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Click Reaction-Mediated Functionalization of Near-Infrared Pyrrolopyrrole Cyanine Dyes for Biological Imaging Applications

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Abstract

A clickable pyrrolopyrrole cyanine (PPCy) dye was synthesized by incorporating an alkyne moiety, followed by click reaction with azide-functionalized molecules of different polarities. The clickable dyes are readily amenable to labelling diverse molecules and exhibit an exceptionally high photostability and an impressive fluorescence quantum yield.

Interests in developing molecular probes that fluoresce in the near-infrared (NIR) wavelengths continue to increase owing to reduced autofluorescence and low attenuation of light in biological tissues.^{1–3} For this reason, extensive research has focused on leveraging the advantages of this tissue “transparent” window for molecular imaging.^{4–7} In spite of the progress made so far, the diversity of NIR dyes available for biological imaging is still limited, and largely confined to NIR fluorescence carbocyanine dyes. In particular, indolium cyanine dyes are the dominant NIR labels for this application, despite their obvious shortcomings which include poor photostability and low quantum yield. Although these dyes are sufficiently stable for macroscopic (>1 mm resolution) in vivo imaging with optical systems that use of low excitation power, current trend to determine the cellular biodistribution and longitudinal measurements of molecular processes in cells and living organisms requires microscopic resolution. Unfortunately, most microscopic techniques utilize high laser power that rapidly photobleaches NIR carbocyanine dyes.^{8, 9} For ease of translation to humans, there is a compelling need to alternative photostable NIR dyes.

Recently, diketopyrrolopyrrole molecules were used to synthesize a new class of NIR cyanine dyes – Pyrrolopyrrole Cyanine (PPCy) Dyes.¹⁰ These dyes were shown to have higher photostability and quantum yields than most organic NIR dyes currently used in biological studies.^{10–12} However, complex formulation strategies were needed to optimize the PPCy dyes for bio-imaging purposes.¹³ In this study, we report the synthesis and characterization of a core PPCy dye that can be readily functionalized for bioimaging through click reaction.

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Copper (I)-catalysed Azide-Alkyne Cycloaddition (CuAAC), also known as click reaction, is widely used to conjugate molecules because of the high selectivity, efficiency, and bioorthogonal reaction conditions.¹⁴ Harnessing these advantages, we developed a CuAAC condition for conjugating the hydrophobic PPCy dyes to molecules with significantly different polarities by incorporating an alkyne group to the PPCy moiety and an azido group in the reacting molecule.

Toward this goal, alkynyl functionality was incorporated into heteroarylacetonitriles (HAAs) by first alkylating the 6-hydroxymethylene group of (2-methyl-6-quinoliny)l)methanol with 5-chloro-1-pentyne, followed by halogenation of the 2-methyl group of **1** with trichloroisocyanuric acid to afford **2**. Subsequent nitrile nucleophilic substitution produced the versatile intermediate compound **3** (Scheme 1), which was used to prepare a variety of compounds. For example, reaction of 3,6-bis(4-butoxyphenyl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione with the HAA **3** afforded the symmetrical PPCy dye **6** as the major compound (see ESI).¹¹

Initial attempts to functionalize the hydrophobic **6**, which are sparingly soluble in common polar solvents such as DMSO, DMF and H₂O, with hydrophilic molecules were difficult because of the disparate solubility of the reactants in different solvents. To improve the reaction conditions, we used CuAAC because solvents have limited effects on this reaction. Thus, a mixture of solvents capable of dissolving all the reactants can readily be used for the click reaction. Generally, the reaction proceeds by stirring **6**, an azide, CuSO₄, (+)-sodium-L-ascorbate, and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in a mixture of H₂O/*tert*-butanol/THF at room temperature until **6** disappears on thin layer chromatography (TLC) plates (typically 24 – 48 h).¹⁵ Compound **6** and its derivatives are also very stable under the click condition. Decomposition products of the dye were not detected by UV-vis or HPLC after the reaction. With this method, reaction of **6** with molecules of diverse polarities afforded the desired compounds **7a–c** in good yields. We illustrated the versatility of this method by reacting **6** with hydrophilic azido-glucose, hydrophobic 1-azido-3-phenylpropan-2-one and a short PEG molecule, 1-azido-2-(2-(2-methoxyethoxy)ethoxy)ethane (Scheme 2).

The absorption and emission spectra of **7b** in DMSO, which is representative of all the compounds prepared, is shown in Figure 1, and the spectroscopic properties of all the compounds prepared are summarized in Table 1, with quantum yields referenced to ICG.¹⁶ The results show that the click reaction products retained the optical properties of the PPCy dye. In contrast to the highly hydrophobic precursor dye **6**, all of the conjugates **7a–c** have excellent solubility and similar quantum yields in DMSO. Interestingly, **7a–c** have similar aggregation levels in DMSO (ESI). This suggests that the triazolium group formed from click reaction plays important role in solubilizing the PPCy molecules. Considering that many *in vivo* molecular recognitions are mediated by hydrophobic interactions, as demonstrated by the abundance of lipophilic drugs, these click reaction products may have the additional advantage of innocuously improving the formulation of the compounds without disrupting the hydrophobic moieties used in molecular interactions.

