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Click Chemistry Based Method for the Preparation of Maleinimide-Type Thiol-Reactive Labels

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Maleinimides are widely used to label proteins and to derivatize thiols. The so-called click reaction was shown to allow the efficient introduction of the maleinimido group into azido-modified fluorophores (benzoxazines) by reacting them with an alkyne-modified maleinimide to yield new thiol-reactive fluorescent labels **5**–**7**. The reaction proceeds under mild experimental conditions and provides a new strategy with which to introduce the maleimide group, as exemplified here for fluorophores. Conceivably, it may be extended to radioactive, electro-active, isotopic, or spin labels. Previously reported methods for the formation of such maleinimides starting from open-ring precursors require rather harsh conditions. The new benzoxazines **5**–**7** are characterized by a fairly long and flexible linker between the chromophore and the maleinimide functional group, and their fluorescence can be photoexcited with diode lasers (which are preferred light sources in fluorometry). They were conjugated to: (a) the aminothiol cysteamine, (b) the peptide glutathione, and (c) to human serum albumin. The fluorescence of the phenoxazinone **5** is strongly solvatochromic, which suggests its use as a polarity-sensitive probe.

Introduction

So-called click chemistry has emerged as an extraordinarily versatile synthetic tool.[1] It involves the 1,3-dipolar cycloaddition of azides to alkynes using Cu^I ion as the catalyst. Click chemistry proceeds in water of pH 7 at room temperature (thus making it a very useful tool in terms of bioconjugation), is regioselective, efficient, simple, and bioorthogonal in that azido groups and alkyne groups are rarely found in biomolecules. As a result, in vitro click chemistry has been used for labeling biomolecules,[2] hormones,[3] and for in-vivo tumor cell targeting[4] with either fluorophores, radiomarkers, spin labels, or nanoparticles made from gold[5] or silica.[6] The success of click chemistry is especially striking in the case of labeling glycans and glycoconjugates.[7]

Site-specific labeling of proteins is of particular interest because it has several advantages over random labeling.[8] One common approach for site-specific labeling is to label solvent-accessible cysteine (Cys) using thiol-reactive reagents.[4–8,9] Cysteine is most attractive in this respect because of its relative rarity throughout the proteome, and because it may even be introduced into proteins that are normally free of the amino acid.[10] Hence, there is substantial interest in the development of labels for thiolated species.[11] Although iodoacetamides and sulfochlorides may play a certain role,[12] the maleinimides form by far the largest group of thiol-reactive labels that are used for selective fluorescent,[9a,11] radioactive,[13] or isotopic labeling of thiols.[14] However, such labels are typically synthesized in only moderate yields,[11b] mainly because the cyclization of the maleamic acids requires rather harsh conditions, which is highly undesirable for chemically less stable markers (fluorophores) such as cyanines and (benz)oxazines. Moreover, most thiol-reactive labels have a maleinimido group that is attached directly to the label, often to the heteroaromatic ring of a fluorophore,[9b,11b,11d,15] which is also undesirable because the buried thiol groups are difficult to access in such cases. We envisioned that the click reaction may provide a facile, novel method with which to introduce maleinimides into a (fluorescent) label.

Among the long-wavelength emitting dyes, the oxazines and the benzoxazines (such as Nile Red and Nile Blue) are attractive because of their stability and brightness.[16] They have been used mainly, for example, as biomarkers for nucleic acid detection in histochemistry,[17] and for labeling proteins.[18] Deeply colored oxazines have a mesomeric donor–acceptor chromophoric system. Oxazinones of type A (Scheme 1) are strongly solvatochromic (e.g., orange and strongly fluorescent in non-protic solvents, red in methanol, but purple and less fluorescent in water solution). Diamino-substituted oxazines of type B are blue and exhibit almost no solvatochromic effects. Being cationic, they have good water solubility, often display an increase in fluorescence

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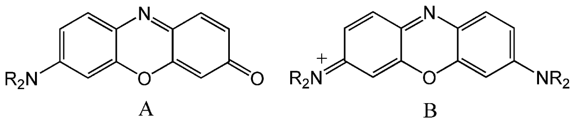
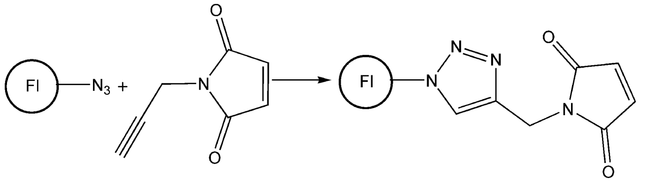
quantum yield upon conjugation, and are amenable to structural modification so that various functional groups can be introduced.



