FLUORESCENT COUMARIN DERIVATIVES: UNDERSTANDING MOLECULAR ARCHITECTURE, PHOTOPHYSICAL, AND CELL-IMAGING RESPONSES

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Abstract. *In this tutorial manuscript, we delve into the fundamental principles behind the design and synthesis of fluorescent coumarin-containing heterocycles, including some hybrid structures, and explore how molecular architecture influences their fluorescent properties. Through strategic substitutions at specific positions on the coumarin framework, it is possible to produce highly fluorescent derivatives that exhibit high fluorescence quantum yields, tunable emission wavelengths, and rapid responses to their microenvironments. These characteristics make coumarin derivatives particularly attractive for bioimaging applications, where precise visualization is crucial. Our focus is specifically on small organic molecules, as polymeric derivatives fall outside the scope of this manuscript. Small-molecule coumarin-containing derivatives have promising applications especially in cell-imaging as fluorescent bioprobes, making them valuable tools in biological research. Their ability to stain cells and multicellular models with precision allows for detailed visualization and analysis of biological processes. We illustrate these points with selected examples analyzed herein, highlighting the versatility and potential of coumarin derivatives in advancing biological research.*

Contents

1. Introduction

The chemical history of coumarin (Figure 1) dates back to 1820, nearly 200 years ago, when Vogel first extracted the heterocyclic coumarin from the tonka bean (*Dipteryx odorata*) and Guibourt independently obtained it from the flowers of Melilot or Sweet clover.[1] The name "coumarin" originated from the French word "coumarou", which was used for tonka beans and was named by A. Vogel. Chemically, coumarin (2*H*-1-benzopyran-2-one) is a benzolactone that can be functionalized at different positions. Coumarin occurs naturally as a secondary metabolite in several plants, microorganisms, and essential oils.[2] According to IUPAC,[3] the recommended nomenclature of the coumarin nucleus corresponds to 2*H*-chromen-2-one (2*H*-1-benzopyran-2-one or benzo-*α*-pyrone).[4] Coumarin-containing derivatives are extensively used in agriculture, pharmacy, and medicine. These naturally occurring compounds are basically classified into six types (Figure 1):[2] i) simple coumarin derivatives (which may encompass hydroxy substitution), ii) furanocoumarins, iii) pyranocoumarins, iv) dihydrofuranocoumarins (linear and angular), v) phenylcoumarins, and vi) bicoumarins. However, there are thousands of synthetic coumarin structures that are not encompassed in this classification, such as aminocoumarins and many others.[5]

Natural coumarins are highly valued for their extensive pharmacological properties, which draw significant interest from medicinal chemists who seek to modify their structure and explore their potential as new therapeutic agents.[2] The biological properties of coumarin-containing compounds have been exhaustively explored,[6] and this subject has been reviewed elsewhere.[7] Based on the importance of this

heterocyclic derivative, several classic and modern synthetic methodologies have been applied and developed[8] to obtain coumarin-based small molecules, and this subject has been recently reviewed as well.[9] These methodologies include catalyzed and non-catalyzed methods,[10] in addition to both metal-containing and metal-free versions and variants.[11] Hybridization and derivatization have been widely used strategies to obtain new derivatives and to improve biological responses.[11,12]

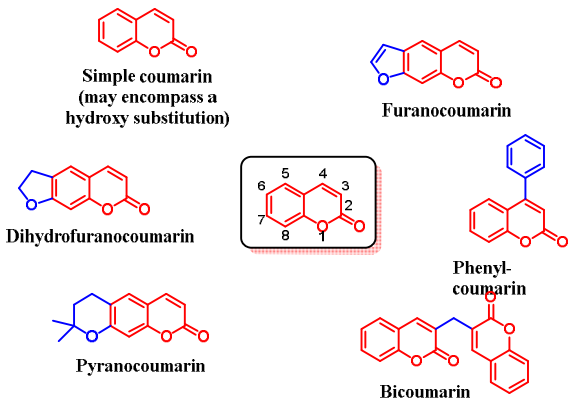


Figure 1. General structure of the coumarin framework and six types of naturally occurring coumarins. Note that these six types may be substituted at different positions. The web version of this manuscript provides the color details for this figure.

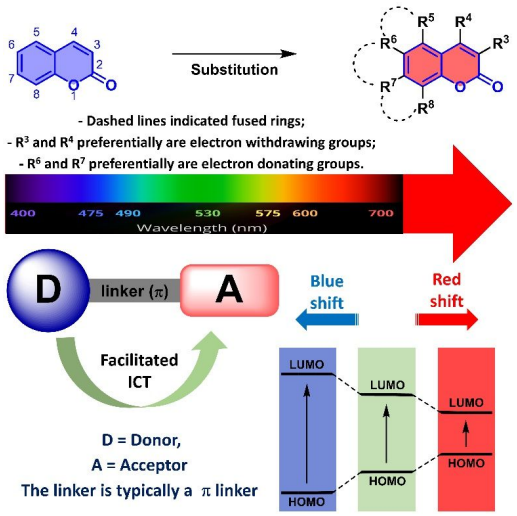
The application of coumarins, however, goes far beyond biological applications. This heterocyclic compound has also been applied in light technology.[13,14] The most basic structure of a coumarin (Figure 1) has nearly zero fluorescence emission,[14] but the appropriate substitution or functionalization of this elementary structure can afford highly fluorescent derivatives, closely related to its structure. Many coumarin compounds exhibit high fluorescence quantum yields, adjustable emission wavelengths, and rapid response to the polarity of their microenvironments. Comprehensive studies on the fluorescence properties of coumarin derivatives have revealed a significant structure-activity relationship, which is essential for designing important fluorescent materials, as we will disclose herein. Indeed, coumarins have been extensively studied for their fluorescence and UV-visible spectroscopy properties. The importance of these spectroscopic techniques in understanding the electronic transitions within coumarins, critical for applications ranging from the analysis of living cells to the design of organic light-emitting diodes and dye-sensitized solar cells, is emphasized in many works.[15-22]

The significant spectral shifts observed in coumarin derivatives due to the introduction of various substituents, which alter the electronic structure and, consequently, the absorption and emission properties, are of vital importance for photo- and biological responses. For example, as we will discuss herein, the replacement of electron-donating or electron-accepting groups at different positions on the coumarin ring can result in red or blue shifts in both absorption and emission spectra. This adaptability allows for the tailoring of coumarin derivatives for specific applications, such as increasing fluorescence efficiency or optimizing the absorption range for electronic device performance. Supported by both experimental data and theoretical studies on their absorption and emission spectra, coumarins are among the most widely used heterocycles in light technologies.

Based on our interest in fluorescent small molecule derivatives and their biological applications,[23-25] in this manuscript, we intend to analyze the basic principles in the design and synthesis of fluorescent coumarin-containing derivatives, including some hybrid structures, and the effect of the molecular architecture on the observed fluorescent properties. We will also focus on small organic molecule compounds, as polymeric derivatives are not of interest for this manuscript. Also, we will only analyze those selected examples of structures developed in the last decade to emphasize the recent development in this field of research.

