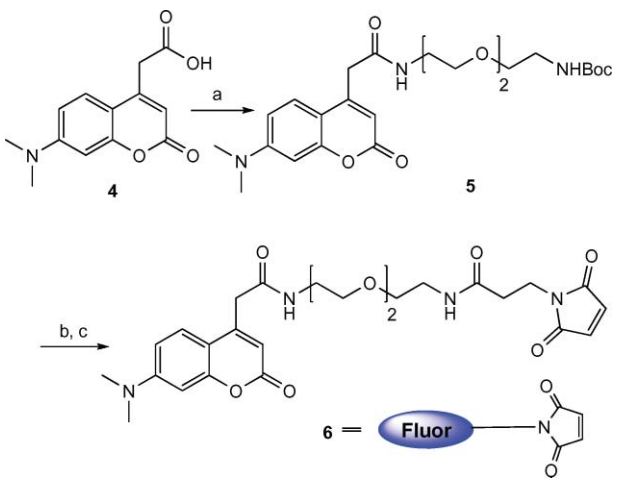


1 3

Entry	-X-	Product	Yield (%)[b]
1	-CH_2CH_2-	**3a**	61
2	-$CH_2CH_2CH_2$-	**3b**	73
3[c]	-$(CH_2)_5$-	**3c**	80
4	-C_6H_4-	**3d**	90

[a] Maleic anhydride **1** (0.03 mol), H_2N-X-COOH (0.03 mol), DMF (30 mL), NHS (0.037 mol) and DCC (0.063 mol). [b] Isolated yield. [c] The product was obtained by DCM extraction.

Synthesis of coumarin probe 6. Having developed practical methods to generate activated NHS-esters **3**, we next designed and prepared a hydrophilic fluorescent probe **6** (Scheme 1). The coumarin carboxylic acid derivative **4** (an immunoaffinity-fluorescent label, IAF)[8] was prepared by the Von Pechmann reaction,[16] by mixing freshly purified *m*-dimethylaminophenol and diethyl 1,3-acetonedicarboxylate with zinc chloride in anhydrous ethanol, refluxing the mixture for 15 h, and hydrolyzing with lithium hydroxide to provide the carboxylic acid **4**. Coupling of **4** with *N*-Boc-1,8-diamino-3,6-dioxaoctane gave the coumarin-linked derivative **5**. Various conditions were attempted for the coupling of coumarin **5** with the activated ester **3a**. Optimal results were obtained by *N*-Boc removal with TFA followed by *N*-methyl morpholine (NMM) promoted coupling in DMF. This afforded the fluorescent probe **6** in 88% yield over 2 steps.



Scheme 1 Reagents, conditions, and yields: (a) *N*-Boc-1,8-diamino-3,6-dioxaoctane, EDC·HCl, DMAP, CH_2Cl_2, rt, 6 h, 79%; (b) TFA, CH_3CN, rt, 0.5 h, 100%; (c) **3a**, NMM, DMF, rt, 5 h, 88%.

The fluorescent probe **6** gave consistent excitation–emission spectra (Fig. 1, λ_{ex} 370 nm, λ_{em} 457 nm), which was found comparable to the free coumarin acid **4** in ethanol.[17] The fluorescence data shows that **6** is suitable for biomolecule labelling and imaging work.

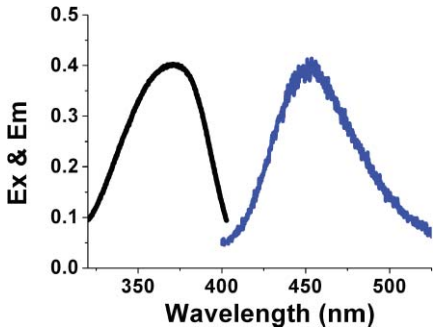
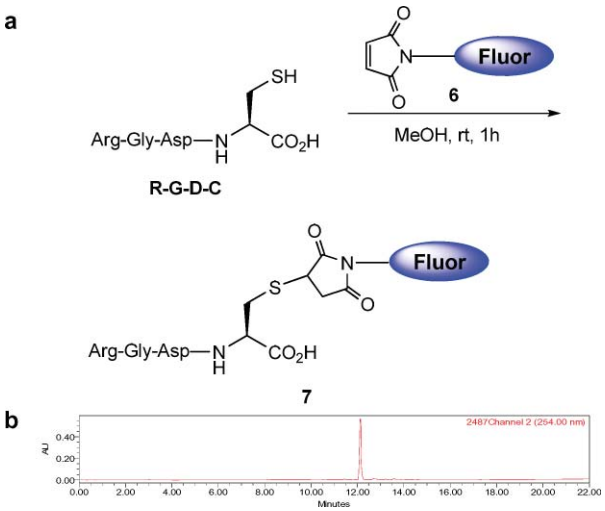


Fig. 1 Fluorescence spectrum of coumarin **6** (normalised). Excitation and emission spectra are shown by black (left) and blue (right) lines, respectively. The spectra were taken with 10 μM of **6** in ethanol.[17]

Labelling RGDC with coumarin probe 6. With the coumarin fluorescent probe **6** in hand, labelling of a bioactive small peptide bearing a cysteine sulfhydryl group was studied (Scheme 2a). To this end, the arginine-glycine-aspartic acid (Arg-Gly-Asp; RGD) ligand was selected, which has a high affinity for the integrin $\alpha_v\beta_3$ receptor that is up-regulated on tumor cell membranes and plays an important role in metastasis.[18] This peptide has also been radio-labelled for PET imaging of tumors.[19] Here, the labelling was conveniently carried out with a 1 : 1 molar ratio of coumarin probe **6** and Arg-Gly-Asp-Cys in methanol.[20] The reaction was completed quantitatively and cleanly within 1 hour. After removal of solvent *in vacuo*, the conjugate **7** was verified by LC-MS[21] and HPLC (Scheme 2b). These results illustrate the clear applicability of the coumarin reagent **6** as an efficient reagent to label biomolecules with sulfhydryl groups.



