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Coumarins as Fluorescent Labels of Biomolecules

António Pereira, Sérgio Martins and Ana Teresa Caldeira

Abstract

Important areas such as environmental sciences, medicine, pharmacy, and cellular biology are dependent on very sensitive analytical techniques. One of the most common methodologies used for their bioanalytical purposes is the fluorescent labelling. The synthesis of new fluorophores and the great development of fluorescent-labelling techniques combined with the enormous technological advances in the field of fluorescence microscopy allowed to deepen the structural knowledge of biomolecules. This new organic fluorophores form covalent bonds with the sample to be analyzed, producing stable bioconjugates that show fluorescence in a wide range of wavelengths, depending on the label used. Coumarin derivatives represent one of the most important chemical classes of organic fluorescent materials being one of the most extensively investigated and commercially significant groups of organic fluorescent materials. In this chapter, it is reviewed the use of fluorescent coumarin derivatives and their application to labelling biomolecules. These fluorescent labels allow researchers to study, and understand, biomolecular assemblies that exhibit complex sensitivity and selectivity. Reactive fluorescent coumarin derivatives are actually widely used in labelling biomolecules as peptides, proteins, oligonucleotides, nucleic acids, and carbohydrates, among other biological molecules.

Keywords: coumarins, fluorophores, labelling, biomolecules, bioconjugation

1. Introduction

Important areas such as environmental sciences, medicine, medicinal chemistry, and cellular biology are dependent on very sensitive analytical techniques to detect and track biomolecules (amino acids, peptides, proteins, antibodies, oligonucleotides, nucleic acids, carbohydrates, and other biological molecules). Many of these techniques often require labelling with reporters or sensors, such as isotope labels [1], radioactive tracers [2], colorimetric biosensors [3], photoswitchable biomaterials [4], photochromic compounds [5, 6], electrochemical sensors [7], or fluorescent labels [8, 9]. The fluorescent labelling presents numerous advantages, when compared to the other techniques, due to the high sensitivity of the fluorescence technique and also due to its non-destructive nature that allows the use of small sample quantities and their fluorescent labels. The fluorescence process occurs in certain molecules called fluorophores or fluorescent dyes, and a fluorescent probe is nothing more than a fluorophore enabled to detect particular components of complex biomolecular assemblies, including live cells, with complex sensitivity and selectivity [10]. The organic fluorophores may form covalent

or non-covalent linkages with the sample to be analyzed, producing the respective bioconjugates (or complexes) that can show fluorescence, from short to very long wavelengths, depending on the label used. The bioconjugation technique depends on two interrelated chemistries: the reactive functionality present on the fluorescent label and the functional groups present on the target biomolecules to be labeled. The knowledge of the basic mechanisms by which the reactive groups couple to target functionalities provides the means to intelligently design the bioconjugation strategy. Choosing the correct fluorescent label that can react with the chemical groups available on target biomolecules forms the basis for successful labelling [11].

In general, the fluorescent label should be small in size and chemically stable, with minimal interference on the structure and biological functions of the unlabeled biomolecules, producing high fluorescence quantum yield bioconjugates.

On the other hand, the labelling reaction should be extremely efficient with high yields, preferably establishing a stable covalent linkage between the fluorescent label and a specific residue in the target biomolecule. The efficiency and selectivity of several fluorescent-labeled biomolecules have been used to study and understand their dynamics, kinetics, and photophysical properties [12–18].

The amine reactive fluorescent labels are the most frequently used to prepare stable bioconjugates to a great number of biological applications since amino groups are either abundant or easily introduced into biomolecules. In contrast, to study some particular protein structures and functions, thiol-reactive reagents are chosen due to the smaller presence of thiol groups, when compared with lysine, in biomolecules [19]. In this context, cysteine is generally the amino acid chosen to label when it is desired to label selectively a protein *in vitro*, due to its relatively low abundance and high nucleophilicity compared to other amino acid side chains. Specific and noninterfering dual fluorescent labelling in a peptide or protein molecule allows conformational investigations in terms of intramolecular distances [20].