2. Basic designing principles to obtain fluorescent coumarin derivatives

Since the basic structure of coumarin has very weak blue fluorescence (virtually non-fluorescent), with small Stokes shifts and low quantum yield, all rationales generally aim to improve these properties, especially targeting cell-imaging applications. Therefore, in the new compound, one should desire to observe large Stokes shifts, high quantum yields, and bathochromic shifts (towards red). In general, most available studies indicate that the substitution in the basic coumarin ring can afford fluorescent derivatives with improved emissions depending primarily on two aspects: the position where the substituent is inserted in the coumarin structure and the nature of the substituent. The substituent may be an electron-donating group, which strengthens the electron density in the coumarin ring, as observed in fused coumarin derivatives.[26] Coumarin-fused-coumarin derivatives have been reviewed and display attractive photophysical properties for different light technology applications.[27] Alternatively, the donating substituent may also facilitate intramolecular charge transfer (ICT) from the excited state, aiding in the structure stabilization from the excited state.[26] Conversely, the substituent can be an electron-withdrawing group, but this substitution should be placed in the correct position to improve the fluorescent properties of the coumarin derivative. As a general rule, modifying the coumarin structure can cause shifts in its fluorescence emission, either towards the blue or red end of the spectrum. When electron-withdrawing groups are attached at positions C3 or C4, and electron-donating groups are at positions C6 or C7, the fluorescence emission tends to shift towards longer wavelengths, that is, towards a red emission (Scheme 1). These rationales are typically also efficient to improve the light absorption properties of the designed coumarins.[28]



Scheme 1. General molecular architecture and ICT (intramolecular charge transfer) in coumarin derivatives. The blue or red shifts are dependent on the nature and position of the substituents. Note the relationship between HUMO-LUMO energy gap and the emission wavelength shift. The web version of this manuscript provides the color details for this figure.

Electron-donating groups, such as $–NH_2$, –OH, and alkylamines (*e.g.* $–NMe_2$, $–NEt_2$), when positioned at the 7-position, tend to lower the energy gap between the HOMO and LUMO, resulting in a red shift in both the absorption and fluorescence maxima. This red shift corresponds to lower energy transitions, which are further enhanced by increased the conjugation within the aromatic system. Conversely, electron-withdrawing substituents like $–CF_3$, –Cl, and –Br generally increase the energy gap, leading to a blue-shift in the absorption and emission spectra (see Scheme 1). The Stokes shift, which is also indicative of conformational changes between the ground and excited states, is reduced by electron-donating groups

and increased by electron-withdrawing groups, reflecting their impact on the photophysical properties of the molecule.[29]

The effects of substituents on the fluorescence quantum yields of coumarins usually correlate well with their electronic nature. Strong electron-donating groups at the 7-position typically enhance the fluorescence quantum yield, while electron-withdrawing or moderately donating groups tend to reduce it. This behavior is linked to changes in the electronic density at the oxygen atom in the coumarin structure, which affects the radiative decay process. Additionally, solvent polarity plays a crucial role, as the sensitivity of absorption and fluorescence to substituent effects increases with the dielectric constant of the solvent, further modulating the spectral properties of many coumarin derivatives.[29]

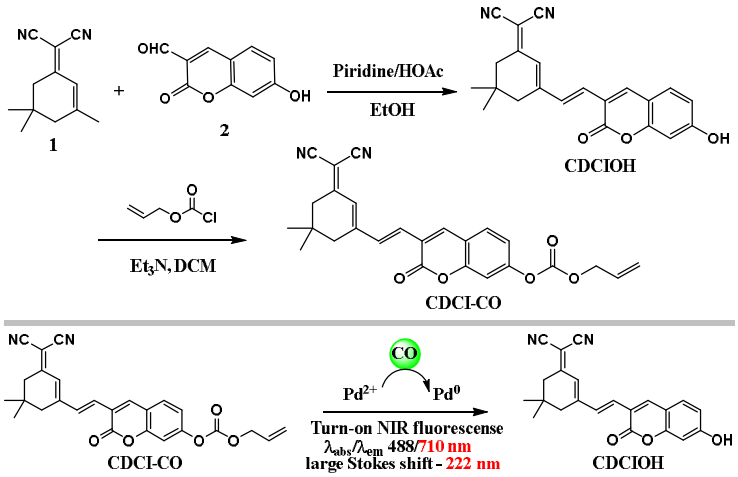
Solvatochromic effects can lead to significant variations in the observed photophysical properties of fluorescent coumarin derivatives. These effects are highly dependent on the solvent used and the conformations that the heterocyclic coumarin structures can adopt. For instance, the ICT process may be hindered in certain solvents, which in turn profoundly impacts the fluorescence and absorption characteristics of the compounds. This phenomenon has been notably observed in 7-NEt_2-substituted coumarins.[30,31] Studies have shown that these coumarins exhibit atypical behavior in non-polar solvents, where they tend to adopt a non-planar, pyramidal configuration due to the 7-NEt_2 group. This non-planarity disrupts the ICT process, leading to altered photophysical properties. In contrast, in solvents with moderate to high polarity, these dyes are generally found to adopt nearly planar structures that are more conducive to ICT. This transition to a planar conformation in polar environments enhances the effectiveness of the ICT process, thereby influencing the fluorescence emission and absorption spectra. The ability of solvents to modulate the molecular conformation and ICT process underscores the importance of solvent choice in the design and application of fluorescent coumarin-based probes.

Non-radiative transitions, such as intersystem crossing (ISC) or internal conversion (IC) from the first excited singlet state (S1) in organic dye molecules, are significant relaxation pathways that compete with photon emission (radiative decay). These processes can substantially influence the photophysical properties of the molecules, often reducing the quantum yield and efficiency of light emission. According to the energy gap law (EGL) of non-radiative transitions, IC (nonadiabatic vibronic coupling between S^1 and S^0) becomes increasingly significant for near-infrared (NIR) emitters.[32] The EGL, which describes the relationship between the energy gap and the rate of non-radiative decay, has been confirmed for IC from S^1 to S^0 in bulk solutions,[33] though deviations can occur depending on the molecular structure and environment. These deviations may arise from factors such as the rigidity of the molecular framework or specific interactions with the surrounding medium. In this context, as we will indicate in this manuscript, red emitters, particularly those designed for the NIR region, may require additional strategies to overcome the inherent drawbacks of the EGL. This is especially relevant since most coumarin derivatives, which are widely used as fluorescent probes, typically emit in the blue-green regions, limiting their applicability for NIR applications without significant structural modifications or the incorporation of additional functionalities to suppress IC and enhance radiative decay.[34]

3. Coumarin derivatives with simple molecular architecture and their fluorescence responses

A novel coumarin-based fluorescent probe called **CDCI-CO** for detecting carbon monoxide in biological systems has been developed (Scheme 2).[35] The probe exhibited near-infrared (NIR) fluorescence, providing distinct advantages such as high sensitivity and selectivity, rapid response, and significant NIR fluorescent turn-on signal changes at 710 nm. The probe's remarkable large Stokes shift of 222 nm effectively reduced interference from excitation and scattered light, making it highly attractive for practical applications. The appropriate substitution with both electron-donating and electron-withdrawing groups afforded large Stokes shifts, thus pointing firmly to the efficacy of the designed molecular architecture. The design of the probe indicates the incorporation of an electron-withdrawing group at the C3 position and the insertion of a quencher attached to oxygen at the C7 position. Upon CO detection, the formation of a hydroxyl group at C7 (electron-donating ability) allowed for the efficiency of the turn-on effect (Scheme 2). The probe was also able to detect CO with a detection limit as low as 33 nM, indicating its high sensitivity. The fluorescent probe **CDCI-CO** successfully demonstrated its capability to selectively image CO in living cells and zebrafish. The turn-on probe showed negligible fluorescence in control conditions but exhibited

