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Conjugation-Induced Fluorescent Labeling of Proteins and Polymers Using Dithiomaleimides

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Supporting Information

ABSTRACT: Dithiomaleimides (DTMs) with alkyl substituents are shown to be a novel class of highly emissive fluorophores. Variable solubility and further functionalization can easily be tailored through the choice of N and S substituents. Inclusion of a DTM unit into a ROP/RAFT initiator or insertion into the disulfide bond of salmon calcitonin (sCT) demonstrates the utility for fluorescent labeling of polymers and proteins. Simultaneous PEGylation and fluorescent labeling of sCT is also demonstrated, using the DTM unit as both a linker and a fluorophore. It is anticipated that DTMs will offer an attractive alternative to commonly used bulky, planar fluorophores.

Site-specific fluorescent labeling of macromolecules is highly desirable for tracking and monitoring purposes.[1] This labeling is often achieved by incorporating a fluorophore through a copolymerization route or introduction into the chain end. Potential advantages of the latter include the decreased effect of self-quenching due to low fluorophore concentration, the availability of the backbone for the introduction of further functionality[2] or stimuli responsiveness (e.g., pH or temperature) and the fact that each molecule has only one fluorophore.[3] Also, the effect of fluorophore incorporation on the properties of the polymer (e.g., solubility, conformation, interactions) are minimized through chain-end incorporation. However, many of the commonly used planar aromatic or ionic dyes are very hydrophobic or hydrophilic, respectively, which can lead to end-labeled homopolymers having amphiphilic character, thus influencing their aggregation and assembly.[1,4]

Polymers with a terminal fluorophore are finding a wide variety of applications as biological probes for intercellular processes[5] and membrane labeling;[6] in the study of polymer dynamics,[7] including the use of single-molecule imaging;[8] for the synthesis of photochromic[9] and light-harvesting[2] materials; and in the study of polymerization mechanisms.[10] One of the advantages of living and controlled radical polymerization[11] is the facile incorporation of desired functionality at chain ends. Labeling of chain ends with a fluorophore can therefore be achieved before the polymerization using a fluorescent initiator or by post-polymerization functionalization. In the latter strategy, highly efficient reactions such as esterifications,[7,12] amidations[6b,13] and "click" reactions[5,14] have been employed. Very high degrees of functionalization can be achieved in this way, with the advantage that a single polymer scaffold can be labeled with many different fluorophores. However, full conversion requires an (often large) excess of the dye, necessitating the frequently difficult removal of unreacted small molecules. Furthermore, as the polymer molecular weight increases and chain ends become less available, the reaction efficiency decreases. Alternatively, the use of a fluorescent initiator ensures that all growing chains are labeled throughout the polymerization, and therefore, high labeling efficiency is achieved without the need for difficult purification. There are many examples of the use of fluorophore-containing initiators in anionic polymerization[15] as well as atom-transfer radical polymerization (ATRP),[9] nitroxide-mediated polymerization (NMP)[8,10] and reversible addition−fragmentation chain transfer (RAFT) polymerization.[2,16]

Conjugation of polymers [e.g., poly(ethylene glycol)] and small molecules to peptides, proteins, and other biological substrates is a powerful method for chemical modification as well as property inducement and enhancement.[17] Research has concentrated on site-specific conjugation, improving the efficiency, expanding the range of sites available, and imparting reversibility to the linking groups.[18] It is often desirable to label the conjugate so its location can be followed. Incorporating fluorescent groups as co-monomers or initiators and/or by post-conjugation is common, with a number of reagents commercially available. Fluorescent labeling of proteins, peptides, and their conjugates can be achieved either chemically through functional group modification or biologically by genetic modification, fluorescent peptide tagging, or enzymatic catalysis.[19] Typically, fluorescent probes are functionalized with reactive moieties suitable for reaction with pendant amino acid functional groups. The ε-amino functionality in lysine residues is reactive toward *N*-hydroxysuccinimide-activated esters, allowing fast and efficient conjugation.[20] Amines can also react efficiently with iso(thio)-cyanate-functionalized molecules such as fluorescein isothiocyanate.[21] However, this approach to labeling often is not site- or protein-specific, especially in the presence of competitive nucleophilic residues, including histidine, serine, threonine, and tyrosine.[22] The labeling specificity can be improved by targeting free cysteine residues, which show efficient conjugation with N-substituted maleimides,[23] pyridyl disulfide[24] and acrylic groups.[25] In particular, reactions with N-substituted maleimides

