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Near IR emitting isothiocyanato-substituted fluorophores: their synthesis and bioconjugation to monoclonal antibodies

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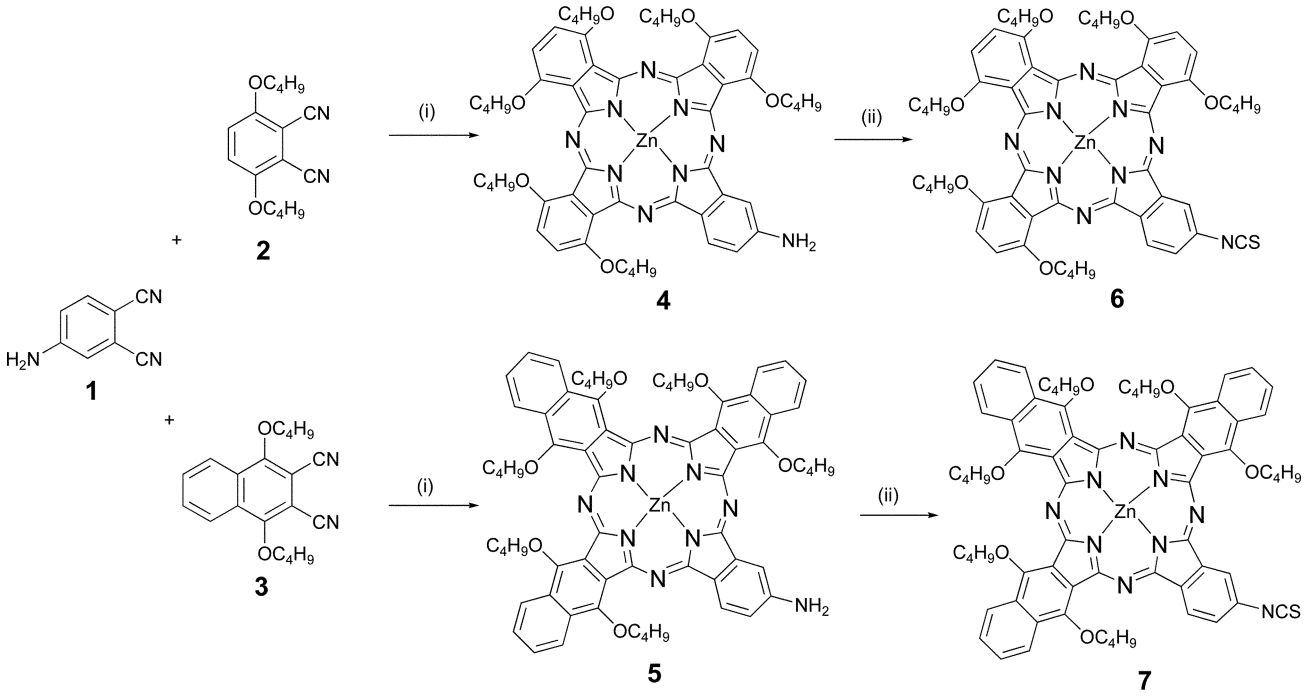
Two near IR emitting fluorophores, based on the phthalocyanine and naphthalocyanine chromophores, which also bear a single isothiocyanato group suitable for conjugation to proteins are reported; their utility as luminescent probes is demonstrated by conjugation to monoclonal antibodies and the ability of these conjugates to selectively bind cells bearing the relevant antigen.