Scheme 2 Labeling of Arg-Gly-Asp-Cys with fluorophore **6**.

Antibody-labelling, cell culture and cell-sorting study

We next applied the strategy to fluorescently label an antibody (antibody #130) specific to the human leukocyte antigen A2 (HLA-A2).[22] Antibody #130 is derived from a murine monoclonal hybridoma, produced in BALB/c mice utilizing a HLA-A2 monomer as an immunogen.[23] The antibody was labelled *via* the sulfhydryl groups liberated by disulfide reduction (Fig. 2).

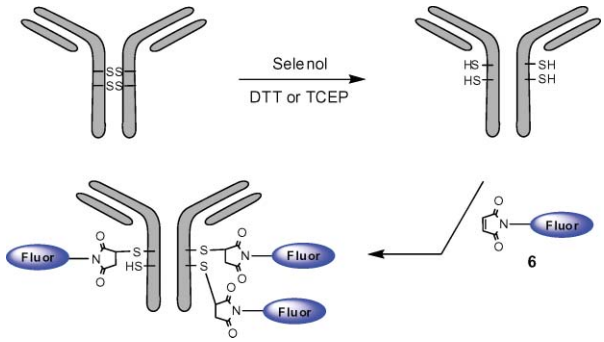


Fig. 2 Illustration of selenol-mediated reduction of antibody disulfide bonds and labelling with fluorophore **6**.

It has been reported that the use of selenol as a promoter can rapidly reduce disulfide bonds in proteins and peptides while retaining their affinity, activity and stability under non-denaturing conditions.[24] In our study, the selenol-mediated reduction of antibody #130 was carried out over 5 min under ambient and physiological-like conditions with dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Here, it was important to employ selenolcystamine as a selenol precursor. Following incubation with the coumarin probe **6** for 30 min, the reductive and bioconjugative processes were quenched with 2-mercaptoethane-sulfonate (MESNA). Dialysation overnight in a phosphate buffered saline solution then delivered the fluorescent-tagged antibody material free from unreacted label **6**.

In our study, a large excess of coumarin reagent **6** was used. The yield of coumarin labelling of the antibody was estimated spectroscopically to be ~15 : 1 (Fig 3). This was established by correlating numerically the experimental spectra of pure coumarin to both that of the pure antibody and that of the coumarin labelled antibody. From the pure spectra, a calculated coumarin labelled antibody spectrum was derived, which could be fitted to the experimental spectrum of the labelled antibody in terms of the spectral shape. Thus, by integrating within the antibody absorption region of ~280 nm, a molar ratio of coumarin to antibody of 15 : 1 was found to correlate the calculated spectrum to that found experimentally. Compared to the Ellman's assay,[25] which is generally used to evaluate the number of thiol groups generated by reduction and attachment of thiol-reactive probes, this method is simple and direct, with no additional antibody required and no need for gel-filtration. For this calculation standard extinction coefficients were used: 22 000 M^{-1} cm^{-1}

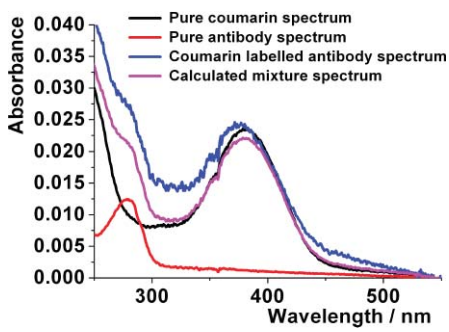


Fig. 3 UV absorption spectra of coumarin, antibody, coumarin labelled antibody and calculated coumarin labelled antibody.

(for pure coumarin[11]) and 210 000 M^{-1} cm^{-1} (for pure antibody, *cf.* Pierce Biotechnology catalogue).

Following antibody labelling with **6**, CIR A2 cells expressing HLA-A2 were incubated with the tagged antibody material for 30 min at 4 °C, and analyzed by flow cytometric analysis (Fig. 4a). The reaction sample showed significant binding to the target protein HLA-A2, as detected by flow cytometry, which showed a significant rightward-shift relative to the negative control. CIR A2 cells with bound coumarin-antibody #130 were also visualized under fluorescence microscopy under both light and dark-field modes (Fig. 4b). Evaluation of the FACS dot plot of labelled CIR A2 cells showed that 70.2% cells were labelled (Fig. 5).

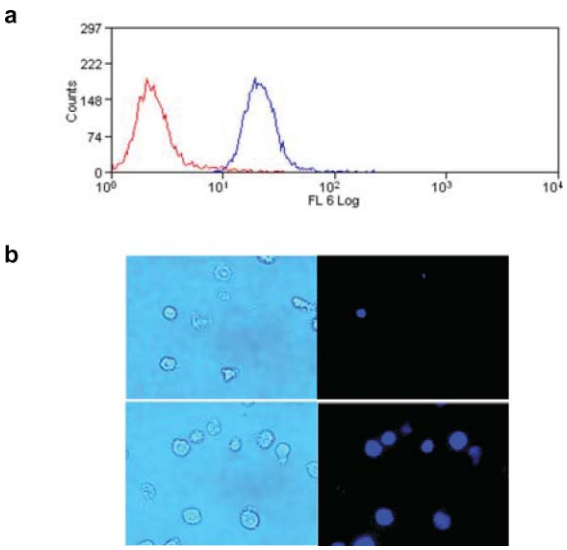


Fig. 4 (a) Flow cytometric analysis of CIR A2 cells after incubation with coumarin **6** labelled antibody #130. A significant detection of fluorescence was observed on CIR A2 cells, which express the HLA-A2 protein (blue). CIR cells, which do not express HLA-A2, were used as a negative control (red). (b) Fluorescence microscopy. There was a significant detection of fluorescence on CIR A2 cells (bottom-right) compared to the negative control CIR cells (top-right).