Scheme 1. Chromophores of the purple oxazines A and the blue oxazines B used in this work.

The presence of a flexible linker between the maleinimide group and the fluorophore is known to facilitate labeling.[11b] We hypothesized that an application of click chemistry to the preparation of thiol-reactive labels according to Scheme 2 may be an elegant way to introduce the maleinimide group in the final step. The results are presented here.



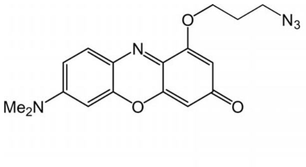
Scheme 2. Strategy for preparation of fluorescent thiol-reactive labels with spacer groups by click chemistry. *Fl* stands for the respective fluorophore.

Results

Synthesis of Azido-Modified Oxazines

Oxazine chromophores were chosen as the fluorophores because they can emit at both red and blue wavelengths, have reasonably good quantum yield (QY; at least in organic solvent) and brightness (defined as molar absorbance multiplied by QY), and are adequately photostable.[15] The blue oxazines are cationic (and thus display good solubility in aqueous solutions), and are compatible with the 635-nm diode laser, which is in widespread use in analytical fluorescence. The three basic chromo(fluoro)phores **1**–**3** were prepared as outlined below and detailed in the Supporting Information. The compounds carry azido groups and – in terms of synthesis – are fairly easily accessible. More specifically, the synthesis of the purple oxazine **1** was accomplished in one step by alkylating 7-(dimethylamino)-1-hydroxyphenoxazin-3-one[19] with 1-azido-3-bromopropane. Oxazine **2** was obtained in four steps by reacting piperidine with phloroglucinol to give 5-(piperidin-1-yl)-benzene-1,3-diol. This was azido-functionalized by (a) alkylation with 1-bromo-3-chloropropane, (b) exchanging the chloro atom with iodine, and (c) converting the iodide into an azide with NaN_3. The resulting 3-(3-azidopropoxy)-5-(piperidin-1-yl)phenol was condensed with *N*,*N*-dimethyl-4-nitrosoaniline to give the blue oxazine **2**.

The blue oxazine **3** was obtained by a different route; in this case the azido functionality was introduced by first alkylating 1-naphthylamine with 3-bromo-1-propanol to give 3-(naphthalene-1-ylamino)propan-1-ol, the hydroxy group of which was then converted into an azido group with NaN_3. The resulting 3-azido-propyl-1-naphthyl-amine



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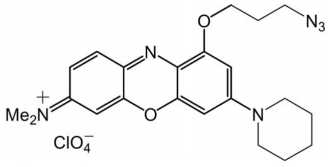

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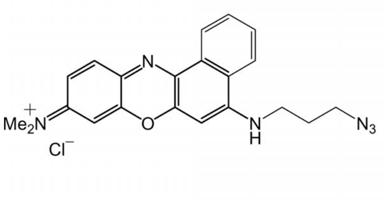

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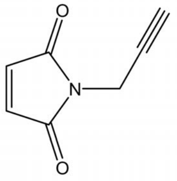

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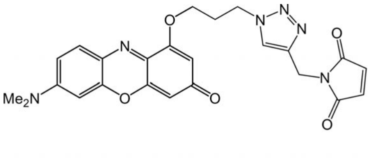
Figure 1. Chemical structures of the oxazine azides **1**–**3**, and of the alkyne maleinimide **4** to which the azides were ‘clicked’.



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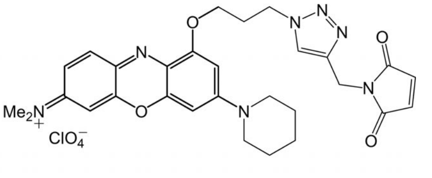

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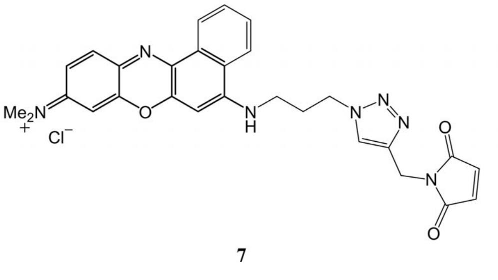

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Figure 2. Chemical structures of the thiol-reactive oxazines **5**–**7**.

was condensed with 5-(dimethylamino)-2-nitrosophenol in acidic medium to give the azidophenoxazine **3**. Compounds **1**–**3** were then 'clicked' to the maleinimide **4** as outlined below (Figure 1).