Considerable interest is currently being shown in luminescent molecules which emit in the near IR region of the electromagnetic spectrum for a variety of analytical applications.[1] Molecules with these spectroscopic properties are especially well suited for biological imaging applications due to the optical transparency of these media at wavelengths greater than 700 nm.[2] In order to be truly useful for such applications it is necessary to have not only an imaging component which absorbs and emits at wavelengths longer than 700 nm, but also a targeting component which binds the luminescent probe selectively to the target tissue.[3] Currently only three probes are commercially available which absorb and emit in this region, Alexa Fluor™ 700, Alexa Fluor™ 750 and Cy7™, these fluorophores are available as amine reactive succinimidyl esters, however, unlike fluorescein based probes, isothiocyanates of these dyes are not available. Bioconjugations with isothiocyanato fluorophores offer the advantage of not generating any by-products, as they react with amines *via* an addition, as opposed to substitution, reaction, thus simplifying conjugate purification. Recently, we have been involved in the development of porphyrins bearing a single isothiocyanato group for conjugation to proteins, both as targeted photosensitisers for photodynamic therapy[4] and also fluorescence imaging of cellular systems.[5,6] The porphyrins however, emit in the red region of the spectrum, typically around 640 nm,[7] whereas human plasma has an intense autofluorescence band ranging from 600–650 nm,[8] leading to poor signal to background ratios.

Phthalocyanines and naphthobenzoporphyrazines have absorption and emission bands which are both bathochromically and hyperchromically shifted relative to the porphyrins, and these spectral properties can be fine tuned by peripheral substitution.[9] We now wish to report that, by judicial choice of peripheral groups, it is possible to synthesise phthalocyanines and naphthobenzoporphyrazines which absorb and emit in the 700–850 nm region, and also bear a single amine-reactive isothiocyanto group suitable for conjugating to protein based biological targeting agents.

The most convenient precursor to the isothiocyanato group is an amine and, as we have previously used this strategy with porphyrin based systems,[5,6] the readily available phthalocyanine precursor 4-amino-1,2-benzenedicarbonitrile **1** was selected to allow introduction of the amino functionality to both macrocycles. As use of this precursor would inevitably mean



Scheme 1 Reagents and conditions: (i) $ZnCl_2$, DBU, *n*-butanol, reflux 24 h (ii) $CSCl_2$, CH_2Cl_2, RT, 4 h.

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that one amino-isoindole unit would be incorporated into both phthalocyanine and naphthobenzoporphyrazine macrocycles, and contribute little to bathochromically shifting the λ_{max} relative to phthalocyanines, the bis-alkoxy substituted precursors, 3,6-dibutoxy-1,2-benzenedicarbonitrile **2** and 1,4-dibutoxy-2,3-naphthalenedicarbonitrile **3** were chosen to provide the remaining sub units. Selection of these latter precursors ensured the presence of electron donating alkoxy groups at positions on the macrocycles which have been shown to result in significant bathochromic shifts relative to the unsubstituted analogues.[9,10,11] Thus **1** and **2** and **1** and **3** respectively, were mixed in 1 : 3 molar ratios with $ZnCl_2$ and DBU in *n*-butanol and heated at reflux under argon for 24 h. After cooling and evaporation of solvent the mixtures were washed with methanol and chromatographed to give the required mono amino phthalocyanato zinc complex **4** and mono amino naphthobenzoporphyrazinato zinc complex **5** in 3.3% and 1.8% yields respectively (Scheme 1). Conversion of **4** and **5** to the corresponding isothiocyanates **6** and **7** was achieved by reaction with excess thiophosgene in dichloromethane at room temperature. Compounds **6** and **7** were characterised by ^{1}H NMR, MS, elemental analysis and UV–visible spectroscopy.†Values of λ_{max} for **6** and **7** were 729 and 796 nm, respectively, and both showed the expected fluorescence bands when excited at these wavelengths with Stokes shifts of 10–20 nm (Figs. 1 and 2). Interestingly, the compounds described here, unlike many conventional fluorophores, have multiple absorptions in the visible and infrared regions, other than the λ_{max}. This allows much larger Stokes shifts to be obtained *e.g.* excitation on the secondary bands at 660 nm for **6** and 700 nm for **7** results in Stokes shifts of approximately 100 nm for each molecule.