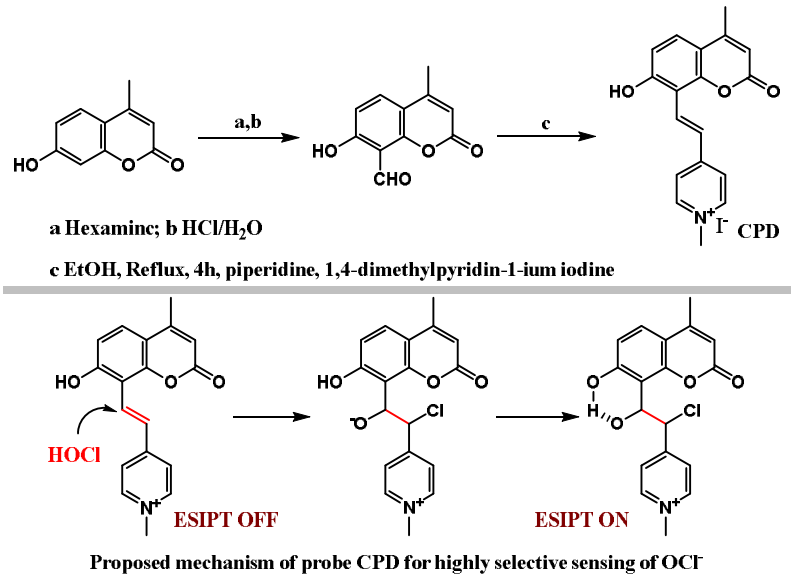
significantly enhanced NIR fluorescence upon CO detection. The imaging experiments in living zebrafish confirmed the probe's potential as an effective tool for in vivo CO detection.



Scheme 2. (Top) Synthesis of the coumarin-containing probe named **CDCI-CO** and (Bottom) the CO detection mechanism mediated by a Pd-catalyst. The web version of this manuscript provides the color details for this figure.

A novel mitochondria-targeted fluorescent probe, **CPD**, based on coumarin, was designed, synthesized, and applied (Scheme 3) to selectively detect hypochlorite (ClO^-).[36] This probe was meticulously engineered to detect ClO^- and target reactive oxygen species (ROS), which are crucial in various physiological processes and disease progression. The **CPD** probe exhibited remarkable photophysical properties: a rapid response time of less than 10 s, dual-channel detection in the blue and green emission ranges, and a notably large Stokes shift of 185 nm. These features significantly enhance the probe performance in complex biological environments by minimizing background interference and improving signal clarity. Additionally, the small-molecule probe demonstrated exceptional selectivity and sensitivity toward ClO^-, with a detection limit as low as 0.012 μM, making it highly effective for detecting trace amounts of this reactive species. In practical applications, the **CPD** probe successfully facilitated imaging of ClO^- within the mitochondria of A549 cells and zebrafish models, highlighting its potential for real-time monitoring of hypochlorite in living systems. This capability is crucial for studying the dynamic roles of ClO^- in biological processes and understanding its involvement in pathological conditions such as oxidative stress and inflammation.

The innovative design of the **CPD** probe incorporated a hydroxyl group at the C7 position of the coumarin scaffold and leveraged this feature to enable the formation of an excited-state intramolecular proton transfer (ESIPT)-prone derivative through a specific reaction with ClO^-. This elegant strategic modification enhanced the probe's sensitivity and contributed to its dual-channel emission, with one emission from the keto form and the other from the enol form of the ESIPT-prone structure. The selective detection of the anion highlights the importance of the molecular design in improving the emissive properties of the coumarin derivative. As noted in Scheme 3, the ESIPT-prone structures is only formed after the reaction of the specific analyte (ClO^-), hence the dual emission is only a possibility after the rection takes place. The molecular architecture designed for the new dye was innovative in the following aspects: first, the dye's structure was tailored for a specific reaction with hypochlorite; second, the in situ-formed coumarin derivative would exhibit the ESIPT signature, therefore allowing the authors to track not only fluorescence changes but also the analyte's presence in a biological environment. Lastly, the probe exhibited nearly zero fluorescence emission before reacting with hypochlorite, allowing the light-up to be observed by the naked eye, which is an always desirable feature in biological analysis.

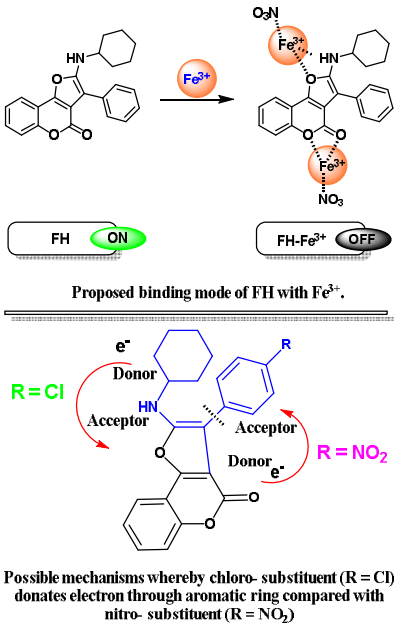


Scheme 3. (Top) Synthesis of the **CPD** fluorescent probe and (Bottom) the ClO^- detection through an ESIPT-prone derivative formation (ESIPT activation). The web version of this manuscript provides the color details for this figure.

The development of novel coumarin fluorescent probes for the selective detection of Fe^{3+} ions applied a fluorescence turn-off strategy (Scheme 4).[37] The research focuses on the synthesis of furocoumarin derivatives, specifically **FH** derivatives (**FCl** and **FNO_2**), which exhibit distinct photophysical behaviors. **FCl** showed intense fluorescence, while **FNO_2** displayed fluorescence quenching. The highly fluorescent **FH** derivative demonstrated significant quenching when interacting with Fe^{3+} ions, forming 1:2 probe-metal complexes. This quenching response was highly selective for Fe^{3+} compared to a broad range of other metal ions, underscoring the probe specificity and potential for targeted sensing applications. The authors explored the practical application of **FH** derivatives in real water samples, validating the probe ability to selectively detect Fe^{3+} and highlighting its potential for environmental monitoring and industrial processes.

The design and synthesis of these furocoumarin derivatives were based on a donor-acceptor molecular architecture, with substitutions at the C3 and C4 positions of the coumarin core crucial in modulating fluorescence properties. In particular, substituting the nitro group with a chlorine atom at one of these positions revealed a pronounced push-pull electronic effect, which "turned on" the fluorescence of the coumarin derivative. This modification allowed for a direct comparison with the **FH** model structure, providing valuable insights into the structure-property relationships governing fluorescence behavior. The ability to fine-tune photophysical properties through strategic chemical modifications highlights the versatility and potential of these furocoumarin derivatives for developing selective and sensitive fluorescent probes for metal ion detection.

The development of a novel fluorescent probe called **BC-OB**, designed for detecting hydrogen peroxide (H_2O_2) in biological systems, based on a π-extended coumarin, has been described (Scheme 5).[38] The **BC-OB** probe was designed to offer a turn-on fluorescence response specifically in the presence of hydrogen peroxide (H_2O_2). This design is particularly noteworthy due to the significant fluorescence enhancement that occurs as a result of an efficient intramolecular charge transfer (ICT) process. This process is triggered by the formation of a fluorescent coumarin derivative following the reaction with the reactive oxygen species (ROS), H_2O_2 in particular. The probe exhibits remarkable selectivity and sensitivity towards H_2O_2, with a detection limit as low as 0.47 μM and a high fluorescence quantum yield of 0.68, making it a highly effective tool for detecting low concentrations of this analyte.