It should be stated at this point that these azides are also useful click labels, for example, for labeling proteins[20] or saccharides[21] containing azido groups, or when labeling alkyne modified nanoparticles.[6,20]

The known synthon **4** was then used to link the maleinimido group to the azido-modified fluorophores. The reaction of **4** with the azidophenoxazines **1**–**3** is characterized by high yields, easy work-up, and proceeds in aqueous solvent at room temperature to give the thiol-reactive labels **5**–**7** shown in Figure 2. The high yield and ease of work-up in the final step makes this approach very powerful in terms of ligating maleimide groups to a fluorophore. We presume that this is a generally applicable approach in that almost any fluorophore containing an azido group can be linked to a maleinimide (and thus become a thiol-reactive label) using the alkyne reagent **4**. Previously reported methods are often tedious.[22] Furthermore, the long linker between the fluorophore and the maleinimide group provides the latter with more flexibility so that thiol groups within biomolecules are more easily accessed.[11b]

Spectral Properties of Labels

The absorption and emission maxima of labels **5**–**7** in buffer solution of pH 7 are summarized in Table 1, and the spectra of **5** and **6** are shown in Figures 3 and 4, respectively. The compounds usually display broad absorption bands and a longwave shoulder. The blue oxazines **6** and **7** almost perfectly match the 635-nm line of the red diode lasers often found in commercial fluorescence instrumentation. The small Stokes' shifts (typically 20–25 nm) are characteristic of such rigid fluorophores.[16c]

Table 1. Absorption and emission maxima (nm), molar absorbances [L/(mol·cm)], and quantum yields (QY; ±20%) of oxazine azides and of oxazine labels (in aqueous buffer of pH 7.0) and in methanol (labels **3** and **7** only). Quantum yields were determined by using the dyes oxazine **1** (QY = 0.11 in ethanol[23]), Nile Red (QY = 0.08 in methanol[24]), and Nile Blue (QY = 0.004 in water[25]) as standards.

Dye (solvent)	Absorption (ε)	Emission (QY)
1 (water)	595 nm (17,300)	629 nm (0.012)
2 (water)	649 nm (53,000)	669 nm (0.014)
3 (water)	636 nm (43,500)	672 nm (0.044)
3 (methanol)	630 nm (49,600)	664 nm (0.21)
5 (water)	597 nm (22,000)	628 nm (0.023)
5 (methanol)	557 nm (21,400)	620 nm (0.16)
6 (water)	650 nm (58,000)	668 nm (0.014)
7 (water)	636 nm (45,000)	669 nm (0.015)
7 (methanol)	628 nm (46,200)	662 nm (0.033)

It is known[16] that the absorption and emission profiles of such oxazine fluorophores are solvatochromic, and that even slight variations in the substitution pattern can have marked effects on their photophysical properties. Indeed, both the absorption and emission maxima in ethanol, methanol, and water are very different, as are the quantum yields. Some data are given in Table 1 for compounds **1**–**3** and **5**–**7**. The bathochromic shift and the lower quantum yield on going from methanolic to aqueous solutions are particularly significant. By analogy to previous studies,[26] lifetimes can be estimated to be in the order of 1–2 ns and are likely to be multi-exponential in the presence of other biomolecules or when other processes (such as electron transfer) can occur.

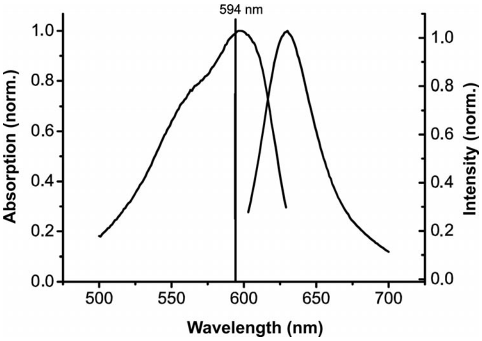

Figure 3. Absorption (left) and emission (right) of label **5** in water. The line of the 594-nm helium/neon laser is also shown.