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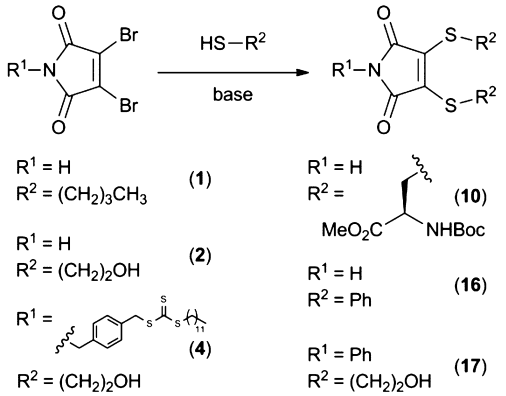
Scheme 1. Synthesis of DTMs via the Addition/Elimination Reaction of 2,3-DBM with Thiols



are highly specific and efficient, forming stable thioether bonds in organic and aqueous media at pH 6.5−7.5.[26] The azide functional group is also useful in bio-orthogonal conjugation reactions through Staudinger ligation[27] and copper-mediated azide−alkyne "click" chemistry.[28] Likewise, tetrazine−alkene Diels−Alder "click" reactions are applied to *in vitro* and *in vivo* labeling and conjugation.[29]

Bridging disubstituted maleimides have been used to prepare highly specific bioconjugates with peptides containing disulfide bonds.[30] Baker et al. showed that dihalo- and dithiophenyl-substituted maleimides are able to bridge somatostatin following *in situ* reduction. This procedure was successfully extended to the conjugation of salmon calcitonin (sCT) without loss of the native structure or activity of the peptide.[31] With respect to fluorescence, Baker et al. also showed that fluorescein-functionalized dibromomaleimide (DBM) could be conjugated to somatostatin to produce a fluorescently labeled bioconjugate.[30a] Here we report a highly versatile approach to orthogonal, chemical fluorescent labeling of polymers and peptides that uses the dithiomaleimide (DTM) motif, negating the need to incorporate a fluorescent tag. We propose this new functional motif is an important new fluorescent marker for applications in materials science and biomedicine.

Conversion of 2,3-DBM to DTMs by an addition/elimination reaction with thiols (Scheme 1) was reported previously.[30a,31b,32] Interestingly, we observed that when an alkyl thiol is used, the product is highly fluorescent. For example, reaction of 2,3-DBM with butanethiol in the presence of triethylamine (TEA) as a catalyst produced the corresponding DTM, **1**. HPLC demonstrated the presence of a single fluorescent species (Figure 1a) whose identity was confirmed by NMR and high-resolution mass spectrometry analysis. The excitation spectrum of **1** in $CHCl_3$ shows maxima at 265 and 415 nm, and the emission spectrum has a maximum at 520 nm, corresponding to Stokes shifts of 255 and 105 nm, respectively (Figure 1b,c). This agrees with a patent report from the 1980s that 2,3-DBM can be used as a "fluorescent stain" for thiol-containing molecules. However, while those authors speculated as to the cause of fluorescence, no attempt was made to characterize the products.[33]

The versatility of DTMs as fluorescent labels, or reporters, is further demonstrated by the variety of substituents that can be incorporated through the simple choice of thiol. Altering the thiol used in the addition/elimination reaction (Scheme 1) can give fluorescent DTMs that are soluble across the entire spectrum of solvent polarities. Reacting mercaptoethanol with 2,3-DBM gives DTM **2**, as previously reported,[30b] with the presence of a single fluorescence species (Figure 1b,c) confirmed by HPLC (Figure S17). While butanethiol derivative **1** is soluble in hexanes, mercaptoethanol derivative **2** is water-soluble, with solution-state fluorescence retained in a wide range of solvents.

Furthermore, simple N-functionalization of 2,3-DBM to give more highly functionalized fluorescent DTMs is possible. N-Functionalization can be achieved by reacting dibromomaleic anhydride with primary amines[30a,32b,34] or alkylating 2,3-DBM with alkyl bromides or alkyl chlorides,[31a,32c] or by Mitsunobu reaction of 2,3-DBM with primary alcohols.[30b,31a] A highly desirable functionality to incorporate is an initiator for controlled radical polymerization (CRP)[35] such as ATRP or RAFT. Whereas the maleimide group must be protected when incorporated into a CRP initiator,[36] we recently showed that initiators functionalized with DBM[32c] or dithiophenol-maleimide[31b] can control the radical polymerization without the need for protecting groups.

Reacting the previously reported 2,3-DBM-functionalized RAFT agent **3**[32c] with mercaptoethanol in the presence of TEA gives the fluorescent RAFT agent **4**. Locating the fluorophore within the R group of the chain-transfer agent means that all of the chains initiated by the RAFT agent will contain the DTM group at their α-terminus. While fluorophores can be incorporated in RAFT-agent Z groups, the susceptibility of the thiocarbonylthio group to hetero- and homolytic degradation make this a less attractive choice.[16] The fluorescence spectrum of **4** is similar to that of non-N-functionalized **2** (Figure 1b,c), which demonstrates that fluorescence is not inhibited by the conversion to a RAFT agent.