Scheme 4. (Top) A turn-off Fe^{3+} detection system based on a fluorescent coumarin derivative and (Bottom) its donor-acceptor molecular architecture. **FH** (R=H), **FCl** (R=Cl), and **FNO$_2$** (R=NO$_2$). The web version of this manuscript provides the color details for this figure.

The **BC-OB** probe has also demonstrated its capability in bioimaging applications, as evidenced by its successful detection of endogenous H_2O_2 in RAW 264.7 cells. The study emphasizes the probe's stability across a range of physiological pH levels, ensuring that it can provide clear fluorescence signals in both neutral and alkaline environments. The well-designed coumarin derivative at the core of this probe features an electron-withdrawing group at the C3 position, along with the functionalization of the oxygen at the C7 position with a phenylboronic acid derivative. This specific functionalization strategy effectively blocks the ICT process, thereby quenching the fluorescence under normal conditions.

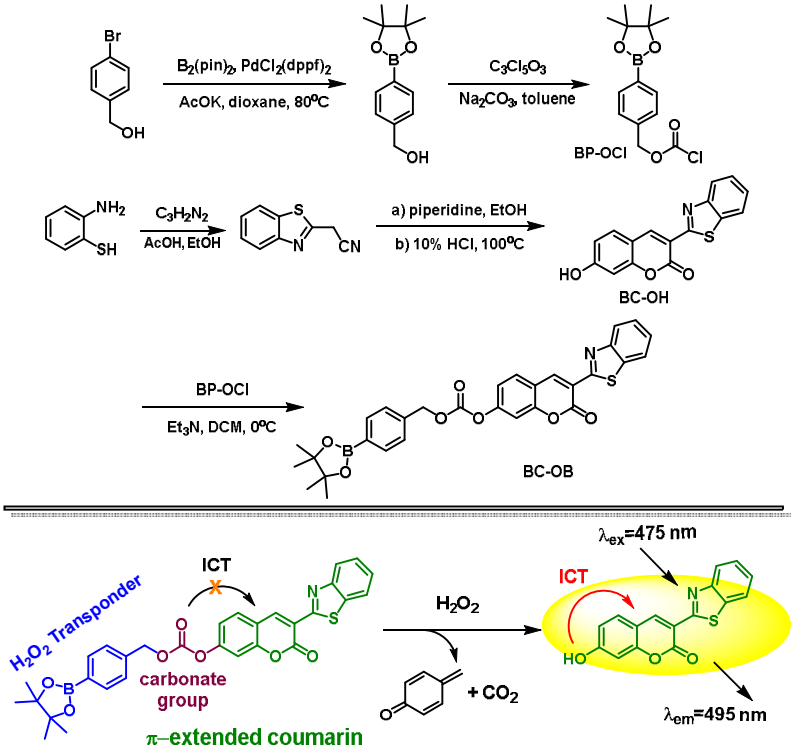
Upon exposure to H_2O_2, the designed coumarin derivative undergoes a reaction that restores the hydroxyl group at the C7 position. This restoration reestablishes the ICT process possibility, thus resulting in a significant increase in fluorescence intensity: a "light-up" effect. This design enabled a highly efficient and specific turn-on fluorescence response, making the **BC-OB** probe a powerful tool for detecting this ROS analyte and studying related biological processes.

All these aforementioned examples clearly demonstrate that the application of the basic principles to improve the fluorescence emissions and Stokes shifts of coumarin heterocyclic derivatives can yield useful bioprobes with designed molecular architectures that exhibit interesting responses.

4. The strategy of hybrid coumarin derivatives

A few words are necessary regarding the use of hybrid fluorophores. Hybrid fluorophores represent a significant advancement in the field of fluorescent probe development, offering unique advantages over traditional single-component fluorophores, as recently reviewed.[39] By combining the properties of two or more different fluorophores at the molecular level, hybrid fluorophores can harness the strengths of each component while mitigating their individual limitations. This hybridization allows for the creation of fluorophores with enhanced photophysical properties, such as improved brightness, larger Stokes shifts, and extended emission wavelengths, particularly in the near-infrared (NIR) region, which is crucial for deep-tissue imaging in biological systems. The ability to fine-tune these properties through strategic molecular design makes hybrid fluorophores versatile tools in bioimaging, enabling more precise and

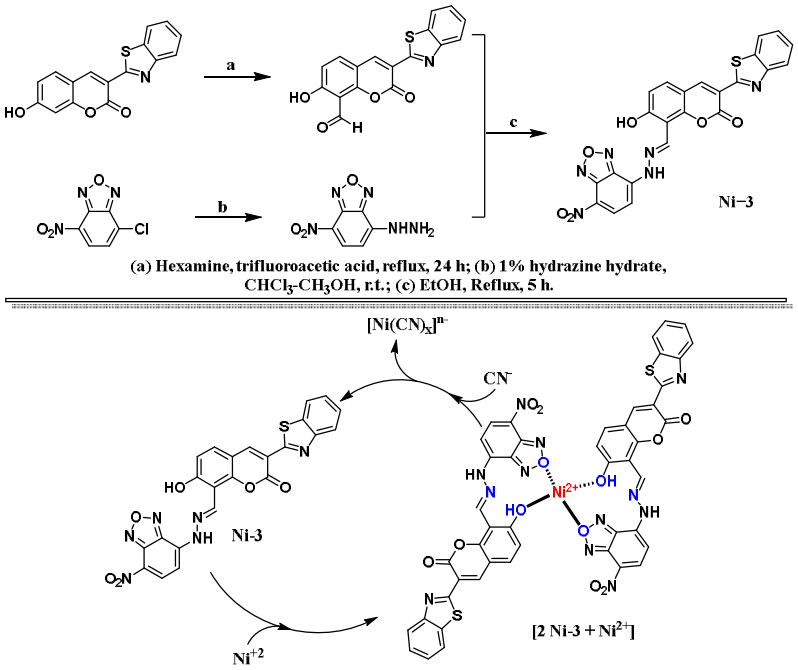
reliable detection of various biological processes. The multifunctionality of hybrid fluorophores opens new routes for their application in complex biological environments. These fluorophores can be engineered to possess targeting capabilities, such as mitochondrial localization, and to respond to specific stimuli or environmental changes, such as pH shifts or the presence of specific ions or molecules. This responsiveness, combined with the ability to perform multiple functions within a single molecular framework, enhances the utility of hybrid fluorophores in both diagnostic and therapeutic applications. The integration of complementary functionalities within a single fluorophore not only streamlines the design of molecular probes, but also reduces potential interference and crosstalk, leading to more accurate and efficient biological assays. As a result, hybrid fluorophores are becoming increasingly important in the development of next-generation fluorescent probes for life sciences and medical research. In this context, with the appropriate molecular architecture and positions of substitution in the coumarin core, hybrid fluorophores may be a powerful tool to overcome most of the limitations observed in fluorophores based solely on the coumarin heterocycle.



Scheme 5. (Top) The synthetic route of probe **BC-OB** and (Bottom) Illustration of designing a "turn-on" fluorescent probe **BC-OB** based on a π-extended coumarin fluorophore for H_2O_2 detection. The web version of this manuscript provides the color details for this figure.