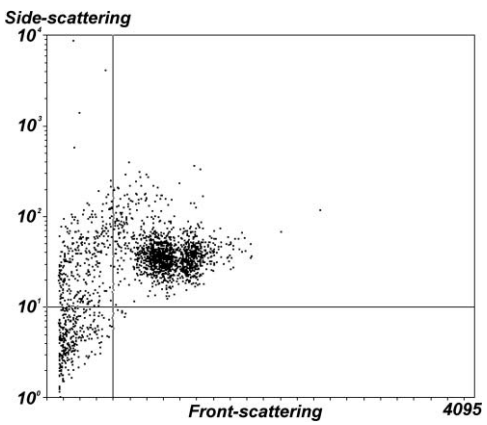


Fig. 5 FACS dot plot of labelled CIR A2 cells (top-right segment comprises 70.16% of labelled cells).

In this study, the yield of the antibody labelling reaction was high (~15 : 1 coumarin–antibody), which may in principle lead to fluorophore quenching and non-linear concentration effects during fluorescence detection at high light intensities. To mitigate photobleaching of the coumarin, lower excitation intensities are recommended. In addition, the amount of fluorophore detected indicated up to half of the disulfide linkages within the antibody were reduced, which could lead to reduced binding ability and activity. Here we found the antibody #130 to still bind well to the cell surface antigen after the reduction process. Care should, however, be given to avoid excess disulfide reduction. It should be noted that this technique is limited to cell surface antibody-labelling as it cannot efficiently label fixed cells nor selectively label cytosolic material.

Conclusions

In conclusion, we have designed, synthesized and successfully demonstrated the applicability of the optical imaging reagent **6** for bioconjugation studies. In particular, **6** can efficiently label cysteine-bearing biomolecules on peptides or proteins, even sulfhydyl groups of native disulfide bonds that were reductively liberated by selenol-reagents. Here it is important to have purified material for fluorescent labelling. For specific labelling of biological mixtures of compounds, GFP labelling and metabolic engineering techniques would be recommended.[3] As a model study, the coumarin-based IAF-labelled[8] Arg-Gly-Asp derivative **7** was successfully prepared, which may find use in monitoring the progress and activity of cancer cells. We further verified that reductive protocols[21] still allowed antibody material to retain their stability and function for additional labelling, biomolecular and cellular studies. Lastly, convenient methods were developed for the synthesis of *N*-substituted maleimides (**2**) and we anticipate our one-pot synthesis of activated maleimide NHS esters (**3**) to find wide synthetic use.

Experimental

General methods

All chemicals were purchased from the Sigma-Aldrich Chemical Co. or Alfa Aesar and were used without further purification, unless indicated otherwise. Experiments were conducted at ambient temperature, unless noted otherwise. Analytical thin layer chromatography (TLC) was performed using a Merck 60 F254 precoated silica gel plate. Subsequent to elution, plates were visualized using UV radiation (254 nm). Further visualization was possible by staining with a $KMnO_4$ water solution. Silica gel 60 (230–400 mesh ASTM) was used for flash column chromatography. Melting points were determined on a Buchi Melting point B-540 apparatus and are uncorrected. Infrared spectra were recorded on a Bio-Rad FTS 165 FTIR spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker ACF 300 or 500 MHz spectrometer. The NMR samples were prepared in a $CDCl_3$, DMSO-d_6 or MeOD-d_4 solution. Chemical shifts are reported as δ in units of parts per million (ppm), calibrated based on the different solvents used ($CDCl_3$ δ 7.26; DMSO-d_6 δ 2.50; MeOD-d_4 δ 3.31). Multiplicities are given as: s (singlet); d (doublet); t (triplet) or m (multiplets). Coupling constants (*J*) are presented in Hz. Mass spectra were recorded on a Finnigan LCQ mass spectrometer, or a Shimadzu LC-IT-TOF spectrometer. Analytical HPLC was carried out on Shimadzu LC-IT-TOF system equipped with an autosampler,

using reverse-phase Phenomenex Luna 5 μm C18(2) 100 Å 150 × 3.0 mm column. 0.1% TFA/H_2O and 0.1% TFA/acetonitrile were used as eluents. The flow rate was 0.6 ml min^{-1}. High resolution mass (HRMS) spectra were obtained using Finnigan MAT95XP GC/HRMS. Molecular biology reagents were obtained from First Base. Solutions for molecular biology were sterilized by autoclaving. Plastic-ware were sterilised by autoclaving or were purchased sterile directly from the manufacturer. Buffer solutions were made using 18 MΩ Milli-Q water (Millipore).

General experimental procedure for the synthesis of *N*-alkylated maleimides

To a solution of maleic anhydride (9.8 g, 0.1 mol) in 100 mL of glacial AcOH were added 7.5 g (0.1 mol) of glycine with vigorous stirring. The resulting slurry was stirred for 8 h under nitrogen at room temperature. The white precipitate was collected by filtration, and washed with H_2O (20 mL) twice. The white solid was dispersed in 200 mL of H_2O, and heated to reflux. The resultant clear solution was refluxed for another 30 min, then cooled down to room temperature and most H_2O removed on a rotary evaporator *in vacuo*. The white precipitate generated was collected by filtration, washed with 20 mL of H_2O, and dried under high vacuum to give 2-maleimidoacetic acid **2a**[26] in 83% yield. mp 110–111 °C (from H_2O) (lit.[27] 112–113 °C). IR ν_{max} (pellet)/cm^{-1} 3208, 1727, 1617. δ_H (300 MHz, DMSO-d_6) 3.67 (2H, s, -CH_2-), 6.08 (2H, s, -CH=CH-). δ_C (75 MHz, DMSO-d_6) 40.3, 135.4, 167.5, 169.4. MS (ESI^+): *m/z* calcd for $C_6H_5NO_4$ $[M + Na + H]^+$ 179.0, found 179.1.

