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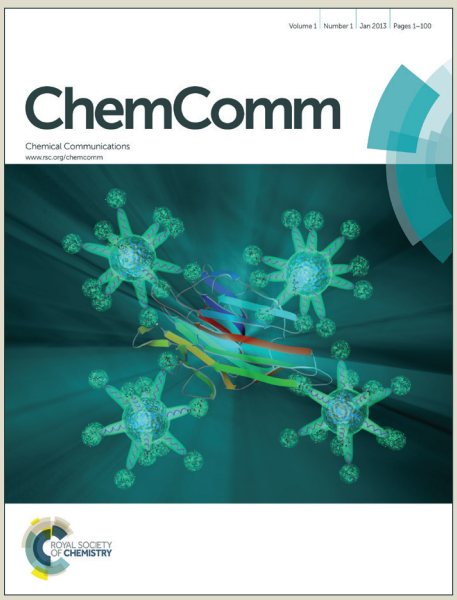

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Two-Color Emissive Probes for Click Reactions

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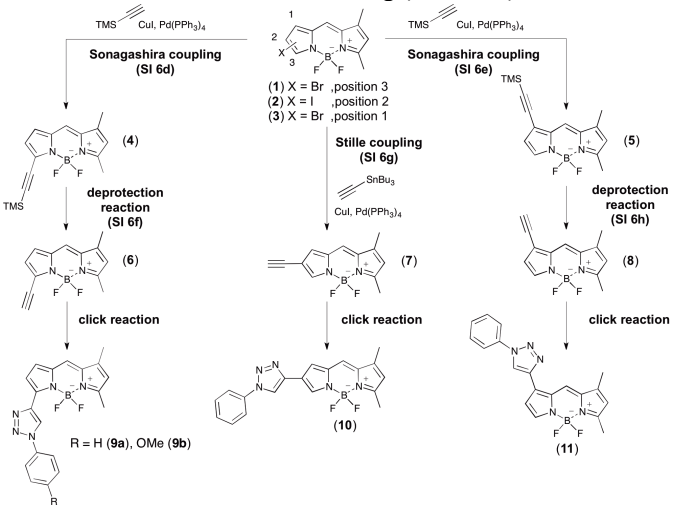
Cu^{I}-catalyzed azide-alkyne cyclization (CuAAC) is the paradigmatic click reaction of continuous interest[1]. Especially fluorogenic and FRET probes became indispensable tools for life sciences[2–4]. Here, we present a fluorescent alkyne for monitoring CuAAC, which undergoes a bathochromic shift upon reaction. Application in single-molecule and catalysis research is foreseen.

In 2001, B. Sharpless defined click reactions as transformations being compatible with a wide variety of functional groups.[5] A prototypic example is Huisgen's 1,3-dipolar cycloaddition of azides with alkynes by Cu^{I} catalysis.[6–12] Especially the development of fluorogenic substrates on its basis stimulated applications in the life science and greatly expanded the application range.[3,12–24] A different approach relies on chromophores expanded by a reactive moiety, which undergo a distinct spectral shift during the transformation.[25,26] In our contribution, we report such a chromophore-containing, "dual-color" reactive BODIPY probe. The presented substrate, undergoing a distinct bathochromic fluorescence shift and thus reducing background signal during CuAAC, allows for following the reaction by spectral and time-resolved fluorescence microscopy.[2,3,13,25–30]

Our interest in click chemistry arose only recently, when we realized that only few fluorogenic substrates with negligible background fluorescence exist for microscopic applications.[2,3,13,27,28] For reaction monitoring by means of fluorescence, a shift in emission wavelength describes the most convenient approach for signal tracking.[25,26,31,32] BODIPY dyes were chosen as scaffold due to their convenient optical properties and easy accessibility via condensation of two pyrroles.[33–35] The benefit of the here proposed reactive probes is twofold: on the one hand, red-shifted electronic spectra of the product lead to unprecedented fluorescence enhancement. Thus, some of the noticed limitations in sensitivity can be overcome without the need of a second chromophore as energy-transfer acceptor.[2] On the other hand, suchlike two-color emissive, reactive probes can act as "molecular chameleons" and are examples among more general attempts to visualize single-molecule chemistry on a microscopic scale.[31,32,36–39] Consequently, the reactive group has to be part of the chromophoric system changing its size (participant approach).[37] As any conjugated ring system, *i.e.* here the triazole after the click reaction, is oriented perpendicular to the chromophore plane when in the *meso* position and thus not conjugated to the latter, fluorescence shifts will be very small or even hypsochromic.[34,36,40] Derivatisation of α, β, or γ position (scheme 1), should therefore induce a presumably bathochromic change in wavelength.[25,34] Accordingly we first investigated the most suitable position on an unsymmetrical BODIPY core for reaction monitoring (scheme 1).



scheme 1: Synthesis of reactive probes for CuAAC-click reaction conditions: 10^{-4} M BODIPY, $8*10^{-6}$ M $CuSO_4$, $2*10^{-5}$ M NaAsc, $3*10^{-2}$ M phenyl azide, EtOH:H_2O – 2:1, rt, min. 6 h (see **SI**, 6 for further details).