A hybrid coumarin-based near-infrared fluorescent probe (named **Ni-3**) that exhibited a large Stokes shift for the sequential recognition of Ni^{2+} and CN^- ions has been described (Scheme 6).[40] The probe was based on 3-benzothiazolyl-7-hydroxycoumarin and featured a highly selective colorimetric and fluorescence quenching response to Ni^{2+} due to its paramagnetic effect and chelation-enhanced quenching (CHEQ) mechanism. The sequential detection of CN^- was achieved through a Ni^{2+} displacement approach. The Stokes shift observed for the probe was 145 nm, which is significant as it reduces the spectral overlap between absorption and emission, enhancing the reliability of fluorescence-based detection. The importance

of the hybrid coumarin structure lies in its ability to extend the π-conjugation system by linking benzofurazan and coumarin through a C=N bond, which allowed the probe to emit in the near-infrared region. This hybridization resulted in improved photophysical properties, such as a better anti-fatigue performance during repeated detection cycles. Additionally, the probe demonstrated highly selective recognition, with a limit of detection for CN^- that was lower than the maximum acceptable concentration level in drinking water set by the World Health Organization (WHO), making it a valuable tool for environmental monitoring and potential biological applications.

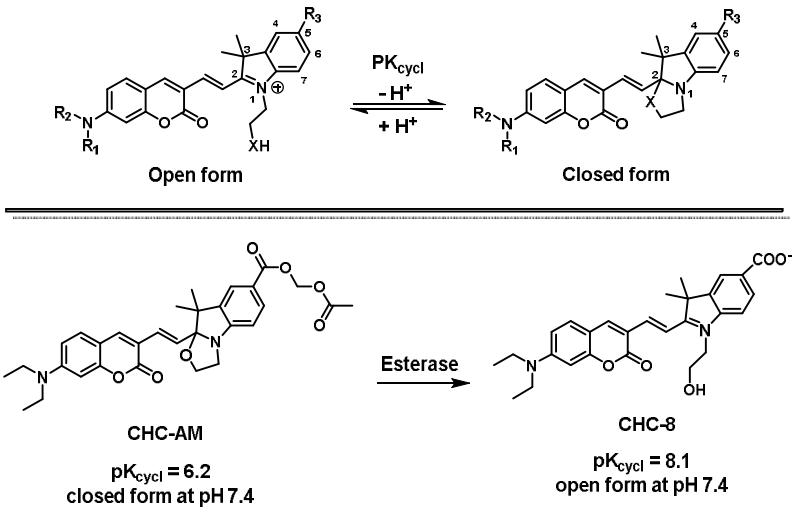


Scheme 6. (Top) Synthesis of the hybrid fluorescent coumarin derivative and (Bottom) mechanism of Ni^{2+} detection followed by CN^- detection. The web version of this manuscript provides the color details for this figure.

An activable fluorescent probes for hydrolase enzymes based on a coumarin-hemicyanine (CHC) hybrid fluorophore has been designed, synthesized and efficiently applied (Scheme 7).[41] The **CHC-1** designed fluorophore was engineered to undergo intramolecular spirocyclization, a process that can be finely tuned by chemical modifications, resulting in significant changes in the optical properties of the probes. This spirocyclization mechanism allowed the designed CHC fluorophores to exhibit large Stokes shifts, which is advantageous for reducing spectral overlap between excitation and emission, thereby improving the signal-to-noise ratio in fluorescence detection. Specifically, the study focused on developing probes for enzymes like γ-glutamyltranspeptidase (GGT) and esterase, which are important in various biological and pathological processes, including cancer.

The importance of the **CHC-1** hybrid lies in its ability to combine the properties of both coumarin and hemicyanine, enabling the design of fluorophores with red-shifted emission wavelengths and large Stokes shifts. For instance, the **CHC-1** probe targeting GGT exhibited a Stokes shift of 96 nm, while the esterase-targeting probe showed a Stokes shift of 66 nm. These large Stokes shifts minimized the overlap between absorption and emission spectra, allowing for more sensitive detection of enzymatic activities in biological samples. The study demonstrated the potential of the **CHC-1** hybrid scaffold as a versatile platform for

developing a wide range of actible fluorescent probes, useful for probing hydrolase functions in various biological contexts.

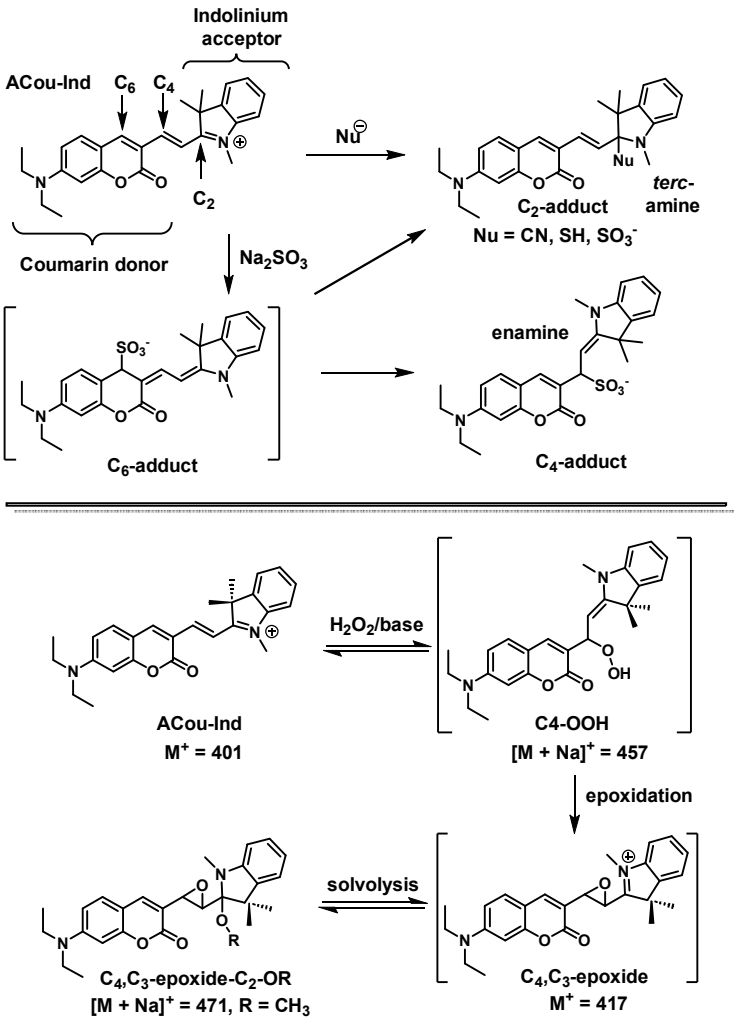


Scheme 7. (Top) Dependence of pH noted in the hybrid fluorescent coumarin derivative and (Bottom) reversible and pH-dependent cyclization and ring opening, changing the fluorescent properties in the presence and absence of Esterase.

The development and investigation of a ratiometric fluorescent probe based on a 7-(diethylamino)coumarin-hemicyanine hybrid (named **ACou-Ind**) for the detection of hydrogen peroxide (H_2O_2) has been described (Scheme 8).[42] The study focused on elucidating the chemosensing mechanism of this hybrid compound, particularly in the presence of reactive sulfur species (RSS) and ROS. The research highlighted that the coumarin-hemicyanine hybrid could serve as a classic ratiometric fluorescent probe by showing a significant shift from red to blue fluorescence upon interaction with specific nucleophilic analytes. The paper revised the previously suggested chemosensing mechanism and provided new insights into the probe's interaction with thiolates and ROS, emphasizing the importance of the hybrid structure for achieving selective and sensitive detection. Note that this example follows the opposite trend of shifting the emission from blue to red; in this case, the red to blue shift was possible because the fluorescent probe was already designed to emit in the red region.