3-Maleimidopropionic acid (2b)[27]. Obtained from β-alanine in 87% yield. mp 97–98 °C (from H_2O) (lit.[26,27] 92–94 °C). IR ν_{max} (pellet)/cm^{-1} 3334, 1710, 1569. δ_H (300 MHz, DMSO-d_6) 2.57 (2H, t, $J = 7$ Hz, -CH_2-), 3.00 (2H, t, $J = 7$ Hz, -CH_2-), 6.04 (2H, s, -CH=CH-). δ_C (75 MHz, DMSO-d_6) 31.8, 35.2, 136.4, 167.7, 172.3. MS (ESI^+): *m/z* calcd for $C_7H_7NO_4$ $[M + H]^+$ 170.0, found 169.9.

4-Maleimidobutyric acid (2c)[28]. Obtained from 4-aminobutyric acid in 85% yield. mp 98–99 °C (from H_2O) (lit.[26] 95–98 °C). IR ν_{max} (pellet)/cm^{-1} 3401, 1707, 1572. δ_H (300 MHz, DMSO-d_6) 1.74 (2H, m, -CH_2-), 2.33 (2H, t, $J = 7$ Hz, -CH_2-), 2.81 (2H, t, $J = 8$ Hz, -CH_2-), 6.01 (2H, s, -CH=CH-). δ_C (75 MHz, DMSO-d_6) 23.0, 30.9, 38.8, 136.6, 167.9, 174.3. MS (ESI^+): *m/z* calcd for $C_8H_9NO_4$ $[M + H]^+$ 184.2, found 184.0.

2-(2-Maleimidoacetamido)acetic acid (2d). Obtained from glycylglycine in 76% yield. mp 113–115 °C (from H_2O). IR ν_{max} (pellet)/cm^{-1} 3315, 1741, 1679, 1493. δ_H (300 MHz, DMSO-d_6) 3.61 (2H, s, -CH_2-), 3.86 (2H, d, $J = 6$ Hz, -CH_2-), 6.03 (2H, s, -CH=CH-), 8.66 (1H, t, $J = 6$ Hz, -NH-). δ_C (75 MHz, DMSO-d_6) 40.5, 41.2, 136.4, 166.7, 167.6, 171.1. MS (ESI^+): *m/z* calcd for $C_8H_8N_2O_5$ $[M + Na]^+$ 235.0, found 235.0.

Maleimidocyclohexane (2e)[29]. Obtained from aminocyclohexane in 68% yield. mp 90–91 °C (from H_2O) (lit.[29] 86–88 °C). IR ν_{max} (pellet)/cm^{-1} 2939, 1579, 1493. δ_H (300 MHz, DMSO-d_6) 1.08–1.88 (10H, m, *c*-hexyl-H), 2.96 (1H, m, *c*-hexyl-H), 6.04 (2H, s, -CH=CH-). δ_C (75 MHz, DMSO-d_6) 24.1, 24.9, 30.7, 50.0, 136.5, 167.7. MS (ESI^+): *m/z* calcd for $C_{10}H_{13}NO_2$ $[M + Na]^+$ 202.1, found 202.2.

General experimental procedure for the one-pot preparation of maleimido-carboxylic NHS-esters

β-Alanine (2.7 g, 30 mmol) was added to a solution of maleic anhydride (2.9 g, 30 mmol) in 30 mL of DMF and the mixture was stirred for 2 h. The resulting solution was cooled in an ice bath and *N*-hydroxysuccinimide (4.2 g, 37 mmol) was added followed by DCC (12.9 g, 63 mmol). After 5 min, the ice bath was removed and the solution was vigorously stirred overnight. The white precipitate (urea) formed was filtered, washed with 5 mL of DMF and the filtrate was poured onto ice. The white precipitate formed in the water was filtered, washed with 10 mL of H_2O and dried *in vacuo* to give succinimido 3-maleimido-propionoate **3a**[30] (4.5 g, 61%). The product can also be isolated in good yield by CH_2Cl_2 extraction after pouring onto ice. mp 158–159 °C (from H_2O) (lit.[30] 158–161 °C). IR ν_{max} (pellet)/cm^{-1} 1718. δ_H (500 MHz, DMSO-d_6) 2.79 (4H, s, -CH_2CH_2-), 3.04 (2H, t, $J = 7$ Hz, -CH_2-), 3.74 (2H, t, $J = 7$ Hz, -CH_2-), 7.04 (2H, s, -CH=CH-). δ_C (125 MHz, DMSO-d_6) 25.9, 29.5, 33.2, 135.1, 167.2, 170.4, 171.0. MS (ESI^+): *m/z* calcd for $C_{11}H_{10}N_2O_6$ $[M + Na]^+$ 289.0, found 289.0.

Succinimido 4-maleimidobutanoate (3b). Obtained from 4-aminobutyric acid in 73% yield. mp 120–122 °C (from H_2O) (lit.[31] 123–129 °C). IR ν_{max} (pellet)/cm^{-1} 1749, 1710. δ_H (500 MHz, DMSO-d_6) δ 1.85 (2H, quin, $J = 7.6$ Hz, -CH_2-), 2.72 (2H, t, $J = 7.6$ Hz, -CH_2CO-), 2.80 (4H, s, -CH_2-), 3.49 (2H, t, $J = 7.0$ Hz, -NCH_2), 6.99 (2H, s, -CH=CH-). δ_C (125 MHz, DMSO-d_6) δ 24.2, 26.4, 28.7, 37.1, 135.5, 169.5, 171.1, 172.0. MS (ESI^+): *m/z* calcd for $C_{12}H_{12}N_2O_6$ $[M + Na]^+$ 303.1, found 303.0.