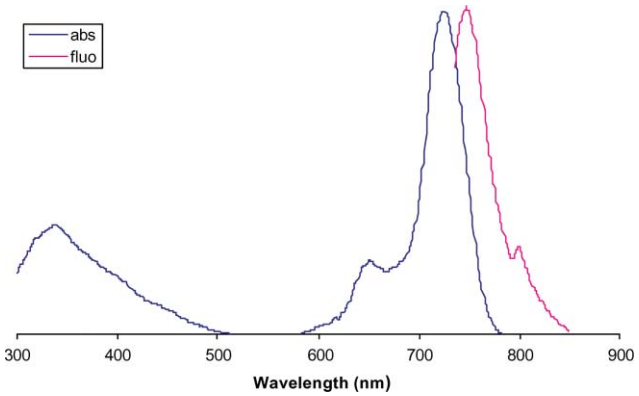

Fig. 1 Absorbance and fluorescence spectra for **6** in DMSO.

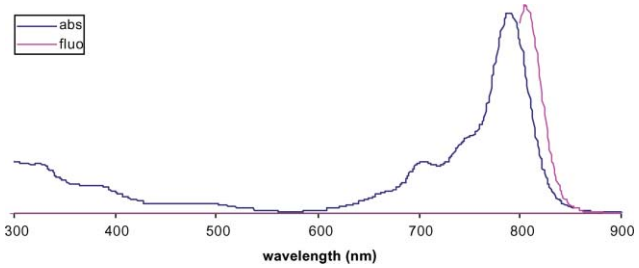

Fig. 2 Absorbance and fluorescence spectra for **7** in DMSO.

In order to demonstrate the utility of these fluorophores for use in biological imaging applications both were conjugated to three different monoclonal antibodies, anti-EpCAM, anti-CD146 and anti-CD104 (Serotec, Oxford, UK) in 0.5 M bicarbonate buffer (pH 9.2) and the conjugates were purified by gel exclusion chromatography.‡§ The degree of labelling (moles of compound per mole of antibody) was determined by UV–visible spectroscopy and was found to be 4.2 : 1, 3.9 : 1 and 13.4 : 1 for anti-EpCAM, anti-CD146 and anti-CD104 respectively with compound **6**, and 4.2 : 1, 7.6 : 1 and 6.3 : 1 for the same antibodies with compound **7**. All three antibodies bind to tumour associated antigens that are upregulated in various cancers. The colon adenocarcinoma (LoVo) and lung large cell carcinoma cell line (COR-L23) were selected to assay binding of the conjugates using flow cytometry. The COR-L23 line expresses the antigens recognised by all three antibodies whereas the LoVo cell line only expresses EpCAM and CD104; thus in addition to positive binding, non-specific binding can also be investigated. Equivalent amounts of antibody were added in every case.

Analysis of flow cytometry data¶ for conjugates of compound **6** shows excellent correlation between conjugated and non-conjugated anti-EpCAM and anti-CD104 antibodies with CORL23 and LoVo cells indicating that the presence of the macrocycle attached to the antibody has not affected binding to the respective antigens (Fig. 3). The anti-CD146 conjugate again shows virtually perfect overlap with binding of its non-conjugated analogue for the antigen positive CORL23 cells, with only a very minor level of non-specific binding to the LoVo cells being introduced by conjugation of the macrocycle. Comparison of these results with those from similar assays using the conjugates of naphthobenzoporphyrazine **7** show some differences for the anti-EpCAM and anti-CD146 antibodies on CORL23 cells, with both showing a slight loss of binding efficiency relative to the non-conjugated antibodies. The same cell line with the anti-CD104 conjugate however shows excellent binding, comparable to the unconjugated antibody. As the loading ratio for the anti-EpCAM conjugate (4.2 : 1) was lower than that for the anti-CD104 conjugate (6.3 : 1), levels of fluorophore bound to the antibody surface cannot be responsible for the slightly lower binding seen with the former conjugate. It is also very unlikely that the linkage of compound **7** itself is inhibiting antigen-binding *per se* as the conjugates all bind identically, when compared with the unconjugated antibodies, against LoVo cells. It would appear therefore that conjugation of different macrocycles can cause subtle differences in the ability of an antibody to recognise its target antigen; however such changes are relatively minor, *i.e.* the anti-EpCAM and anti-CD146 conjugates still effectively bind all cells.