Polymerizing *tert*-butyl acrylate (tBA), triethylene glycol monomethyl ether acrylate (TEGA), styrene (Sty), and *N*-isopropylacrylamide (NIPAM) gave the corresponding polymers with good control over molecular weight (Table S1).

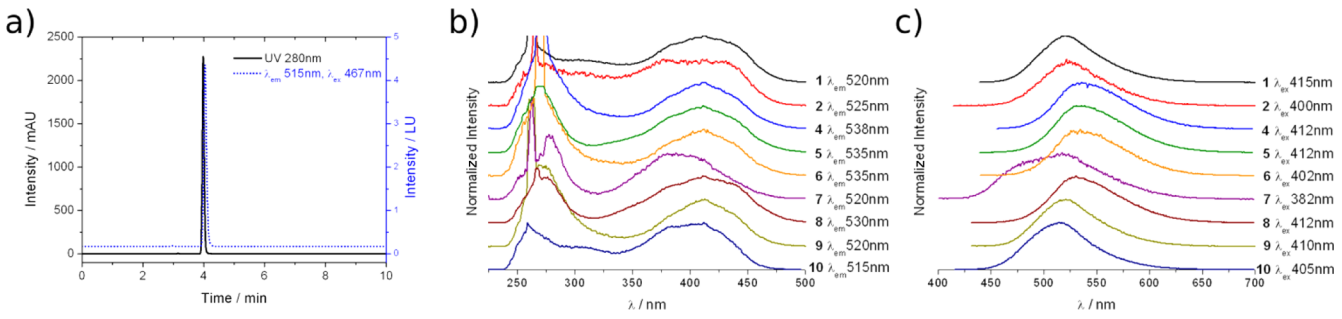


Figure 1. (a) HPLC chromatogram of **1** with simultaneous UV (λ = 280 nm) and fluorescence (λ_{ex} = 467 nm, λ_{em} = 515 nm) detection. (b) Excitation spectra and (c) emission spectra of fluorescent DTMs **1**−**10**.

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Interestingly, while polymerizations of Sty and NIPAM were previously found to be inhibited when DBM-functionalized RAFT agent **3** was used, conversion of the DBM group in **3** to the DTM group in **4** enabled successful polymerization of both monomers. Furthermore, as **4** contains two hydroxyl groups, it can also be used as an initiator for organocatalytic ring-opening polymerization (ROP)[37] of *rac*-lactide to give poly(D,L-lactide) (**9**) with low dispersity. For polymers **5**−**9**, the presence of both the trithiocarbonate and DTM end groups, with UV/vis absorption maxima at ∼309 and ∼400 nm respectively, was confirmed by size-exclusion chromatography. Use of a photodiode array detector allowed simultaneous collection of the entire UV/vis spectrum at all elution times, demonstrating the presence of both of these characteristic absorptions for the polymers (Figure S18). In addition, MALDI-TOF MS of polymers **6**, **8**, and **9** showed the presence of polymeric species with masses corresponding to the end groups resulting from **4** (Figure S19).

As expected, all polymers containing the DTM group were highly fluorescent, with fluorescence spectra in $CHCl_3$ similar to that of **4** (Figure 1b,c). These results demonstrate that the use of the novel fluorescent dual ROP/RAFT agent **4** allows the facile synthesis of a range of hydrophobic, hydrophilic, and thermoresponsive fluorescent polymers. Interestingly, the fluorescence of the polymeric DTM appeared to be much brighter than that of the small-molecule precursor. Integrated emission intensities for the polymeric DTM systems were found to be 2−10 times greater than that of either **1** or **2** at similar concentrations. This can be attributed at least in part to two effects on the environment of the polymeric DTM. The first is the inability to readily form self-quenching dimers or larger aggregates in the presence of a polymeric chain, which may shield the DTM chain end from other DTM units while also placing large steric restrictions on the DTM.[38] The second is a decrease in the amplitude and frequency of emission-decreasing collisional events with solvent molecules.[39]

It was previously shown that 2,3-DBM can be used to bridge the disulfide bonds in the proteins somatostatin and sCT.[30a,31a] Reducing the disulfide bond and then reacting with 2,3-DBM forms a DTM bridge with retention of the protein's native structure. Following our observation of DTM fluorescence with **1**, we hypothesized that this disulfide-bridging reaction should also form a fluorescent product, allowing for peptide labeling. As an initial test, we reacted the commercially available protected cysteine *N*-Boc-Cys-OMe with 2,3-DBM according to a previously reported procedure[32a] to give DTM **10**. As expected, **10** is also fluorescent, sharing the same spectral characteristics as **1** (Figure 1b,c), while HPLC confirmed the presence of a single fluorescent product (Figure S20). Similarly, conjugation of 2,3-DBM with higher-order peptides, including the tripeptide glutathione (GSH) and 32-amino acid sCT was investigated. Conjugation of GSH was achieved by adding 2,3-DBM to 2.2 equiv of GSH in pH 6.2 buffer (Scheme S1). Reversed-phase (RP) HPLC after 1 h showed nearly quantitative conversion with correlating UV and fluorescence traces (Figure S21). The major peak was found to correspond to a >95% yield of the disubstituted GSH−DBM conjugate **11**.