Succinimido 4-maleimidohexanoate (3c)[32]. Obtained from 6-aminohexanoic acid in 80% yield. mp 79–80 °C (from CH_2Cl_2) (lit.[31] 70–73 °C). IR ν_{max} (thin film)/cm^{-1} 3624, 3459, 2942, 1815, 1784, 1696. δ_H (500 MHz, $CDCl_3$) δ 1.40 (2H, m, -CH_2-), 1.61 (2H, quin, $J = 7.6$ Hz, -CH_2-), 1.76 (2H, quin, $J = 7.6$ Hz, -CH_2-), 2.58 (2H, t, $J = 7.6$ Hz, -CH_2CO-), 2.81 (4H, s, -CH_2-), 3.51 (2H, t, $J = 6.9$ Hz, -NCH_2-), 6.67 (2H, s, -CH=CH-). δ_C (75 MHz, $CDCl_3$) δ 24.0, 25.5, 25.8, 28.0, 30.7, 37.4, 134.0, 168.3, 169, 170.8. MS (ESI^+): *m/z* calcd for $C_{14}H_{16}N_2O_6$ $[M + Na]^+$ 331.3, found 331.0.

Succinimido 4-maleimidobenzoate (3d)[30]. Obtained from 4-aminobenzoic acid in 90% yield. mp 195–197 °C (from H_2O) (lit.[30] 205–207 °C). IR ν_{max} (pellet)/cm^{-1} 1767, 1730. δ_H (300 MHz, DMSO-d_6) 2.91 (4H, s, -CH_2CH_2-), 7.26 (2H, s, -CH=CH-), 7.70 (2H, d, $J = 9$ Hz, Ar-H), 8.22 (2H, d, $J = 9$ Hz, Ar-H). δ_C (75 MHz, DMSO-d_6) 26.0, 123.3, 126.9, 131.3, 135.5, 138.3, 161.7, 169.8, 170.8. MS (ESI^-): *m/z* calcd for $C_{15}H_{10}N_2O_6$ $[M - H]^+$ 313.0, found 312.9.

***N*-Boc-1,8-diamino-3,6-dioxaoctane.** A solution of $(Boc)_2O$ (2.206 g, 10.1 mmol) in CH_2Cl_2 (50 mL) was slowly added to a solution of 1,8-diamino-3,6-dioxaoctane (10.7 g, 72.3 mmol) in CH_2Cl_2 (70 mL) at 0 °C. The reaction mixture was stirred from 0 °C to room temperature for 3 h. The organic layer was successively washed with water (2 × 60 mL), brine (60 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford *N*-Boc-1,8-diamino-3,6-dioxaoctane (2.01 g, 80%) as a colourless oil. IR ν_{max} (thin film)/cm^{-1} 3540, 2869, 1712. δ_H (300 MHz, $CDCl_3$) 1.43 (9H, s, *t*-Bu), 1.76 (2H, br s, NH_2), 2.68 (2H, br s, -CH_2NH_2-), 3.31 (2H, dt, $J = 5.1$, 5.1 Hz, -CH_2NHBoc), 3.53 (4H, dt, $J = 5.1$, 5.1 Hz, -OCH_2CH_2O-), 3.61 (4H, s, -OCH_2-),

5.17 (1H, br s, NH). δ_C (75 MHz, $CDCl_3$) 28.3, 40.2, 41.4, 70.02, 70.08, 73.1, 79.0, 155.9. MS (ESI^+): *m/z* calcd for $C_{11}H_{24}N_2O_4$ $[M + H]^+$ 249.2, found 249.0.

7-Dimethylaminocoumarin-4-acetic acid (4). Coumarin **4** was synthesized from *m*-dimethylaminophenol and diethyl 1,3-acetonedicarboxylate as reported,[17] but by using freshly purified starting materials. *m*-Dimethylaminophenol (12 g, 87.5 mmol), diethyl 1,3-acetonedicarboxylate (19.46 g, 96.2 mmol, 1.1 equiv.) and $ZnCl_2$ (14.31 g, 105 mmol) were dissolved in absolute ethanol (50 mL). The reaction mixture was heated at reflux for 15 h. The reaction mixture was cooled to room temperature and the yellow precipitate formed was filtered. The filtrate was poured into ice-water. The precipitate formed was filtered and air-dried. The product was recrystallized to give ethyl 7-dimethylaminocoumarin as orange needles (15.26 g, 63%). IR ν_{max} (pellet)/cm^{-1} 2981, 1721, 1600. δ_H (300 MHz, $CDCl_3$) 3.05 (1H, s, -$N(CH_3)_2$), 3.67 (2H, s, -CH_2-CO-), 4.17 (2H, q, $J = 7.1$ Hz, -OCH_2CH_3), 6.05 (1H, s, Coum-CH-), 6.52 (1H, d, $J = 2.7$ Hz, Coum-CH-), 6.62 (1H, dd, $J = 8.9$, 2.5 Hz, Coum-H), 7.40 (1H, d, $J = 8.9$ Hz, Coum-H). δ_C (75 MHz, $CDCl_3$) 14.0, 38.1, 40.0, 61.5, 98.2, 108.4, 108.9, 110.5, 125.2, 148.4, 152.8, 155.8, 161.6, 169.0. MS (FAB): *m/z* calcd for $C_{15}H_{17}NO_4$ $[M + H]^+$ 276.1, found 276.1.