The fluorescent probes described here are hydrophobic, a factor which can lead to aggregation of antibody conjugates. However, this would be expected to result in significantly increased binding to cells which do not express the corresponding antigen, and would be indicated by flow cytometry curves displaced significantly to the right of those for the corresponding non-conjugated antibody. Although his effect can be seen to a minor degree in the case of compound **6** with the CD146 antibody and LoVo cell line, it cannot be seen at all for compound **7** with the same antibody and cells. As **7** is the more hydrophobic of the two fluorophores, this physicochemical property does not seem to result in aggregation of antibody conjugates.

Finally, as both phthalocyanines[12] and naphthobenzoporphyrazines[13] have been shown to be capable of photocytotoxicity, and this would clearly have implications for imaging of live cells, the photocytotoxicity of all conjugates was screened against the cell line to which they demonstrated positive binding. In all cases no difference was observed between irradiated and non-irradiated cells, with 100% cell survival in both cases, even at significantly higher concentrations than those used for imaging.

In conclusion, we have shown that fluorescent macrocycles based on the phthalocyanine and naphthobenzoporphyrazine core can be synthesised which absorb and emit in the spectral region most favourable for biological imaging. The macrocycles incorporate isothiocyanate groups which allows facile bioconjugation to monoclonal antibodies. The ability of these conjugates to selectively bind to and identify cells which express the corresponding antigens has also been demonstrated.

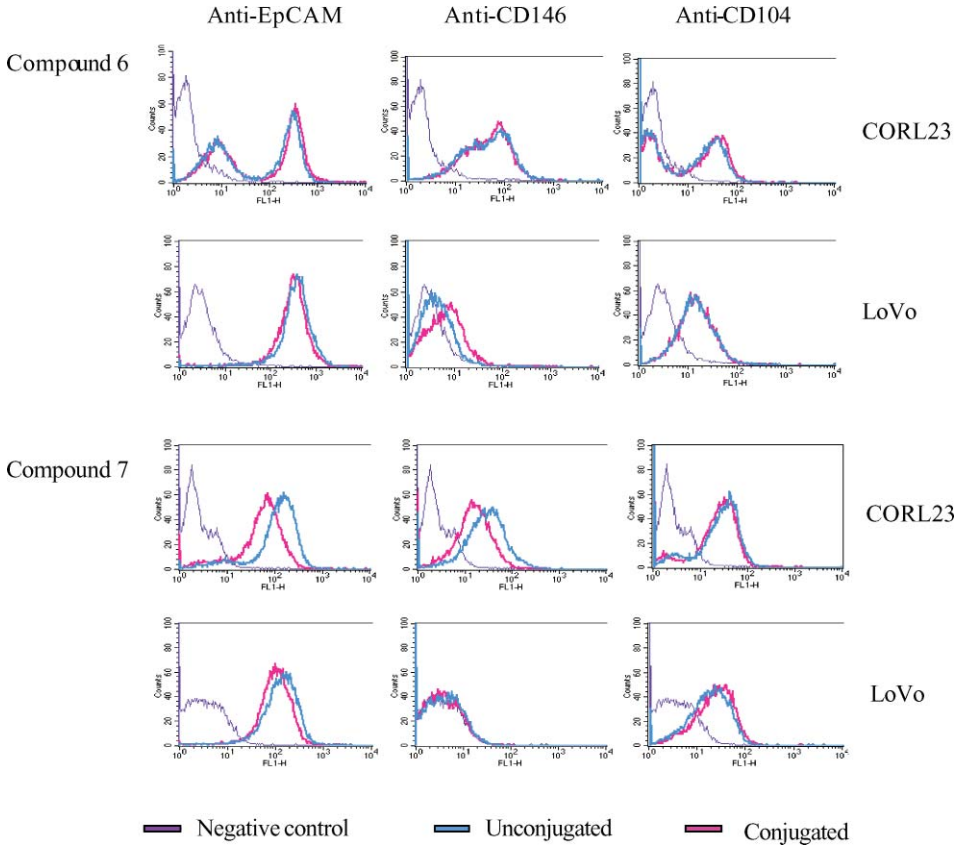

Fig. 3 Flow cytometry of conjugated antibodies.