An important feature of PPCy dyes is their high photostability in live cells, suggesting that these NIR dyes can withstand the intense light used in microscopy. The rapid photobleaching of most NIR carbocyanine dyes under these conditions prevents their use in time-lapse microscopy. Accordingly, we used **7b** to assess the photostability of the PPCy dye conjugates in cells. Expectedly, we were able to solubilize **7b** in lower concentration of surfactant (0.03% DMSO, 0.3% Tween80) than the amount needed for the parent dye, **6**. The breast cancer cells MCF-7 were incubated with **7b** (5 μ M) in Dulbecco's modified Eagle's medium (DMEM) containing 0.03% DMSO, 0.3% Tween80 and GeneJuice¹⁷ at 37

°C for 18 h. The cells were then washed (3×) with 0.01 M phosphate buffered saline (pH 7.4) before fixing with 4% paraformaldehyde for 15 min. For nuclear staining, the cells were treated with 0.01% Triton-X for 2 min at room temperature followed by TOTO1 (1:2000) for 30 min. Images were acquired on a fluorescence microscope by using 488/540 nm and 710/810 nm excitation/emission filters for TOTO1 and **7b**, respectively. The bright fluorescence of the dye was retained in cells after prolonged exposure to normal light used in microscopy (Figure 2). Based on this information, we studied the photostability of **6b** in live cells. Indocyanine green (ICG), a standard NIR carbocyanine dye widely utilized in optical imaging studies,^{2, 6} was solubilized in the same solvent mixture as **7b** and used as a reference. The microscope was set to its maximum excitation power (120 watts) for this study and the relative fluorescence intensity was determined by normalizing the average FI value in total of cells from the average FI value of the background using the NIH ImageJ software. The PPCy dye conjugate retained 80% of the initial fluorescence after irradiating for 90 min, while ICG fluorescence was almost completely photobleached within 15 min (Figure 3). This result demonstrates the feasibility of using the new PPCy dyes for longitudinal microscopy studies such as time-lapse microscopy and kinetic studies.

In summary, we have successfully developed a method to functionalize PPCy dyes using click reaction. Click reaction is particularly suitable for conjugating diverse clickable compounds by using solvent mixtures that ensures initial solubility of the reactants and reagents. The additional 1,2,3-triazole ring formed by CuAAC improved the solubility of the conjugates in polar solvents such as DMSO. This enhancement facilitated the use low concentrations of DMSO and surfactant to solubilize the PPCy conjugates in aqueous media for biological imaging study. These dyes are about 700× more stable than ICG under similar exposure conditions using an epifluorescent microscope. Demonstration of the enhanced photostability of a representative PPCy dye in cells paves the way for potential application of NIR dyes in time-lapse experiments, which has been confined to dyes in the visible wavelengths. Future studies will focus on labelling biomolecules with these PPCy dyes for a variety of biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The normalized absorption (—) and emission (- - -) of **7b** in DMSO at room temperature

Figure 2.

Live cell imaging using **7b**. MCF-7 cells were incubated with **6b** (5 μ M) in DMEM containing 0.03% DMSO, 0.3% Tween80 and GeneJuice (5 μ M) at 37 $^{\circ}$ C for 18 h. (A): Bright field; (B): TOTO1 stained cell nuclei; (C): **7b** stained cell; (D): composite image of cells with TOTO1 and **7b** staining.

Figure 3.
Photostability study of **LS729** compared to ICG in live MCF-7 cells.

Scheme 1.

Synthesis of the clickable HAA **3**: i) NaH, DMF, r.t., 2 h, then, 5-chloro-1-pentyne, r.t., overnight; ii) trichloroisocyanuric acid, CHCl_3 , r.t., overnight; iii) NaCN, NaI, DMF, 60 °C, 2 h

Scheme 2.

Structure of the PPCy dye **6** and the reagents and condition of the click conjugation: i) $CuSO_4$, (+)-sodium-L-ascorbate, TBTA, H_2O / *tert*-butanol/THF, r.t., 24 – 48 h

Table 1

Spectroscopic data of **5** and its derivatives of $S_0 \leftrightarrow S_1$ in CHCl_3 and DMSO at room temperature

	$\lambda^{\text{A*}}$ [nm]	ϵ [M ⁻¹ cm ⁻¹]	λ^{F} [nm]	Φ [%]	τ [ns]	
6	DMSO	N/S	N/S	N/S	N/S	
	CHCl ₃	754	169 000	766	42	4.0
7a	DMSO	755	146 000	770	31	3.6
	CHCl ₃	N/S	N/S	N/S	N/S	N/S
7b	DMSO	755	138 000	769	37	3.6
	CHCl ₃	754	135 000	767	50	4.0
7c	DMSO	755	103 000	770	33	3.6
	CHCl ₃	754	110 000	767	48	4.0

* λ^{A} : absorption; ϵ : extinction coefficient; λ^{F} : emission; Φ : quantum yield referenced to ICG¹⁶; τ : fluorescence lifetime; N/S : Not Soluble