Ethyl 7-dimethylaminocoumarin (10 g, 36.32 mmol) was dissolved in THF–H_2O (3 : 1) (60 mL) and cooled to 0 °C. 2 M LiOH solution (36.3 mL, 72.64 mmol, 2.0 equiv.) was added dropwise. The reaction mixture was stirred at room temperature for 0.5 h. Water (60 mL) was added, the aqueous layer was extracted with Et_2O (3 × 50 mL). The aqueous layer was acidified to pH 2 by a 2 M HCl solution. The precipitate formed was filtered and air dried to give coumarin **4** as a yellow solid (8.20 g, 91%). IR ν_{max} (pellet)/cm^{-1} 1706, 1621. δ_H (300 MHz, MeOD) 3.09 (1H, s, -$N(CH_3)_2$), 3.81 (2H, d, $J = 1$ Hz, -CH_2-CO-), 6.08 (1H, s, Coum-H), 6.58 (1H, d, $J = 3$ Hz, Coum-H), 6.79 (1H, dd, $J = 9$, 3 Hz, Coum-H), 7.55 (1H, d, $J = 9$ Hz, Coum-H). δ_C (75 MHz, DMSO-d_6) 37.2, 39.8, 97.4, 108.1, 109.1, 109.6, 126.0, 150.2, 152.8, 155.4, 160.7, 170.8. MS (FAB): *m/z* calcd for $C_{13}H_{13}NO_4$ $[M + H]^+$ 248.1, found 248.0.

Boc 2-(2-(2-(coumarin-4-acetamido)ethoxy)ethoxy)ethyl-carbamate (5). To a solution of 7-dimethylaminocoumarin-4-acetic acid **4** (766 mg, 3.1 mmol) and *N*-Boc-1,8-diamino-3,6-dioxaoctane (843 mg, 3.4 mmol) in 15 mL of anhydrous CH_2Cl_2 were added EDC·HCl (680 mg, 3.6 mmoL) and DMAP (75 mg, 0.6 mmoL) at 0 °C. The mixture was stirred from 0 °C to room temperature for 6 h. After the reaction completed (as monitored by TLC), it was diluted with 5% $KHSO_4$/ice (20 mL) and extracted with CH_2Cl_2. The combined organic layer was washed with water, 5% $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4 and concentrated in *vacuo.* The residue was purified by flash column chromatography on silica gel (hexane–ethyl acetate, 1 : 8 to 0 : 1) to give Boc-coumarin-carbamate **5** (1.12 g, 79%) as a light yellow solid. IR ν_{max} (thin film)/cm^{-1} 3348, 3275, 2931, 1732, 1720. δ_H (500 MHz, DMSO-d_6) 1.36 (9H, s, -$C(CH_3)_3$), 3.01 (6H, s, -$N(CH_3)_2$), 3.06 (2H, dt, $J = 6$ Hz, 6 Hz, -CH_2NH-), 3.23 (2H, dt, $J = 6$ Hz, 6 Hz, -CH_2NH-), 3.37 (2H, t, $J = 6$ Hz, -CH_2-), 3.42 (2H, t, $J = 6$ Hz, -CH_2-), 3.49 (4H, s, -CH_2CH_2-), 3.61 (2H, s, CH_2CO-), 6.00 (1H, s, Coum-H), 6.54 (1H, d, $J = 3$ Hz, Coum-H), 6.71 (1H, dd, $J = 3$ Hz, 9 Hz, Coum-H), 6.73 (1H, m, -NH-), 7.54 (1H, d, $J = 9$ Hz, Coum-H), 8.27 (1H, t, $J = 6$ Hz, -NH-). δ_C (125 MHz, DMSO-d_6) 28.2, 38.7, 38.8, 38.9, 40.1, 68.9, 69.1, 69.4, 69.5, 77.5, 97.4, 108.2, 108.9, 109.3, 125.9, 151.3, 152.8, 155.3, 155.5, 160.6, 167.9. HRMS (ESI^+): *m/z* calcd for $C_{24}H_{35}N_3O_7$ $[M + Na]^+$ 500.2367, found 500.2389.

***N*-(2-(2-(2-(2-(Coumarin-4-acetamido)ethoxy)ethoxy)ethyl-)-3-(maleimido)propionamide (6).** Boc-coumarin-carbamate **5** (200 mg, 0.4 mmol) was dissolved in 3 mL of CH_3CN, cooled to 0 °C and 1 mL of TFA was added. After 30 min, as monitored by TLC, no more starting material was observed, and the solvent was removed *in vacuo.* The residue was dissolved in 5 mL of DMF, followed by addition of 3-maleimidopropionoic acid **3a** (111mg, 0.42 mmol) and 25 μL of NMM under nitrogen. The mixture was stirred at room temperature for 5 h, then poured into 5 mL of ice water. The solution was acidified to pH 3~4, extracted three times with CH_2Cl_2. The combined organic layer was washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated in *vacuo.* The residue was purified by flash column chromatography on silica gel (MeOH–CH_2Cl_2, 1% to 4%) to give coumarin 3-(maleimido)propionamide **6** (186 mg, 88%) as a light yellow sticky oil. IR ν_{max} (thin film)/cm^{-1} 3321, 2924, 1712, 1659, 1647, 1612, 1531. δ_H (500 MHz, DMSO-d_6) 2.33 (2H, t, $J = 8$ Hz, -CH_2-), 3.01 (6H, s, -$N(CH_3)_2$), 3.15 (2H, m, -CH_2-), 3.23 (2H, m, -CH_2-), 3.36 (2H, m, -CH_2-), 3.42 (2H, t, $J = 6$ Hz, -CH_2-), 3.49 (4H, s, -CH_2CH_2-), 3.59 (2H, t, $J = 8$ Hz, -CH_2-), 3.61 (2H, s, -CH_2-), 5.99 (1H, s, Coum-H), 6.54 (1H, d, $J = 3$ Hz, Coum-H), 6.70 (1H, dd, $J = 3$ Hz, 9 Hz, Coum-H), 6.99 (2H, s, -CH=CH-), 7.54 (1H, d, $J = 9$ Hz, Coum-H), 8.00 (1H, t, $J = 5$ Hz, -NH-), 8.29 (1H, t, $J = 6$ Hz, -NH-). δ_C (125 MHz, DMSO-d_6) 34.4, 34.5, 38.9, 39.1, 39.2, 40.5, 69.4, 69.5, 69.9, 70.0, 97.9, 108.7, 109.4, 109.7, 126.5, 135.0, 151.8, 153.2, 155.8, 161.2, 168.4, 170.0, 171.2. HRMS (ESI^+): *m/z* calcd for $C_{26}H_{32}N_4O_8$ $[M + Na]^+$ 551.2112, found 551.2101.