The expeditious development of the fluorescent-labelling techniques allowed to explore and discover several cellular functions. To study, and understand, the activity of signal transduction by visualizing protein binding or folding, the fluorescence correlation spectroscopy (FCS) and the fluorescence resonance energy transfer (FRET) are widely used [21]. Molecular tags that specifically bind to particular membrane-permeable dyes [22] allow to study protein dynamics and trafficking by fluorescence recovery after photobleaching (FRAP) as well the protein turnover [23, 24].

The great development of fluorescent-labelling techniques combined with the enormous technological advances in the field of fluorescence microscopy allowed to study, *in vivo* and *in vitro* systems, the protein distribution as well as their translocation and their interactions [25]. With specific and efficient fluorescent labelling, the proteins can be visualized in real time for the elucidation of their functions in a complex biological network, which also allows the detection of the protein-protein interactions, fundamental to understand intra- and intercellular communications [26].

Coumarins (benzopyranones or 2H-chromen-2-ones), whether natural products or synthetic ones, have also aroused a growing interest of the scientific community in the last decades due to their very significant pharmacological activity [27–37]. The nature and substitution pattern in the coumarins grant them diversified and exceptional optical properties with high fluorescence quantum yields [38]. Coumarins constitute the major class of fluorescent dyes [39–63], used as fluorescent labels and probes for physiological measurement [43–47], fluorescent whiteners [48], optical brighteners [49, 50], nonlinear optical chromophores [51–53], emission layers in organic light-emitting diodes (OLED) [54–57], and more recently, in caging [58–61], and labelling [62, 63]. Due to strong blue fluorescence of coumarin, it is easy to distinguish its light from green, yellow, and red,

an enormous advantage in multicolored fluorescence investigation. Developments from the last decade show that the introduction of appropriated substituents into the coumarin ring contributes to structures with improved photophysical and spectroscopic properties [64–66]. The synthesis of new fluorophores, with absorption and emission at long wavelengths, is of extreme importance for biological purposes, and the coumarins may play a leading role in this field.

2. Chemical labelling

Of all different fluorescent-labelling techniques, the chemical labelling is actually one of the most used as it allows novel types of experiments in biomolecules using a wider range of reactive fluorescent chromophores available. The covalent attachment of the chemical probes with specific amino acid has the advantage of being an irreversible process when compared to the non-covalent binding [67]. The chemical labelling methodology produces very stable bioconjugates, easy to manipulate with high efficiency, in a great number of available fluorophores that can be coupled covalently to the target biomolecule. Chemical labelling methods produce better results in *in vitro* studies rather than *in vivo* [18]. The most used methods in chemical labelling, in the biomolecules' native functional groups, under mild aqueous conditions, and using fluorescent coumarins, are discussed below.

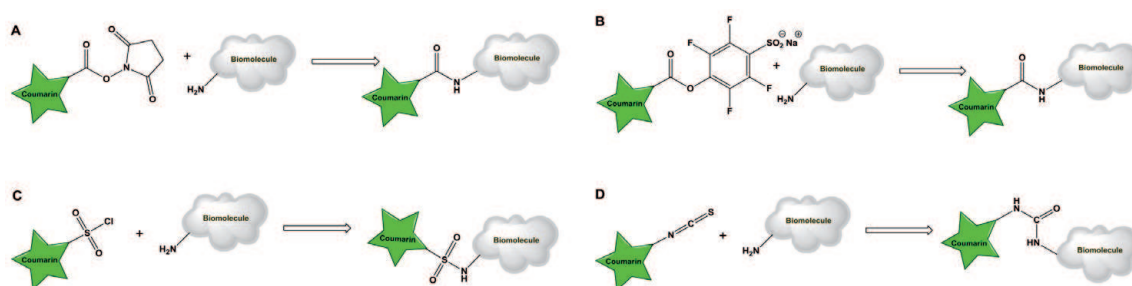
2.1 Amine reactive fluorescent coumarins