All BODIPY compounds were synthesized according to existing procedures.[41–45]

UV-Vis and fluorescence spectroscopic characterization of all components was done (table 1; **SI** 6). CuAAC was investigated at micromolar dye concentration. Under these conditions, almost stoichiometric amounts of Cu^{II} and ascorbic acid were employed and large excess of the azide is added for establishing

reasonable conversion kinetics. The reaction process was measured following fluorescence signal of each alkyne compound and the corresponding reaction product. Excitation of weakly absorbing, spectrally broad higher excitation states at 415 nm ensured that all chromophores could be excited at the same time despite unknown spectral shifts during the conversion.

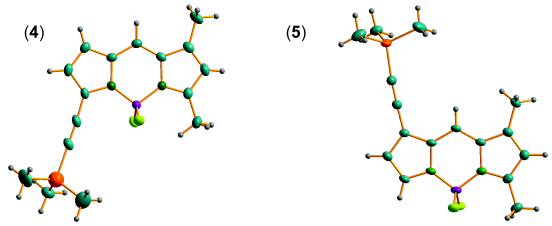


figure 1: X-ray crystallographic structures of the stabilized precursors of two ethynyl BODIPYs. The fluorescent alkynes (**6**) – (**8**) did not withstand crystallization without decomposition but were unambiguously identified in NMR by formation of the characteristic alkyne-H signal at δ = 3.1 – 3.6 ppm (**SI**, 3)[41,45].

Conversions of the different substrates are compared (figure S1). First, the reaction of α-ethynyl BODIPY (**6**) with phenyl azide was investigated. A change in fluorescence wavelength from λ = 528 nm of (**6**) to λ = 548 nm of the product is visible. The spectral shift is large enough for spectral separation, as the spectra are rather narrow ($\Delta\lambda_{FWHM} \approx 20$ nm) over the whole course. At 538 nm an isoemissive point appears after approximately 2 hours. β-ethynyl BODIPY (**7**) was converted under same conditions. Despite a noticeable spectral shift during the reaction from λ_{em} = 540 nm to λ_{em} = 556 nm (figure S1), this change is smaller. Additionally, due to overlapping emission spectra, which even become broader during the reaction ($\Delta\lambda_{FWHM} \approx 42$ nm → 60 nm)[41], an optical separation appeared in vain. We therefore excluded compound (**7**) from further investigations. The third investigated compound (**8**), exhibits optical properties similar to compound (**6**). During the conversion, no optical shift is detected, but the emission band of γ-ethynyl BODIPY (**8**) slowly diminished (figure S1). For verifying that any reaction except from decomposition of the BODIPY compound (**8**) occurred, fluorescence lifetime analysis was performed at the beginning and after 24 h. A prolongation from $\tau_{fl(8)}$ = 4.7 ns to $\tau_{fl(corr.\ product)}$ = 5.7 ns (both in EtOH/H_2O) indicated a successful conversion, in agreement with previously noticed fluorescence lifetime prolongations due to substitution at this position.[45] We conclude from these experimental data that CuAAC of compound (**8**) hardly affects the steady-state spectroscopic properties and (**8**) was consequently discarded from further experiments. As a result of the comparison, (**6**) exhibits the optimal parameters for reaction monitoring.

For further improving optical separation, three different azide moieties, benzyl azide, phenyl azide and anisyl azide, were tested for conversion (figure S1). Every species is expected to enlarge the chromophoric system by a different amount. Comparison of the resulting spectra reveals, however, that only the triazole ring as common structural element, is sufficient to shift the emission by roughly 20 nm to the red. Exhibiting the largest fluorescence change, anisyl azide was used for following the kinetics (figure 2A).

After some initial reduction due to putative Cu^I-acetylide formation, the fluorescence intensity of the starting compound diminished in the same manner as the product fluorescence emerged (figure 2A, inset: kinetic analysis of substrate conversion and product formation†). A suchlike isoemissive point at λ_{em} = 540 nm was already found in figure S1 after the initial phase.