The importance of this hybrid coumarin structure lies in its ability to combine the fluorescent properties of coumarin with the reactive nature of hemicyanine, creating a versatile probe that responds to a wide range of biological analytes. This dual functionality enables the probe to detect hydrogen peroxide with a high degree of sensitivity, making it valuable for studying oxidative stress and other ROS-related cellular processes. The Stokes shift observed in this study is particularly significant because it ensures minimal overlap between the excitation and emission spectra, thereby reducing background noise and improving the overall performance of the probe in complex biological environments.

The synthesis and evaluation of a ratiometric fluorescent probe based on a phenothiazine-coumarin-pyridine hybrid for the detection of hypochlorous acid (HOCl), named **Probe1**, has been described (Scheme 9).[43] The researchers designed this hybrid molecule to exploit the electron-donating properties of phenothiazine and the electron-accepting properties of pyridine, coupled with the fluorescent characteristics of coumarin. Upon exposure to HOCl, the bioprobe exhibited a significant shift in its emission spectrum from deep-red (~630 nm) to cyan (~500 nm), enabling the accurate ratiometric sensing of HOCl without interference from spectral crosstalk. Once again, from red to blue shifts. This design provided a robust method for the selective and sensitive detection of HOCl, which is crucial for understanding its role in various biological processes.



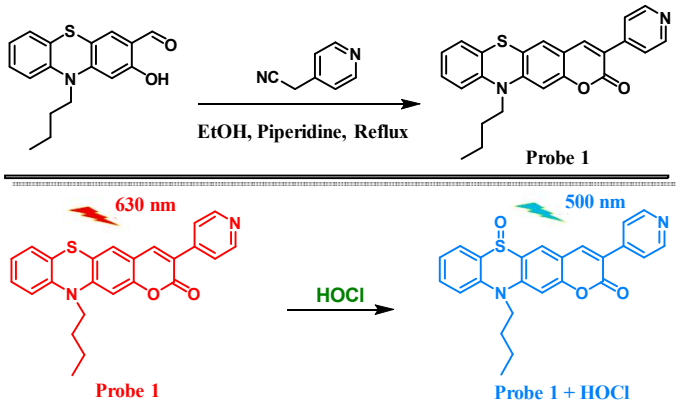
Scheme 8. (Top) Molecular architecture and reactivity of the of the hybrid fluorescent coumarin **ACou-Ind**. (Bottom) Sensing mechanisms of **ACou-Ind**. The web version of this manuscript provides the color details for this figure.

The importance of the coumarin hybrid lies in its ability to combine multiple functional groups into a single molecule, thereby enhancing its photophysical properties and enabling efficient detection in complex biological environments. The Stokes shift observed was large, approximately 130 nm, which is advantageous because it minimized any overlap between the excitation and emission spectra, reducing background interference and improving the probe's performance. This feature is particularly important for imaging applications, where clear and accurate fluorescent signals are essential for visualizing HOCl in live cells and organisms.

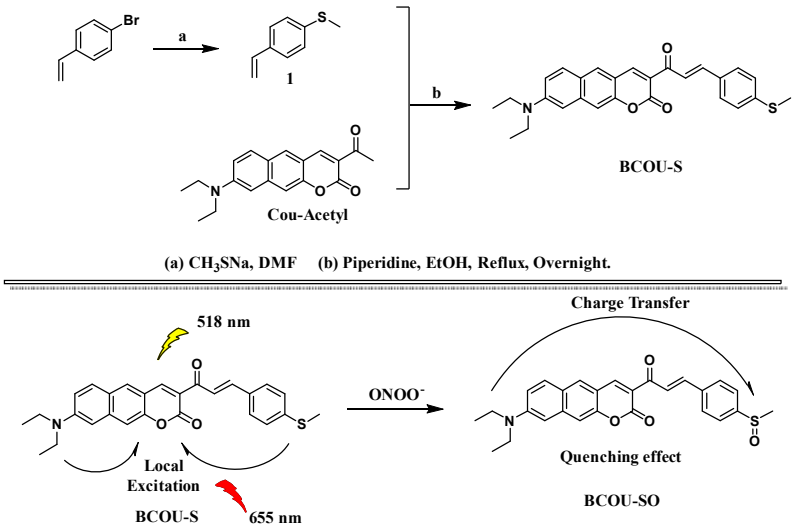
5. Highlighted cell-imaging applications

The synthesis of a coumarin derivative named **BCOU-S** probe has been described (Scheme 10).[44] While successful, it presented some challenges typical of creating complex fluorescent probes. The primary

challenge involved designing and constructing a stable and efficient molecular structure capable of performing dual functions: targeting lipid droplets (LDs) and selectively responding to peroxynitrite ($ONOO^-$) in living cells. The molecular architecture was key to the efficiency of the new structure. Direct π-extension at positions C6 and C7 resulted in a lowered energy band gap and a red shift, while maintaining structural stability. The probe, also used to evaluate drug-induced liver injury (DILI), integrated a π-extended coumarin core, providing high lipophilicity for targeting LDs, and a methyl thioether group as a recognition site for $ONOO^-$. **BCOU-S** exhibited a large Stokes shift of 137 nm and a low detection limit of 27 nM, making it highly sensitive for detecting subtle changes in $ONOO^-$ levels and LDs status in live cells. The study demonstrates that **BCOU-S** can effectively differentiate between various levels of DILI caused by drugs like acetaminophen, establishing clear time- and dose-dependent relationships. This dual-parameter monitoring approach significantly reduces false positives in early DILI detection compared to single-parameter methods.



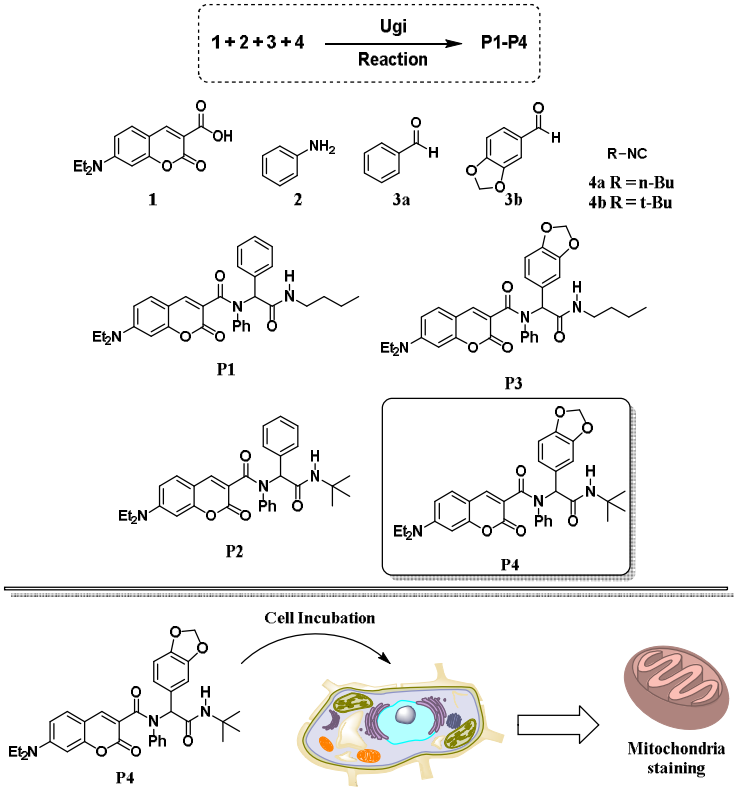
Scheme 9. (Top) Synthesis of the hybrid fluorescent coumarin **Probe1** and (Bottom) sensing mechanism for hypochlorous acid detection. The web version of this manuscript provides the color details for this figure.