Presently, amine reactive fluorescent coumarins are widely used to label biomolecules, as peptides, proteins, oligonucleotides, and nucleic acids, among others. The fluorescent bioconjugates obtained are very useful in fluorescence *in situ* hybridization (FISH), receptor labelling immunochemistry, cell tracing, and fluorescent analog cytochemistry studies. Almost all of the techniques used in these tests implicate a robust fluorescent conjugate able to support rigorous incubation, hybridization, and washing steps, which is provided by the stability of the covalent bond between the amine reactive dye and biomolecule. Chemically, the amine labelling reaction proceeds usually through acylation pathway producing stable amide (or thiourea) bonds. The “ideal” reactions are those which require the same conditions as proteins, like functional group tolerance, compatibility, selectivity, water as solvent (or pH ~ 7), room temperature, high reaction rates, low reactant concentration, and nontoxic reagents.

A number of fluorescent amino-reactive coumarins have been developed to label various biomolecules, and the resultant conjugates are widely used in biological applications. Four major classes of amine-reactive fluorescent reagents are currently used to label biomolecules: succinimidyl esters (SE), 4-sulfotetrafluorophenyl (STP) esters, sulfonyl chlorides, and isothiocyanates [68]. **Figure 1** represents, in a general schematic diagram, the referred labelling reactions, between an amine group of a biomolecule and a fluorescent amino-reactive coumarin.

2.1.1 Fluorescent coumarin succinimidyl esters

Succinimidyl esters (SE) are proven to be very good reagents for amine modifications. These kinds of reagents are generally stable and show good reactivity and selectivity with aliphatic amines, such as the amine group of lysine side chain. Some of these kinds of reactive dyes are hydrophobic molecules and should be previously dissolved in anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO), but the sulfo-succinimidyl esters are water soluble. The amine labelling

**Figure 1.**

Schematic diagram of amine labelling techniques using succinimidyl esters (A), 4-sulfotetrafluorophenyl esters (B), sulfonyl chlorides (C), and isothiocyanates (D).

reaction with succinimidyl esters has a handicap, due to its great pH dependence. Succinimidyl esters react with non-protonated aliphatic amine groups, and the amine acylation reaction must be carried out at pH > 7.5. In the specific case of protein labelling by succinimidyl esters, the reactions require a pH between 7.5 and 8.5. Buffers used in labelling reactions shall not contain nucleophilic compounds because they may react with the labelling reagent to form unstable intermediates that could destroy the reactive dye. Most conjugations are done at room temperature, but either high or low temperature may be required for a particular labelling reaction. Some of the fluorescent coumarin succinimidyl esters contain a seven-atom aminohexanoyl spacer between the fluorophore and the reactive group, providing better solubility and spatial separation between the fluorophore and the target molecule being labeled. This separation potentially reduces the quenching that typically occurs upon conjugation and makes the dye more available for recognition by secondary detection reagents [68]. The most important fluorescent coumarin succinimidyl esters used for labelling biomolecules are shown in **Table 1**, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications [19, 68].