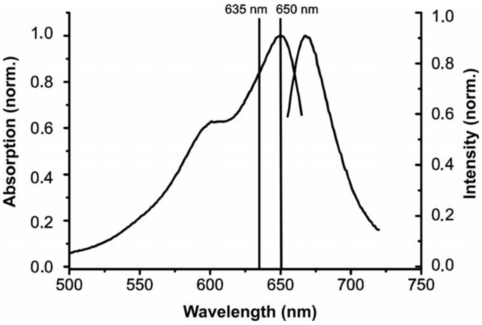

Figure 4. Absorption (left) and emission (right) of label **6** in water. The lines of the 635- and 650-nm laser diodes are also shown.

Stability and Effects of pH on Fluorescence

The fluorescence intensity of the maleinimides **5**–**7** in aqueous buffer slowly increased over time. This may be attributed to the known[27] tendency of maleinimides to undergo hydrolysis to give the corresponding (open-ring) maleamic acid. The increase in fluorescence is due to the fact that the (cyclic) maleinimides are efficient triplet quenchers of many fluorophores, whereas (non-cyclic) maleic acid monoamides and the thiol conjugates of maleinimides, being succinimides and cyclic, are not.

The effect of pH on the fluorescence of the labels was studied in solutions (ca. 3 μM) with pH values of between 5 and 10. The red emission of the blue oxazines **6** and **7** did not depend on the pH value in the range tested. The yellow fluorescence of the purple oxazinone label **5**, in contrast, started to decrease at pH values of below pH 6. This is clearly due to the fact that the oxazinone is uncharged, whereas compounds **6** and **7** are positively charged and are thus unlikely to be protonated.

Labeling of Thiols

The maleinimides **5**–**7** were first used to label human serum albumin (HSA; a 65 kD protein), which is known to have one single thiol group [along with more than 30 dithio (–S–S–) groups, which do not react with maleinimido groups]. Labeling was accomplished in buffer solutions of pH 7 by adding the labels as concentrated solutions in either *N*,*N*-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO). After reacting HSA with either of the labels for 3–6 h at room temperature, the labeled protein was purified by size exclusion chromatography (SEC). In some cases, they were submitted to capillary electrophoresis.

The dye-to-protein ratio (DPR), which is defined as the number of labels statistically bound to one HSA, was determined by spectrophotometric analysis to be 1.0. This is in agreement with the fact that native HSA has one single thiol group. The fluorescence spectra of the conjugate revealed that it is stable for at least one week in buffered solution at room temperature, which indicates both the stability of the fluorophore and the spacer (in essence the triazole). The MALDI-TOF mass spectrum of the HSA conjugate also confirmed that single-labeling had occurred (see the Supporting Information).

Label **7** also was conjugated to two smaller thiols: cysteamine (an amine) and L-glutathione (GSH; a peptide). Both were found to undergo quantitative reaction in pH 7 buffer after a one hour reaction, whereas labeling of HSA required at least three hours. The reaction was monitored by spectrophotometric analysis. Addition of cysteamine or GSH to label **7** in pH 7 buffer resulted in a small increase in absorption at around 600 nm and a moderately strong increase in emission (Figure 5).

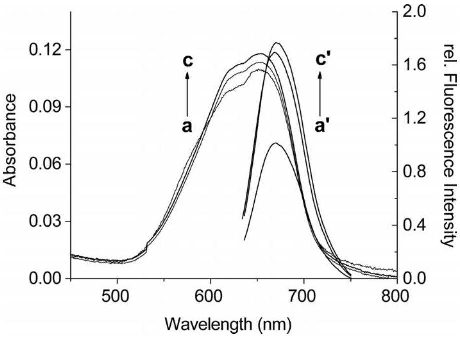

Figure 5. Absorption spectra (a–c) and emission spectra (a′–c′) of label **7** after addition of cysteamine and glutathione in pH 7 buffer; (a, a′): label only; (b, b′): label plus cysteamine; (c, c′): label plus glutathione.

It can be seen in Table 1 that the maleimide **7** has a rather small QY (0.015), whereas Figure 5 shows that the fluorescence increases by around 69 % within 5 min after addition of cysteamine to **7**. Addition of GSH also caused an increase (by 73 %) in fluorescence while control reactions performed in parallel (label **7** in the same concentration but without cysteamine or GSH added) showed virtually no change in fluorescence. The product of the reaction with cysteamine was subjected to LC-MS analysis (see MS spectra in the Supporting Information), and the results confirmed that the addition reaction occurred as outlined in the Scheme shown in the Supporting Information.