A closer inspection of the kinetics is obtained by a biexponential fit function. It is interesting to note that no time lag for product formation was found indicating, partially, rather rapid Cu^I-acetylide formation. The analysis yields rate-constants, which were slightly larger for the substrate depletion than for the product emergence†. Evidence for a side reaction is provided by appearance of a hypsochromically shifted emission band at λ_{em} = 470 nm concomitant with product formation. The broad emission band was traced back to BODIPY-decomposition, that was only noticed under the use of anisyl azide. We learn, however, from these kinetic experiments that a successful visualization of the conversion appears feasible in microscopy between 15 min and 2 h. After reaction completion, the product was isolated, identified by its typical ^{1}H NMR

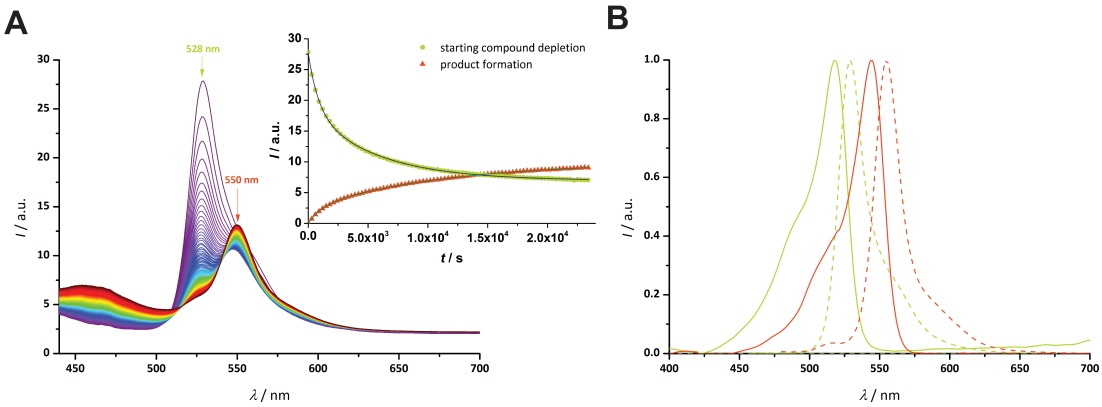


figure 2:
A: Conversion of (**6**) with anisyl azide to (**9b**) (reaction progress over 6 h, spectra taken every 5 min);
B: Comparison of compounds (**6**) and (**9b**).

Table 1: Spectroscopic details of the favored dyes (see **SI** chapter 6 for data of all compounds).

compound	λ_{abs} / nm	λ_{ex} / nm	λ_{em} / nm	τ / ns (DCM)	Φ (DCM)
α-TMSAc-BODIPY (4)	529	529	540	5.3	1.00 – 0.02
α-Ethynyl-BODIPY (6)	518	518	529	6.5	0.89 ± 0.02
Click product (9)	544	544	555	6.1	0.73 ± 0.05

signal (δ = 8.83 ppm)[46] and subsequently characterized by optical spectroscopy (table 1, **SI** 6.i, figure S30, S31, S37). A bathochromic shift of 26 nm with respect to the substrate (figure 2B) is observed. Both molecules slightly differ in their fluorescence lifetimes as well (table 1).

The obtained spectroscopic characteristics from ensemble measurements can be exploited for microscopic visualization (figure 3). Commercially available azide-functionalized agarose beads were adsorbed on a glass slide. The first detection channel uses excitation with a pulsed diode laser at λ_{ex} = 470 nm and λ_{det} = 525/50 nm, thus ensuring detection of the starting compound (**6**) (column A, figure 3). A second channel only addresses the triazole product by means of a pulsed fiber laser operating at λ_{ex} = 550 nm and λ_{det} = 585/50 nm. Background fluorescence was negligible (figure S2, S6).

α-ethynyl BODIPY (**6**) was added (figure 3, 1st row). Green emission shows BODIPY accumulation within the bead. τ_{fl} over all pixels is 4.5 ns, distinctly reduced compared to the lifetime of BODIPY (**6**) in the solvent (τ_{fl}(EtOH) = 5.6 ns)