2.1.2 Fluorescent coumarin 4-sulfotetrafluorophenyl (STP) esters

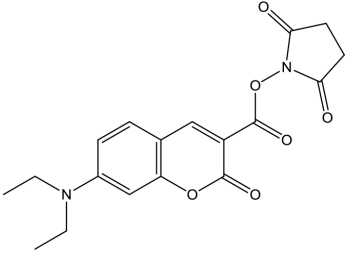
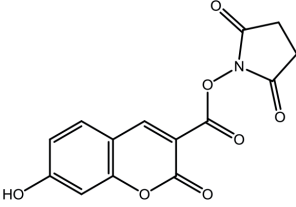
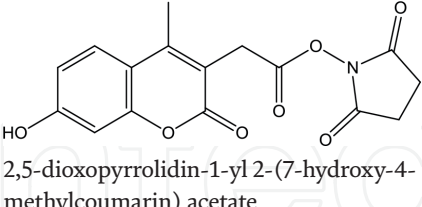
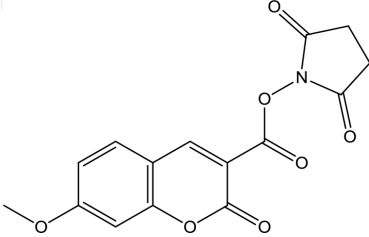
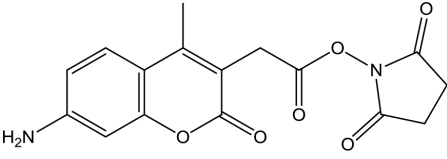
Some succinimidyl esters may not be compatible with a specific application due to their insolubility in aqueous solution. To overcome these limitations, the 4-sulfotetrafluorophenyl (STP) ester can be used. These sulfonated esters have higher water solubility than simple succinimidyl esters and sometimes eliminate the need for organic solvents in the conjugation reaction, which is a great advantage to maintain the native characteristics of biomolecules. They are, however, more polar than succinimidyl esters, which makes them less likely to react with buried amines in proteins or to penetrate cell membranes [68, 94]. **Table 2** presents the single fluorescent coumarin 4-sulfotetrafluorophenyl (STP) ester used for labelling biomolecules, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications [95, 96].

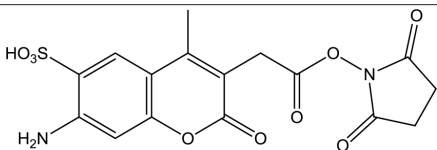
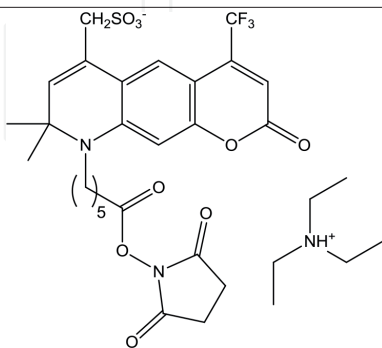
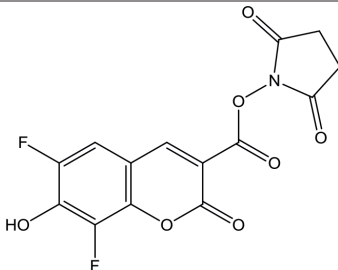
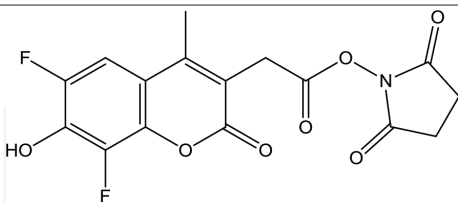
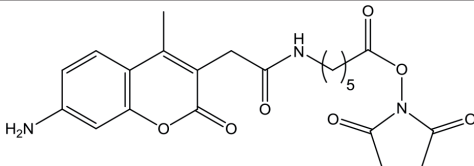
2.1.3 Fluorescent coumarin sulfonyl chlorides

Sulfonyl chlorides (SC) are highly reactive and are unstable in water, especially at high pH required for reaction with aliphatic amines. The labelling reactions with sulfonyl chlorides must be performed, carefully, at very low temperature in a place with local exhaust ventilation. Sulfonyl chlorides present a major reactive handicap as they can also easily react with other reactive groups present in biomolecules as phenols, thiols, aliphatic alcohols, imidazoles, and many others. Fortunately, this

kind of reactions rarely occurs in proteins or in aqueous solution, allowing the use of this type of chromophores to label proteins. Sulfonyl chloride dyes are generally hydrophobic molecules and should be dissolved in anhydrous dimethylformamide (DMF), but never in dimethylsulfoxide (DMSO) due to their highly instability in this solvent.