sCT is used in the treatment of a number of bone diseases,[40] and the presence of a reductively labile disulfide bond between the Cys^1 and Cys^7 residues is ideal for inserting the bridging DTM moiety. Consequently, the conjugation of sCT and 2,3-DBM was realized by adopting conditions developed by Jones et al.[31a] (Scheme S2). A sample of the crude mixture was then analyzed by fluorescence spectroscopy, and excitation and emission spectra were obtained (λ_{ex} = 341 nm, λ_{em} = 502 nm; Figure S22). Additionally, the reduction and conjugation profiles were again followed by RP-HPLC. The sCT and reduced sCT traces showed response in the UV (λ = 280 nm) but no fluorescence response at the given wavelengths. However, upon conjugation to 2,3-DBM, the shift in the UV response was complemented by the appearance of a corresponding fluorescence peak (Figure 2), with quantitative conversion achieved

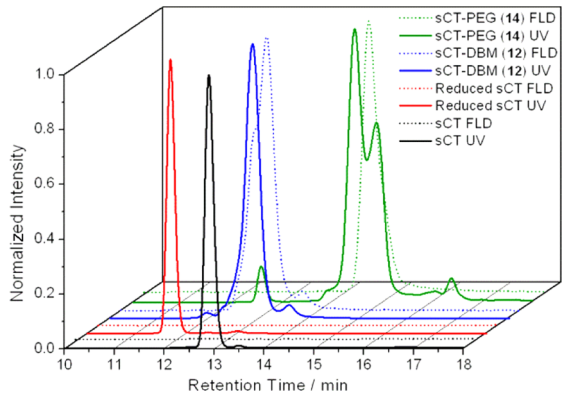

Figure 2. RP-HPLC analysis of the sCT-DBM conjugation reactions, with simultaneous UV (λ = 280 nm) and fluorescence (λ_{ex} = 341 nm, λ_{em} = 502 nm) detection.

using just 1.1 equiv of 2,3-DBM with respect to sCT. Hence, this represents a convenient and facile approach to protein conjugation, resulting in conjugation-induced fluorescent labeling, without the need for additional functionalization of either moiety. The utility of this approach could be further expanded by introducing functionality at the maleimide nitrogen. For example, an alkyne group was introduced to give a "clickable" fluorescent product, **13** (Figure S24). Furthermore, using an *N*-poly(ethylene glycol)maleimide allowed PEGylation of sCT with simultaneous fluorescence labeling in a one-pot reaction (**14**; Figure 2). While some unreacted PEG was seen in the RP-HPLC UV trace as a high RT shoulder, a single peak in the fluorescence trace corresponding to PEGylated sCT was observed.

Adding a single thiol to 2,3-DBM also gave a fluorescent product. Although the thiobromomaleimide of *N*-Boc-Cys-OMe (**15**) showed a significantly reduced fluorescence intensity relative to **1** (Figure S25), this result demonstrates the utility of this conjugation-induced fluorescence for monocysteine-functionalized proteins.[30a] Interestingly, conjugating aromatic rings to the DTM unit drastically decreases its fluorescence intensity. For example, when thiophenol was used in the addition/elimination reaction, the product, **16**, had vastly decreased fluorescence. Likewise, adding mercaptoethanol to *N*-phenyl-DBM gave a significantly less fluorescent product, **17** (Figure S25). We believe that this reduction in fluorescence occurs because the lone pairs of the N and S atoms of the DTM moiety must be free to contribute to the resonance structure of the DTM ring in order to achieve optimum fluorescence.

In this work, we describe the development of a range of new fluorescent small molecules (dithiomaleimides, DTMs) that have bright emissive properties (∼500 nm) in a range of solvents. We demonstrate that DTMs can be readily introduced into polymers and peptides and used as both a conjugation site and also a fluorescent label, allowing simultaneous PEGylation and

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fluorescent labeling of disulfide-containing proteins. We propose that a key advantage of this new reporter group is its versatile chemistry as well as most notably its small size, which allows for ready incorporation without affecting or disrupting the scaffold. Given the built-in fluorescence properties and chemical diversity of this class of molecules, we propose that they will serve as a functional platform for a range of applications. Further studies are exploring the scope of these probes as well as their application as labeling groups for other biomolecules.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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