Labelling Arg-Gly-Asp-Cys (RGD-C) with coumarin 6. RGD-C (5 mg, 0.01 mmol) and coumarin **6** (5.3 mg, 0.01 mmol) were dissolved in 0.75 mL of MeOD-d4. The mixture was stirred at room temperature for 1 h. ^1H NMR analysis showed that no more starting material was left, and pure product was verified by LC-MS and HRMS. MS (ESI^+): *m/z* 979.4 ($[M + 2H]^+$, 100%). HRMS (ESI^+): *m/z*: calcd for $C_{41}H_{59}N_{11}O_{15}{}^{32}S$ $[M + 2H]^+$ 979.4064, found 979.4043. HPLC showed quantitative labelling of RGD-C (*cf.* Scheme 2b).

Antibody thiol liberation by selenol/DTT disulfide reduction and fluorescent labelling using coumarin 6. The antibody #130 was generated by a hybridoma technique as previously reported.[23] To a solution of antibody #130 in PBS (250 μl, 0.34 mg/mL), PBS buffer (32 μL, 0.5 M with 1 mM EDTA, pH 7.4) and EDTA (3 μL, 0.1 M) were added, followed by selenocystamine solution (7.5 μL, 20 mM) and aqueous dithiothreitol (DTT) solution (7.5 μL, 40 mM). The mixture was stirred at room temperature for 5 min, after which the coumarin probe **6** (80 μL, 10 mM) was added. After 30 min of the labelling reaction, 2-mercaptoethanesulfonate (MESNA) (15 μl, 0.1 M) was added to quench the reaction for 1 min. The reaction was dialyzed against 1 × PBS overnight at 4 °C. Next, the reaction mixture (20 μL) was added to 1×10^6 CIR A2 cells for 30 min at 4 °C, followed by three washes in 1 × PBS before being analyzed separately *via* a CyanADP flow cytometric machine (Dako) or *via* an Axioimager Z1 fluorescence microscope

(Zeiss). The same results and observations were obtained with antibody #130 when reduced by selenocystamine/TCEP.

Acknowledgements

We thank the Singapore Bioimaging Consortium and A*STAR for funding (SBIC 005/2005 and INN-006/2006) and a postdoctoral scholarship (to H.Y.S.). We further thank the National University of Singapore for a Graduate Scholarship (to M.H.N.). This work was funded in part by the Competitive Research Program of the National Research Foundation of Singapore (NRF-G-CRP 2007–04).

Notes and references

1 T. Suzuki, S. Hisakawa, Y. Itoh, N. Suzuki, K. Takahashi, M. Kawahata, K. Yamaguchi, H. Nakagawa and N. Miyata, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 4208; R. J. Mart, K. P. Liem, X. Wang and S. J. Webb, *J. Am. Chem. Soc.*, 2006, **128**, 14462; G. Pastorin, W. Wu, S. Wieckowski, J. P. Briand, K. Kostarelos, M. Prato and A. Bianco, *Chem. Commun.*, 2006, **11**, 1182.

2 I. Chen, M. Howarth, W. Lin and A. Y. Ting, *Nat. Methods*, 2005, **2**, 99; Y. Hu, X. Huang, G. Y. Chen and S. Q. Yao, *Mol. Biotechnol.*, 2004, **28**, 63; A. Miyawaki, A. Sawano and T. Kogure, *Nat. Cell Biol.*, 2003, (Suppl.), S1 – S7.

3 G. U. Nienhaus, *Angew. Chem., Int. Ed.*, 2008, **47**, 8992; H. Mizuno, A. Sawano, P. Eli, H. Hama and A. Miyawaki, *Biochemistry*, 2001, **40**, 2502.

4 L. W. Miller, J. Sable, P. Goelet, M. P. Sheetz and V. W. Cornish, *Angew. Chem., Int. Ed.*, 2004, **43**, 1672; K. M. Marks, P. D. Braun and G. P. Nolan, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 9982.

5 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004; J. F. Lutz, *Angew. Chem., Int. Ed.*, 2007, **46**, 1018.

6 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596; J. P. Collman, N. K. Devaraj, R. A. Decréau, Y. Yang, Y. L. Yan, W. Ebina, T. A. Eberspacher and C. E. D. Chidsey, *Science*, 2007, **315**, 156.

7 N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046; J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793; J. A. Codelli, J. M. Baskin, N. J. Agard and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2008, **130**, 11486.