The labelling reactions of amines with SC reagents are strongly pH dependent, and the sulfonylation-based conjugations may require a pH 9.0–10.0 for optimal conjugations, which potentiates the sulfonyl chlorides' degradation by hydrolysis reactions. In general, sulfonylation-based conjugations have much lower yields than the succinimidyl ester-based conjugations. As in the case of succinimidyl esters, the buffers used in sulfonyl chloride reactions shall not contain nucleophilic compounds, because they may react with the labelling reagent to form unstable

Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 2,5-dioxopyrrolidin-1-yl-7-diethylaminocoumarin-3-carboxylate (DEAC SE)	432/472	Strong blue-fluorescent bioconjugates. Quite hydrophobic fluorescent dye, used for labelling live cells	[19, 69–72]
 2,5-dioxopyrrolidin-1-yl 7-hydroxycoumarin-3-carboxylate	363/447	One of the most popular blue-fluorescent dyes for labelling proteins and nucleic acids and increasingly used to label peptides, nucleotides, and carbohydrates	[19, 73]
 2,5-dioxopyrrolidin-1-yl 2-(7-hydroxy-4-methylcoumarin) acetate	364/458	Widely used for preparing bioconjugates of blue fluorescence but pH-dependent and environment-sensitive fluorescence	[19, 74, 75]
 2,5-dioxopyrrolidin-1-yl 7-methoxycoumarin-3-carboxylate	358/410	Used to label peptides and nucleotides with strong blue fluorescence and also used to label cell membranes although its fluorescence is quite short	[19, 72, 76, 77]
 2,5-dioxopyrrolidin-1-yl 2-(7-amino-4-methylcoumarin-3-yl)acetate	350/450	Used for fluorohistochemical examination of human kidney glomeruli. Reacts under mild conditions	[78, 79]

Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 <p>7-amino-3-(2-((2,5-dioxo-pyrrolidin-1-yl)oxy)-2-oxoethyl)-4-methylcoumarin-6-sulfonic acid (Alexa Fluor™ 350 SE)</p>	346/442	Blue-fluorescent dye, water soluble and pH insensitive from pH 4 to pH 10, used for stable signal generation in imaging and flow cytometry	[68, 80–83]
 <p>Triethylammonium (9-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-8,8-dimethyl-2-oxo-4-(trifluoromethyl)-8,9-dihydro-2H-pyrano[3,2-g]quinolin-6-yl)methanesulfonate (Alexa Fluor™ 430 SE)</p>	430/545	Bright green-fluorescent dye, water soluble and pH insensitive from pH 4 to pH 10. Used for stable signal generation in imaging and flow cytometry	[68, 80, 81, 84, 85]
 <p>2,5-dioxopyrrolidin-1-yl 6,8-difluoro-7-hydroxycoumarin-3-carboxylate (Pacific Blue™ SE)</p>	410/455	Conjugates of this dye are strongly fluorescent even at neutral pH. Ideally suited for 405 nm violet diode laser excitation on the Applied Biosystems® Attune™ Acoustic Focusing cytometer and similarly equipped fluorescence microscopes	[68, 86–88]
 <p>2,5-dioxopyrrolidin-1-yl 2-(6,8-difluoro-7-hydroxy-4-methylcoumarin-3-yl)acetate (Marina Blue™ SE)</p>	365/460	Conjugates that are strongly fluorescent, even at neutral pH. Optimally detected using optical filters configured for 4',6-diamidino-2-phenylindole (DAPI)	[68]
 <p>2,5-dioxopyrrolidin-1-yl 6-(2-(7-amino-4-methylcoumarin-3-yl)acetamido)hexanoate (AMCA-X SE)</p>	353/442	Conjugates yield blue fluorescence that can be used as a contrasting color in multicolor applications. Because its fluorescence may not be as bright as that of other dyes or may be obscured by autofluorescence, it is only recommended for use with highly abundant targets	[68, 89–91]