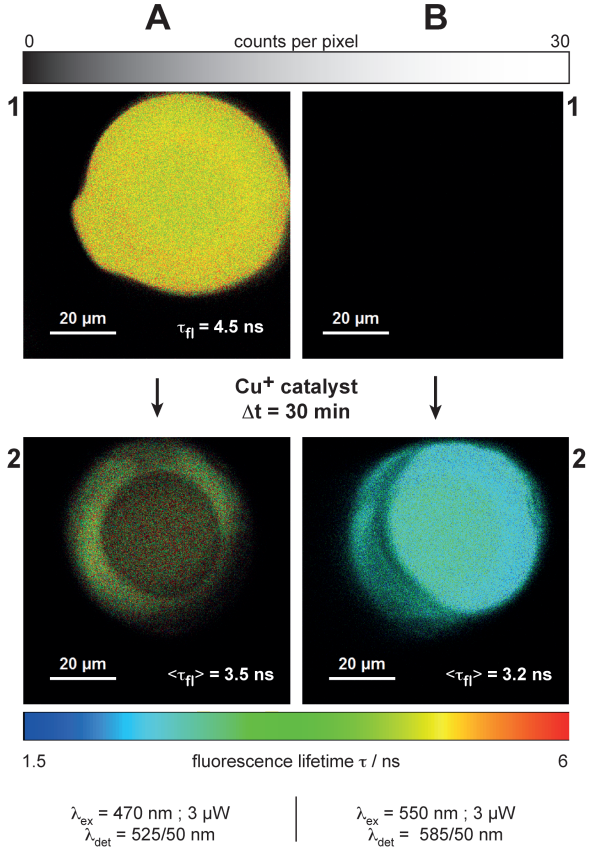


figure 3: Visualization of click reaction between α-ethynyl BODIPY (**6**) and azide agarose on a glass surface with corresponding fluorescence lifetimes; column **A**: excitation with λ_{ex} = 470 nm @ 20 MHz, emission filter λ_{det} = 525/50 nm; column **B**: excitation with λ_{ex} = 550 nm @ 80 MHz, emission filter λ_{det} = 585/50 nm. Time lag between **2** and **3** is 30 min.

In agreement with the control experiment (figure S6), this is the result of a changed micro-environment, *e.g.* an altered refractive index[47]. Analyzing the same bead with λ_{ex} = 550 nm, no signal was noticed. This result hints to negligible light-induced AAC, as B1 was recorded immediately after A1.

After addition of $CuSO_4$/NaAsc in H_2O, green fluorescence is visible deriving from unreacted α-ethynyl BODIPY adsorbed to the agarose network (averaged lifetime $\langle\tau_{fl}\rangle$ = 3.5 ns, A2). Noteworthy, the control experiment (figure S6) exhibited he same tendency in lifetime reduction. We therefore attribute this common shortening to the locally increased concentration of cuprous and cupric ions as triazole compounds are known for their good complexation behavior of Cu^{I}.[48] Fluorescence in the product channel (figure 3 B2), which was not detected in the control experiment, perfectly matches the spectroscopic behavior found in the cuvette experiments. Together with an even further reduced fluorescence lifetime (averaged lifetime $\langle\tau_{fl}\rangle$ = 3.2 ns), these findings unambiguously proved triazole formation.

In our work, we established a fluorescent reaction system for studying CuAAC and its visualization by fluorescence microscopy. Fluorophores bearing C-C triple bonds at different positions as target for click chemistry were synthesized and characterized. It turned out that the α-position at the BODIPY core is most sensitive for following CuAAC by fluorescence spectroscopy (figure 2). Comparison of several azide moieties revealed that only the triazole ring is mostly responsible for the bathochromic fluorescence shift by more than 20 nm. Finally, we visualized the click reaction using azide agarose (figure 3). A careful combination of excitation and detection conditions easily separates product and substrate fluorescence. After catalyst addition, fluorophores located within the beads changed their emission color from green to yellow as in the cuvette experiment.

Conclusions

A two-color fluorescent probe for investigating CuAAC is presented. Due to the distinct bathochromic shift, our compound considered as fluorogenic substrate for application in heterogeneous catalysis, when only the product is excited.[39] It should be noted that exactly these latter features are the main advantages over all other yet available substrates for single-molecule chemistry investigation, where the chromophore extension was shortened leading to a hypsochromic shift.[25,31,32] The presented data serve as basis for ongoing single-molecule experiments and will be continued similarly to previous work.[25]

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Notes and references

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† α-ethynyl BODIPY depletion: k_{SC-1} = 1,17 * 10^{-3} s^{-1}, k_{SC-2} = 1,77 * 10^{-4} s^{-1}
product formation: k_{P-1} = 0,86 * 10^{-3} s^{-1}, k_{P-2} = 0,87 * 10^{-4} s^{-1}

†† Experimental details, used reagents, instruments, procedures and analytical data are given in the Electronic Supplementary Information (ESI).

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