Scheme 10. (Top) Synthesis of the fluorescent coumarin **BCOU-S**. (Bottom) Sensing mechanisms for $ONOO^-$ detection. The probe was also efficient in detecting LDs dynamics in live cells. The web version of this manuscript provides the color details for this figure.

The research highlights the probe ability to independently track $ONOO^-$ fluctuations and LDs accumulation, crucial for understanding DILI mechanisms. The probe showed high selectivity toward $ONOO^-$ over other reactive species and maintained stability across a wide pH range. Cellular experiments revealed that **BCOU-S** could visualize both exogenous and endogenous $ONOO^-$ in HepG2 cells, with substantial fluorescence changes upon $ONOO^-$ exposure (Scheme 10). Additionally, the probe effectively monitored LDs dynamics during oleic acid-induced expansion and starvation-induced depletion. In an acetaminophen-induced DILI model, **BCOU-S** detected significant LDs accumulation and $ONOO^-$ level increases, demonstrating its potential for early DILI diagnosis and prevention by simultaneously addressing two independent biomarkers.

The synthesis and application of four new fluorescent peptoids (**P1-P4**) has been described using the Ugi multicomponent reaction (U4CR) to obtain the structures of interest (Scheme 11).[45] The synthesis of the fluorescent derivatives primarily employed a 7-NET_2-containing coumarin carboxylic acid derivative as the main strategy. This simple yet elegant approach facilitated the direct production of synthetic derivatives with reasonable photophysical properties, such as emission in the blue to green regions. However, the Stokes shifts were generally small, approximately 45-60 nm. The study focused on evaluating the photophysical properties of peptoids and their potential as biomarkers for live cell imaging, specifically in breast cancer cells (MCF-7). The peptoids were tested at nanomolar concentrations to minimize cytotoxicity, ensuring accurate subcellular localization without disrupting cellular homeostasis. This approach was crucial for obtaining reliable imaging results while maintaining cell viability of the tested model.

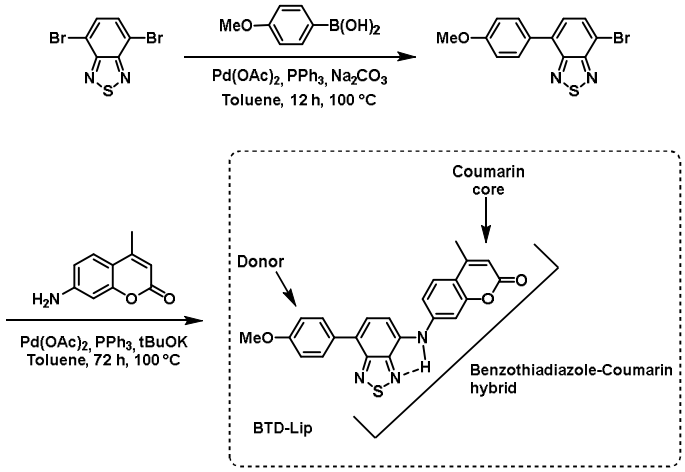


Scheme 11. Multicomponent synthesis of fluorescent coumarin-based peptoids (**P1-P4**) applied in cell imaging experiments. **P4** showed a high preference for mitochondria inside live cells. The web version of this manuscript provides the color details for this figure.

Among the four blue fluorescent peptoids examined **P1-P4**, only **P4** exhibited specific affinity for mitochondria, as confirmed by co-staining experiments with the established mitochondrial marker MitoTracker Red. This suggested that **P4** could serve as a selective and reliable mitochondrial marker, providing a valuable tool for studying mitochondrial dynamics and function, with imaging conducted in the blue channel. The other three peptoids **P1-P3** exhibited minimal selectivity and were dispersed throughout the cytosol of the cells. The synthesis of these peptoids involved challenges, particularly in optimizing the incorporation of a fluorescent coumarin tag while maintaining desired photophysical properties, such as brightness and stability. It was essential for the peptoids to efficiently penetrate cell membranes and selectively target specific organelles. Balancing these factors was critical for the success of the imaging probes.

In addition to targeting capabilities, the photostability and cellular selectivity of the peptoids were rigorously evaluated. Among them, **P4** once again demonstrated superior photostability and selectivity compared to commercial dyes like MitoTracker Red. This enhanced performance underscores the **P4** potential as a highly selective and stable probe for live cell imaging, especially for studies requiring long-term imaging or high-resolution subcellular localization. Overall, this study highlights that fluorescent peptoids, particularly **P4**, offer significant promise for live cell imaging. They provide high selectivity, photostability, and minimal cytotoxicity, making them valuable tools for diverse biological and medical research applications.

The synthesis, characterization, and application of a novel fluorescent hybrid coumarin-containing compound, named **BTD-Lip**, has been published (Scheme 12).[46] The molecular architecture was crucial for developing a new and stable red emitter based on the coumarin heterocycle. Substitutions were designed to incorporate an *N*-group at the C7 position of the coumarin core, facilitating intramolecular hydrogen bonding with the 2,1,3-benzothiadiazole heterocycle, thereby creating a hybrid structure. This approach promoted planarity and conjugation. The strategy proved effective, resulting in large Stokes shifts observed in all tested solvents, including water, with shifts of up to 120 nm.



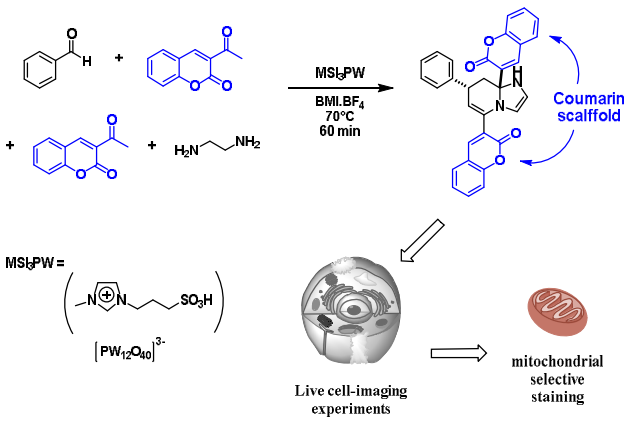
Scheme 12. Synthesis and rationale of the molecular architecture in the design of the hybrid fluorescent benzothiadiazole-coumarin derivative **BTD-Lip**.

The challenges in synthesizing **BTD-Lip** included optimizing the structure to balance its lipophilicity and photophysical properties, essential for efficient intracellular staining and imaging. The BTD core was chosen for its stable emission properties, while the coumarin moiety was selected to enhance lipophilicity and extend π-conjugation, as reviewed elsewhere.[47,48] Despite these challenges, the two-step synthesis was successful, yielding a compound that not only surpassed BODIPY in performance but also demonstrated impressive selectivity in the more complex *C. elegans* model. **BTD-Lip** was able to selectively stain lipid-

rich regions in the nematode, where BODIPY showed nonspecific staining, highlighting the **BTD-Lip** potential as a superior tool for lipid structure visualization in both simple and complex biological systems. The biological study focused on the selective staining of LDs in both live cells and the nematode *Caenorhabditis elegans*. **BTD-Lip**, the hybrid benzothiadiazole-coumarin derivative, demonstrated significant advantages over commercially available dyes like BODIPY, including the ability to stain a broader range of lipid structures. In live cell experiments, **BTD-Lip** stained approximately 32% more LDs than BODIPY, while in fixed cells, it stained about 40% more. The compound also exhibited excellent photostability and a large Stokes shift, which facilitated its use in imaging without photobleaching.