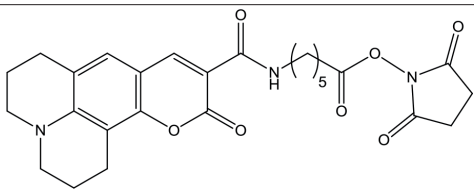
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 2,5-dioxopyrrolidin-1-yl 6-(11-oxo-2,3,5,6,7,11-hexahydro-1H-pyrano[2,3-f]pyrido[3,2,1-ij]quinoline-10-carboxamido)hexanoate (Coumarin 343 X SE)	437/477	Blue-emitting coumarin can be used to design fluorescence resonance energy transfer (FRET)-based assays with fluorescein amidite (FAM) as acceptor and to construct systems which harvest blue light energy	[92, 93]

Table 1.
Fluorescent coumarin succinimidyl esters used for biomolecule labelling.

intermediates that could destroy the reactive dye [19, 97–99]. **Table 3** shows fluorescent coumarin sulfonyl chlorides used for labelling biomolecules, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications. In addition to the coumarins presented in **Table 3**, new sulfonyl chloride coumarins have been developed, with high potential as fluorescent probes [100, 101].

2.1.4 Fluorescent coumarin isothiocyanates

Isothiocyanates form thioureas upon reaction with amines, but some thiourea products are much less stable than the conjugates that are prepared from the corresponding succinimidyl esters. Most part of isothiocyanate-reactive dyes are hydrophobic molecules and should be dissolved either in anhydrous dimethylformamide (DMF) or in dimethylsulfoxide (DMSO), and their reactions may require a pH 9.0–10.0 for optimal conjugations. As in the previous cases, the buffers used shall not contain nucleophilic compounds. The isothiocyanate conjugations are done at room temperature, but either high or low temperature may be required for a particular labelling reaction [19, 102]. The unique fluorescent coumarin isothiocyanate used for labelling biomolecules is shown in **Table 4**, but new isothiocyanate coumarins have been synthesized, with high potential as fluorescent probes [103, 104].

2.2 Thiol-reactive fluorescent coumarins

Cysteine is, in comparison with lysine, a rare amino acid present in biomolecules, and, for this reason, thiol-reactive reagents are used to label selectively a biomolecule at a defined site, probing their function, interaction, and biological structure. A great number of thiol-reactive dyes have been developed to analyze the proteins' topography in biological membranes, to measure the distances within (or between) proteins, and to observe and understand the changes in protein conformation using environmental sensitive probes.

Maleimides and iodoacetamides are the principal types of thiol-reactive coumarin dyes reported in the literature. Despite many similarities in their reactivity and selectivity toward thiol-reactive moieties, maleimides have a great advantage in relation to iodoacetamides, due to their high stability, solubility in simple solvent mixtures, and their high reactivity in the neutral pH range. Air oxidation of thiol compounds (to

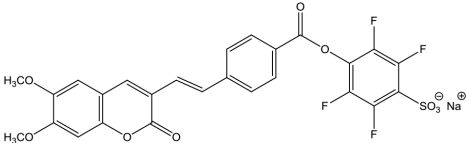
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 Sodium (E)-4-((4-(2-(6,7-dimethoxycoumarin-3-yl)vinyl)benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate	392/490	Used to label proteins and nucleotides with strong blue fluorescence. Blue-fluorescent dye, water soluble and pH insensitive with excellent photostability	[95, 96]

Table 2.
Fluorescent coumarin 4-sulfotetrafluorophenyl (STP) ester used for biomolecule labelling.