The fluorescence spectra recorded in the absence and presence of cysteamine were also acquired in presence of histamine, tyramine, dopamine, and tryptamine, none of which carried thiol groups. No further increase in fluorescence intensity was detectable. This is additional proof that label **7** reacts with the thiolated amine selectively, and not with other amines.

Discussion

The method presented here for the introduction of a maleinimido group involves a small number of synthetic steps and the critical step, whereby the preformed fluorophore is linked to a maleimide functional group, occurs in the final step of synthesis using the efficient click reaction. Although not shown here, we presume that the method can be extended to other fluorescent labels, such as rhodamines, fluoresceins, and dipyrromethenones (e.g., the Bodipy™ dyes) or to other popular labels (such as the Alexa™ dyes), and to ketocyanines.[28] Moreover, this approach may be extended to labels other than fluorescent compounds, for example to spin labels, radiomarkers, or markers for use in NMR spectroscopy.

Maleinimides may also be obtained by reacting fluorophores with amino-reactive functions, such as *N*-hydroxysuccinimide esters, with 2-aminoethylmaleinimide. This reaction, although proceeding smoothly, requires more tedious work up and, in particular, requires a carboxy derivative of the dye to be prepared and then made amino-reactive, typically by activation with a carbodiimide and *N*-hydroxysuccinimide (NHS) (or its sulfo derivative) to yield the NHS ester, which is sensitive to moisture and has limited storage stability. Synthesis of maleinimides by using NHS esters is therefore deemed to be more tedious (at least in most cases) and less straightforward.

Labeling of typical small thiols was found to proceed under the conditions used to introduce the maleimide labels and yielded conjugates with longwave absorption and fluorescence maxima. This characteristic is useful if background fluorescence from biological samples is to be suppressed. In the case of cysteamine, labeling with **7** may also be considered to be an example of the derivatization of thiols, which then may be quantified following separation techniques such as HPLC or CE (capillary electrophoresis).

The merits of labeling a thiol group are particularly clear in case of HSA. This protein contains 35 cysteines, 34 of which are present in the disulfide form, whereas only one is freely available for labeling.[29] This is in contrast to the number of amino groups: HSA contains 60 lysines, 35 of which are freely accessible.[30] Labeling of amino groups not only occurs randomly,[31] but may also lead to a range of DPRs, whilst thiol labeling of HSA results in highly site-specific and strictly single labeling as demonstrated by a DPR of 1. Thiol labeling also eliminates the risk of self-quenching of fluorophores due to multiple labeling. Moreover, the functionality of the protein is likely to be better retained in the case of thiol labeling. In cases where no free cysteine is available, disulfide bonds (if present) may first be reduced to thiols. Alternatively, site-directed mutagenesis or heterobifunctional crosslinkers may be applied to introduce a cysteine group. Hence, thiol labeling has a wide scope and is generally to be preferred over amine labeling.

The labels presented here (compounds **5**–**7**) have the typical properties of oxazine dyes in terms of molar absorbances and fluorescence emission profiles. The effect of substituents on the molar absorbances of the oxazines under consideration is worth noting (Table 1). The molar absorbances of the blue diamino-substituted oxazine labels of type B are significantly smaller if the nitrogen of the mesomeric system is directly substituted than if the label is substituted at position 3. For example, the molar absorbance of label **6** (58,000 L/mol·cm) is much higher than that of label **7** (46,000 L/mol·cm) although both possess similar mesomeric π-electron systems. This effect is corroborated by Frade et al.,[16d,32] who synthesized various derivatives of the Nile Blue compound functionalized at the nitrogen atom of the mesomeric system. The molar absorptions of these dyes are in the range 13,000–22,000 L/mol·cm, which is much smaller than the molar absorption of Nile Blue (76,800 L/mol·cm in ethanol[33]). The beneficial effect of an increase in the quantum yield of maleimide fluorophores upon conjugation to a thiol is also observed in this case. Maleinimides have a low-lying triplet state that is capable of deactivating the excited singlet states of fluorophores by enhancing radiationless dissipation of excited-state energy. This results in rather poor quantum yields (3.3% for label **7** in air-equilibrated methanol). Upon conjugation, the π-electron system of the maleimide is converted into a succinimide (as shown in the Supporting Information). As a result, the level of the triplet state increases and leads to less efficient radiationless deactivation, i.e., an increase in fluorescence. This is reflected by a QY of 3.8% for the conjugate to cysteamine, 4.1% for labeled GSH (both in air-equilibrated methanol), and 3.4% for HSA (in water).