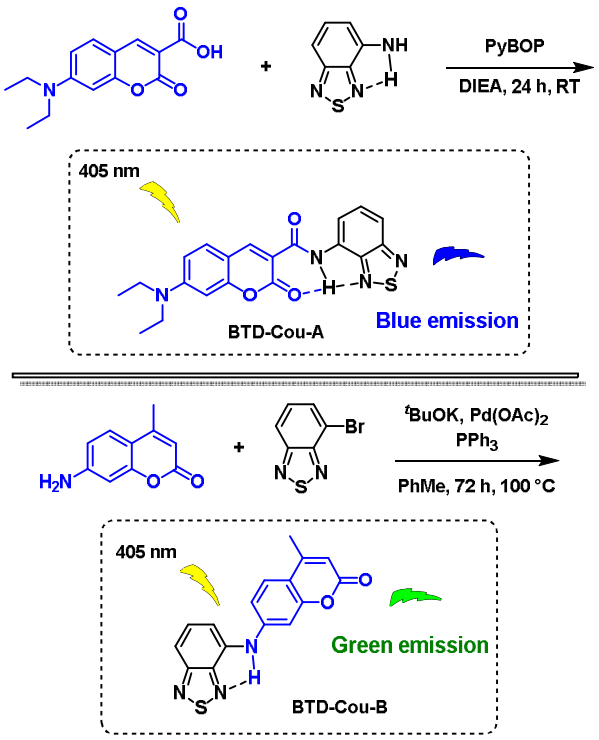
A multicomponent synthesis of bridgehead nitrogen heterocycles, *i.e.* hexahydroimidazo[1,2-*a*]pyridine (**HImP**) derivatives has been described (Scheme 13).[49] The study optimized the reaction conditions, achieving the exclusive formation of the *trans* isomer in just 1 h. A fluorescent derivative incorporating a coumarin scaffold was also synthesized, which demonstrated preferential accumulation in mitochondria when tested as a live-cell imaging probe. The incorporation of the coumarin scaffold was particularly significant due to its well-known photophysical properties, including high fluorescence efficiency and large Stokes shift, which make it an ideal candidate for bioimaging applications. The coumarin moiety not only provided the **HImP** derivative with desirable fluorescent properties but also ensured selective mitochondrial targeting in live cells, as evidenced by the probe's performance in fluorescence microscopy experiments. This study highlighted the dual utility of the coumarin-containing HImP derivative as both a valuable chemical scaffold for synthesis and a potent tool for biological imaging.

The coumarin-containing derivative **HImP 4e** displayed large Stokes shifts across various solvents, ranging from 105 to 194 nm, which is advantageous for imaging applications as it minimizes spectral overlap and autofluorescence issues. The molar extinction coefficients were also notably high, with values between 3.78 and 4.26 mM^{-1} cm^{-1}, indicating strong absorption. The emission maxima were in the blue to green region, making the compound well suited for fluorescence microscopy using standard filters. These properties allowed **HImP 4e** to be used effectively as a live-cell imaging probe, where it showed intense green fluorescence and preferential accumulation near the nuclei of MCF-7 breast cancer cells, suggesting mitochondrial localization. It is noted that both coumarins in the structure of **HImP 4e** are substituted with a bridgehead nitrogen heterocycle at the C3 positions, which imparts electron-withdrawing properties. This substitution aligns with the expected red shift and the electronic distribution that favors the stability of the entire molecule.



Scheme 13. Multicomponent synthesis of hexahydroimidazo[1,2-*a*]pyridine incorporating the coumarin scaffold to activate the fluorescence of the bridgehead nitrogen heterocycle. Derivative **HImP 4e** had a preference for mitochondrial selective staining. The web version of this manuscript provides the color details for this figure.

Another hybrid benzothiadiazole-coumarin was disclosed with interesting results. The article focused on the synthesis and characterization of hybrid benzothiadiazole-coumarin dyes, specifically **BTD-Cou-A** and **BTD-Cou-B**, designed for photophysical applications and bioimaging (Scheme 14).[50] The photophysical evaluation of the synthesized dyes revealed that they exhibit strong π-π* transitions, characterized by large molar extinction coefficients and relatively small Stokes shifts, particularly in the case of **BTD-Cou-A**. **BTD-Cou-B**, however, demonstrated favorable Stokes shift values, highlighting a distinct difference between the two derivatives. The coumarin moiety played a crucial role in stabilizing the ICT process, significantly impacting the emission properties and photostability of both fluorophores. Notably, the designed **BTD-Cou-B** exhibited a stronger ICT character and larger Stokes shifts compared to **BTD-Cou-A**, making it more suitable for specialized imaging applications. In **BTD-Cou-A**, substitution at the C3 position with an amide containing the electron-withdrawing benzothiadiazole core, resulted in relatively modest Stokes shifts (21-77 nm), despite its excellent performance in cellular and multicellular responses. Conversely, the C7 substitution in **BTD-Cou-B** led to the development of a new fluorescent derivative with considerably larger Stokes shifts (100-150 nm). This derivative exhibited outstanding performance across all bioimaging experiments, both in vitro and in vivo, and was effectively monitored in the green channel, displaying a bright and consistent emission.



Scheme 14. Hybrid benzothiadiazole-coumarin derivatives synthesized and applied for selective plasma membrane staining and imaging in zebrafish. The web version of this manuscript provides the color details for this figure.

Coumarin role was pivotal in enhancing the lipophilic nature of dyes and ICT processes, thus improving their photophysical properties, particularly for **BTD-Cou-B**. Both dyes showed excellent photostability and low cytotoxicity in cellular bioimaging tests, with **BTD-Cou-A** emitting intensely in the blue channel and **BTD-Cou-B** in the green channel. The selective staining of cellular membranes highlights their potential for bioimaging applications, with coumarin significantly contributing to the efficient cellular

interaction and high emission intensities observed during imaging experiments. **BTD-Cou-A** and **BTD-Cou-B** were evaluated for their staining capabilities in zebrafish embryos, a complex multicellular model. The dyes demonstrated excellent selectivity, particularly staining the cerebral and somite regions with minimal background fluorescence. Compared to the commercial dye CellMask, the hybrid dyes offered more precise and selective staining, especially in specific areas of the brain, highlighting their potential as effective tools for detailed imaging in complex biological systems such as zebrafish embryos.

6. Concluding remarks

In this manuscript, we have explored the complexities of designing and synthesizing fluorescent coumarin-containing derivatives, with a particular focus on their potential applications as bioprobes in biological systems. The studies presented here underscore the critical role that molecular architecture plays in modulating the photophysical properties of coumarin derivatives, such as fluorescence quantum yields, Stokes shifts, and emission wavelengths. By analyzing various coumarin-based probes, we have demonstrated that strategic substitutions on the coumarin core can lead to significant improvements in fluorescence emission characteristics. The introduction of electron-donating and electron-withdrawing groups at specific positions enables fine-tuning of the electronic properties, thereby enhancing the fluorescence response.

The diverse examples of coumarin derivatives discussed in this manuscript highlight the importance of understanding the structure-activity relationships inherent in these compounds. This understanding is crucial for the rational design of new fluorescent materials tailored for specific applications. In conclusion, the insights gained from the studies on coumarin derivatives not only advance our knowledge of their photophysical properties but also open new pathways for the development of innovative fluorescent materials. As research in this field continues to evolve, coumarin derivatives will undoubtedly remain at the forefront of fluorescence technology, serving as valuable tools in biological research and beyond. This manuscript provides a foundation for future explorations, encouraging the continued pursuit of novel coumarin-based probes that can address emerging challenges in biochemistry and medicine.

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