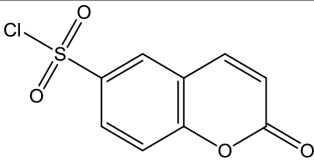
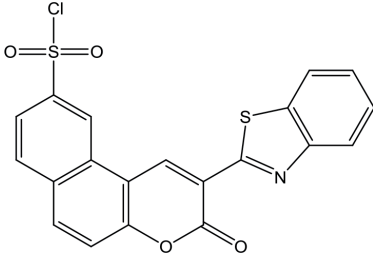
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 Coumarin-6-sulfonyl chloride	360/460	Used to label amines, amino acids, and phenols in mild conditions. Fluorescence produced in alkaline solution or in the presence of β -cyclodextrin	[99, 100]
 2-(benzo[d]thiazol-2-yl)-3-oxo-3H-benzo[f]chromene-9-sulfonyl chloride	405/435	Biosensor sensitive toward polarity changes in bio environments	[101]

Table 3.
Fluorescent coumarin sulfonyl chlorides used for biomolecule labelling.

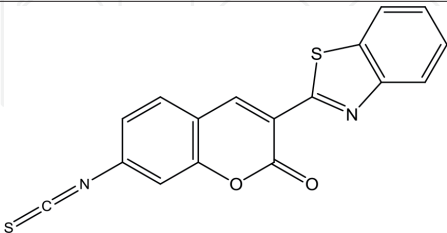
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 3-(benzo[d]thiazol-2-yl)-7-isothiocyanatocoumarin	485/535 (conjug.)	Selective determination of flu antigen	[102]

Table 4.
Fluorescent coumarin isothiocyanate used for biomolecule labelling.

disulfides) is a major competing reaction for the iodoacetamide modifications of thiol compounds [18, 19, 105]. Due to the disinterest on the development of new coumarin iodoacetamides, for the above reasons, only the fluorescent coumarin maleimides will be focused in this section. **Figure 2** represents, in a general schematic diagram, the thiol-labelling reaction with fluorescent coumarin maleimides.

2.2.1 Fluorescent coumarin maleimides

Maleimides readily react with thiol moieties of biomolecules to form thioether conjugates even under neutral conditions. The thioether bond formed is quite stable and is known to be responsible for the light produced, especially in the solution. Maleimides require conjugation conditions less rigorous than those of iodoacetamides and do not react with histidine and methionine under physiological conditions. Most labelling reactions can be done at room temperature at neutral pH. However, either elevated or reduced pH or temperature may be required for a particular labelling reaction [18, 19, 68]. In **Table 5**, the most important fluorescent coumarin maleimides used for labelling biomolecules are presented, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications.

2.3 Tyrosine-reactive fluorescent coumarins

The hydroxyl groups of the amino acids can be labeled with the same reagents used for the lysine residues, but the labelling reaction is carried out in organic solvent, like anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO), which absorbs the formed water molecule avoiding possible hydrolysis reactions. The amino acid

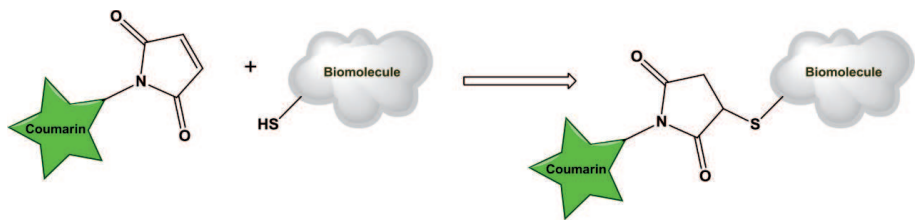
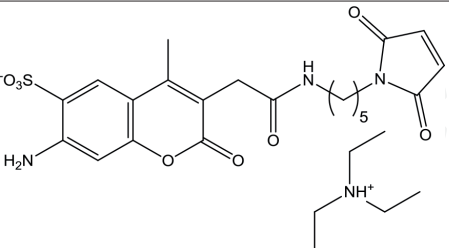
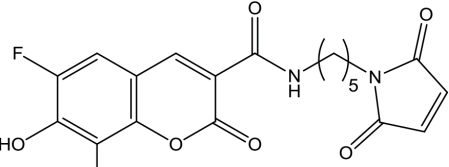


Figure 2.
Schematic diagram of thiol-labelling technique using maleimides.

Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Reference
 Triethylammonium 7-amino-3-(2-((5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)amino)-2-oxoethyl)-4-methylcoumarin-6-sulfonate (Alexa Fluor™ 350 C5 Maleimide)	345/444	Blue-fluorescent dye, with moderate photostability, water soluble and pH insensitive from pH 4 to pH 10, used for stable signal generation in imaging and flow cytometry	[68, 80, 81, 106–108]
 N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-6,8-difluoro-7-hydroxycoumarin-3-carboxamide (Pacific Blue™ C5-Maleimide)	410/455	Excellent reagent for thiol-selective modification, quantitation, and analysis and usually requires a higher pH than reaction of maleimides with thiols. Does not react with methionine, histidine, or tyrosine	[68, 80, 109]

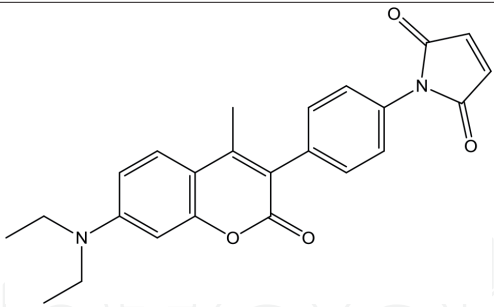
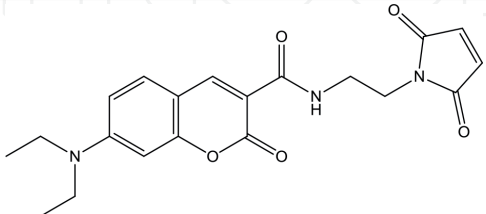
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Reference
 1-(4-(7-(diethylamino)-4-methylcoumarin-3-yl)phenyl)-1H-pyrrole-2,5-dione	391/472	Labelling of protein thiol groups in tissue sections. Fluorescence probe for glutathione intact cells Used to monitor release of thiols, to quantitate thiol in microplate reactions, and to distinguish proliferating cancer cells by nuclear protein staining	[68, 105]
 7-(diethylamino)-N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)coumarin-3-carboxamide	419/467	Used as a fluorescent biological sensing device and for real-time measurements for the release of inorganic phosphates during enzymatic reaction. Also, used for intramolecular fluorescence energy transfer (FRET) experiments	[68, 110–112]

Table 5.
Fluorescent coumarin maleimides used for biomolecule labelling.

hydroxyl groups do not allow highly specific labelling reactions due to the existence of several hydroxyl groups in biomolecules (serine, threonine, and tyrosine) [113].

One of the well-known labelling methods is the reaction with diazonium salts resulting in the formation of azo compounds, as 4-trifluoromethylcoumarin-7-diazonium chloride [114]. Although these aryl diazonium ions are promising for the desired application, their storage and delivery are challenging, and they often require in situ generation. The pH range should be between 8 and 10 for the formation of a phenolate anion [115].

3. Concluding remarks

Reactive fluorescent coumarins have been increasingly attracting special interest as fluorescent labels, with a wide range of applications in bioimaging and biolabelling, due to their extremely attractive and stable scaffold. Coumarins will allow the development of new low-cost fluorescent dyes due to its easy synthesis with high yields, large Stokes shift, pH independence of absorbance and emission, and excellent photostability, which represents a great value for the biological fluorescence imaging techniques.

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Conflict of interest

There are no conflicts of interest to declare.

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