The selectivity of maleinimide for thiol groups over amino groups is more than 1000:1 at pH 7, and the best specificity is found[34] at pH values of between 6.5 and 7.5. This enables selective labeling of thiol groups in most cases. However, it needs to be kept in mind that maleinimides often suffer from hydrolysis, which yields a luminescent product that is not conjugated to the thiol.

In conclusion, we have developed a versatile method for introducing the widely used maleimido group, which, previously, has only been introduced by using thermal methods and then only in poor yield. We believe that this reaction, which is shown here for fluorescent labels of the benzoxazine group, can be extended to various other fluorescent labels, to electrochemical labels, and to radiomarkers.

Experimental Section

Chemicals and Reagents: All reagents were purchased from Sigma–Aldrich unless noted otherwise. Buffer salts and solvents were purchased from Merck. Deuterated solvents, such as [D$_6$]dimethyl sulfoxide were obtained from Deutero GmbH (www.deutero.de). Tetramethylsilane was used as an external standard for NMR measurements.

Instruments: Fluorescence spectra were recorded with a Hitachi F-2500 fluorometer in 1 × 1 × 3 cm quartz cuvettes, with excitation and emission slit widths set to 10 nm. Absorption spectra were recorded in glass cuvettes of the same size with a Techcomp UV-8500 spectrophotometer (Shanghai, China; www.techcomp.com.cn). ESI mass spectra were acquired with a ThermoQuest TSQ 7000 mass spectrometer (www.thermo.com), and the MALDI mass spectra with a model 4700 Proteomics Analyzer (Applied Biosystems; www.appliedbiosystems.com). ^{1}H NMR spectra were recorded with an Avance 300 MHz NMR spectrometer (Bruker-BioSpin; www.bruker-biospin.com). Emission spectra were acquired with a 635-nm diode laser as the excitation light source. IR spectroscopic analysis of the intermediates and new labels were carried out in the attenuated total reflection mode on an Excalibur FTS 3000 spectrometer (Biorad; www.bio-rad.com), equipped with a Golden Gate Diamond Single Reflection ATR System (Specac; www.specac.com).

Protocol for Labeling HSA: HSA (3.0 mg, 4.6 nmol) was dissolved in phosphate buffer (300 µL, 22 mM, pH 7). Labels **5**, **6**, or **7** (0.2 mg) were dissolved in DMSO or DMF (20 µL). The two solutions were mixed and allowed to react for 6 h at r.t. in the dark with gentle stirring. The labeled HSA was purified by size exclusion chromatography on a Sephadex G-25 column with an i.d. of 2 cm using phosphate buffer (22 mM, pH 7) as the eluent. The eluted fractions containing the label were dialyzed for five days at 4 °C (two days with 5 L of 10 mM phosphate buffer of pH 7; three days with 5 L of a 1 mM phosphate buffer of pH 7) to remove free and non-covalently bound label. MALDI-TOF spectra of the labeled proteins are given in the Supporting Information.

Determination of the Dye-to-Protein Ratio (DPR): The HSA concentrations and DPRs of the HSA conjugates were determined by assuming the additivity of the absorbances at 280 nm of both the protein and the labels. Dye concentrations in solutions of labeled HSA were determined according to the Lambert–Beer law from the absorbance of the label at (or near) the peak wavelength (590–630 nm). It was assumed that the bound and the unbound labels have identical molar absorbances.

Protocol for Labeling Cysteamine and L-Glutathione: Label **7** (0.2 mmol) was dissolved in methanol (100 µL) and mixed with cysteamine or glutathione (30 µmol) in pH 7 buffer (0.5 mL). The solution was stirred for 20 min and then loaded onto a short C18 reverse-phase column. After chromatography, the volume of the solution was adjusted to 3 mL, transferred to a quartz cuvette and submitted to absorptiometric and fluorometric analyses.

Supporting Information (see also the footnote on the first page of this article): The synthetic route to *N*-propargylmaleimide (**4**), and to labels **5**, **6**, and **7**. MALDI-TOF characterization of unlabeled and labeled HSA. Schematic of the reaction of label **7** with cysteamine, and mass spectrum of the product. Four additional references are also included.

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