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Notes and references

† Selected experimental and spectral data : **6** δ_H(400 MHz DMSO-D_6) 1.16 (18H, m, CH_2CH_3), 1.51 (12H, m, $CH_2CH_2CH_3$), 2.10 (12H, m, $OCH_2CH_2CH_2CH_3$), 4.65 (12H, m, $OCH_2CH_2CH_2CH_3$), 7.7 (9H, m, Ar*H*); *m*/*z* (MALDI) 1068 ($M + H^+$); λ_{max} (DMSO/nm) 336, 660, 729; **7** δ_H(400 MHz DMSO-D_6) 1.17 (18H, m, CH_2CH_3), 1.75 (12H, m, $CH_2CH_2CH_3$), 2.21 (12H, m, $OCH_2CH_2CH_2CH_3$), 5.12 (12H, m, $OCH_2CH_2CH_2CH_3$), 6.35 (3H, m, benzene-*H*), 7.72(6H, m, naphlalene-*H*), 8.85 (6H, m, naphthalene-*H*); *m*/*z* (MALDI) 1217 (M^+); λ_{max} (DMSO/nm) 348, 700, 796.

‡ Cell lines and antibodies: The LoVo human colon adenocarcinoma and CORL23 human lung large cell carcinoma cell lines (ECACC) were grown in DMEM and RPMI respectively. All media was supplemented with 10% v/v fetal calf serum, 2 mM L-glutamine, 100 μg ml^{-1} streptomycin, 100 μg ml^{-1} penicillin and 0.25 μg ml^{-1} amphotericin B (Invitrogen). All cells were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. The murine antibodies, anti-CD104 and anti-CD146, were purchased from Serotec. The anti-EpCAM antibody was a generous gift from Prof. S. Warnaar (University of Leiden). Anti-CD104 binds to a 205 kDa glycoprotein involved in cell–cell adhesion and is upregulated in colorectal and bladder carcinomas. Anti-CD146 binds to a cell adhesion molecule upregulated in melanoma cells. Anti-EpCAM recognises a 40 kDa cell–cell adhesion molecule upregulated on a variety of carcinomas, particularly colorectal anti-EpCAM and anti-CD104 shows positive binding to both the LoVo and CORL23 cell lines whereas the anti-CD146 only shows binding to CORL23.

§ Conjugation of antibodies: Conjugation is carried out in a 1 ml reaction volume containing 1 mg ml^{-1} antibody in 0.5 M bicarbonate buffer (pH 9.2) and a 20 × molar excess of compound **6** or **7**. The reaction vessels were agitated gently at room temperature for 1 h and protected from light. The conjugates were purified using Sephadex G25 columns (Amersham) and eluted with phosphate buffered saline pH 7.4 (PBS). The degree of labelling (the number of moles of compound **6** or **7** conjugated per mole of antibody) was calculated using spectroscopic methods.

¶ Flow cytometry: Cells were removed from culture vessels with 5 mM EDTA. After washing in PBS the cells were counted, re-suspended in PBS–0.25% w/v bovine serum albumin (BSA) and 2 × 10^5 cells were added to each tube. The cells were labelled with 50 μl (5 μg ml^{-1}) of anti-EpCAM, anti-CD104 or anti-CD146 (conjugated/unconjugated) for 1 h at 4 °C. After washing with PBS–BSA, cells were labelled with 50 μl (10 μg ml^{-1}) of rabbit anti-mouse IgG-FITC (Serotec) for 1 h at 4 °C. A further wash with PBS–BSA was performed before analysis of cells in a FACScalibur cytometer (BD Biosciences). Data is presented as histograms plotting cell number against fluorescence intensity.

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