8 C. C. Hughes, J. B. MacMillan, S. P. Gaudêncio, W. Fenical and J. J. La Clair, *Angew. Chem., Int. Ed.*, 2009, **48**, 728; A. D. Rodríguez, M. J. Lear and J. J. La Clair, *J. Am. Chem. Soc.*, 2008, **130**, 7256; M. D. Alexander, M. D. Burkart, M. S. Leonard, P. Portonovo, B. Liang, X. Ding, M. M. Joullié, B. M. Gulledge, J. B. Aggen, A. R. Chamberlin, J. Sandler, W. Fenical, J. Cui, S. J. Gharpure, A. Polosukhin, H. R. Zhang, P. A. Evans, A. D. Richardson, M. K. Harper, C. M. Ireland, B. G. Vong, T. P. Brady, E. A. Theodorakis and J. J. La Clair, *ChemBioChem*, 2006, **7**, 409.

9 R. J. Moots, M. Matsui, L. Pazmany, A. J. McMichael and J. A. Frelinger, *Immunogenetics*, 1991, **34**, 141.

10 J. P. Goddard and J. L. Reymond, *Trends Biotechnol.*, 2004, **22**, 363; N. C. Lim and C. Brückner, *Chem. Commun.*, 2004, 1094; S. R. Trenor, A. R. Shultz, B. J. Love and T. E. Long, *Chem. Rev.*, 2004, **104**, 3059; N. C. Lim, J. V. Schuster, M. C. Porto, M. A. Tanudra, L. Yao, H. C. Freake and C. Brückner, *Inorg. Chem.*, 2005, **44**, 2018; M. S. T. Gonçalves, *Chem. Rev.*, 2009, **109**, 190.

11 W. Jarzeba, G. C. Walker, A. E. Johnson, M. A. Kahlow and P. Barbara, *J. Phys. Chem.*, 1998, **92**, 7039.

12 M. G. Fritz and D. Seebach, *Helv. Chim. Acta*, 1998, **81**, 2414.

13 A. A. Souto, A. U. Acuna, J. M. Andreu, I. Barasoain, M. Abal and F. Amat-Guerri, *Angew. Chem.*, 1995, **107**, 2910; A. A. Souto, A. U. Acuna, J. M. Andreu, I. Barasoain, M. Abal and F. Amat-Guerri, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 2710.

14 M. Galvani, M. Hamdan, B. Herbert and P. G. Righetti, *Electrophoresis*, 2001, **22**, 2058; L. K. Rogers, B. L. Leinweber and C. V. Smith, *Anal. Biochem.*, 2006, **358**, 171.

15 R. Frings and G. Grahe, 1998,EP0847991 A1; D. H. Rich, P. D. Gesellchen, A. Tong and C. K. Buckner, *J. Med. Chem.*, 1975, **18**, 1004; N. J. Ede, G. W. Tregear and J. Haralambidis, *Bioconjugate Chem.*, 1994, **5**, 373.

16 B. B. Dey, *J. Chem. Soc.*, 1915, **107**, 1606; P. Portonovo, X. Ding, M. Leonard and M. M. Joullié, *Tetrahedron*, 2000, **56**, 3687.

17 Fluorescent experiment: 10 μl of coumarin probe **6** (1 mM in EtOH) was diluted to 1 ml with EtOH, and its excitation and emission spectra were recorded using a Shimadzu RF-5301PC Spectrofluorophotometer.

18 E. Ruoslahti, *Annu. Rev. Cell. Dev. Biol.*, 1996, **12**, 697.

19 A. J. Beer, R. Haubner and M. Sarbia *et al.*, *Clin. Cancer Res.*, 2006, **12**, 3942; R. Haubner, W. A Weber and A. J. Beer *et al.*, *PLoS Med.*, 2005, **2**, e70.

20 Y. Okamura, I. Maekawa, Y. Teramura, H. Maruyama, M. Handa, Y. Ikeda and S. Takeoka, *Bioconjugate Chem.*, 2005, **16**, 1589; C. Sun, S. E. Aspland, C. Ballatore, R. Castillo, A. B. Smith and A. J. Castellino, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 104.

21 HRMS (ESI): *m/z* calcd for $C_{41}H_{61}N_{11}O_{15}{}^{32}S_1$ $[M + 2H]^+$ 979.4064, found 979.4043.

22 R. Singh and L. Kats, *Anal. Biochem.*, 1995, **232**, 86; *Techniques in Protein Chemistry*, ed. D. R. Marshak, Academic Press, San Diego 1996, pp. 221–230; R. Singh and G. M. Whitesides, *J. Org. Chem.*, 1991, **56**, 6931.

23 G. Köhler and C. Milstein, *Nature*, 1975, **256**, 495.

24 R. B. del Rosario and R. L. Wahl, *Cancer Res.*, 1990, **50**(Suppl.), 804s; D. D. Schroeder and M. L. Dumas, *Am. J. Med.*, 1984, **76(3A)**, 33; M. S. Collins and J. H. Dorsey, *Am. J. Med.*, 1984, **76**(3A), 155.

25 G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70; P. W. Riddles, R. L. Blakeley and B. Zerner, *Methods Enzymol.*, 1983, **91**, 49.

26 V. Ondrus, L. Fisera and V. Bradac, *ARKIVOC*, 2001, 60–67.

27 E. Kassianidis, R. J. Pearson and D. Philp, *Chem. Eur. J.*, 2006, **12**, 8798.

28 L. D. Lavis, T.-Y. Chao and R. T. Raines, *ACS Chem. Biol.*, 2006, **1**, 252.

29 J.-C. Hsieh and C.-H. Cheng, *Chem. Commun*, 2005, **36**, 4554.

30 M. J. Paterson and I. M. Eggleston, *Synth. Commun.*, 2008, **38**, 303.

31 F. Satoshi, P. L. Redi and T. Takeshi, *JP Pat.*, 11071347, 2007.

32 M. J. Swanson, P. I. Guire, *US Pat.*, 200302604, 2003.

