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Cryptocyanine-Based Fluorescent Probes for DNA Detection: Controlling Solubility and Aggregation Through Side Chain Design

Farkas Domahidy^{1,2}  | Levente Cseri¹  | Gábor Turczel^{1,3}  | Blanka Huszár¹ | Balázs J. Rózsa^{1,4,5}  | Zoltán Mucsi^{1,6}  | Ervin Kovács^{1,7} 

¹BrainVisionCenter, Budapest, Hungary | ²Hevesy György PhD School of Chemistry, Eötvös Loránd University, Budapest, Hungary | ³NMR Research Laboratory, HUN-REN Research Centre for Natural Sciences, Budapest, Hungary | ⁴Laboratory of 3D Functional Network and Dendritic Imaging, HUN-REN Institute of Experimental Medicine, Budapest, Hungary | ⁵Faculty of Information Technology and Bionics, Pázmány Péter Catholic University, Budapest, Hungary | ⁶Faculty of Material and Chemical Sciences, University of Miskolc, Miskolc, Hungary | ⁷Institute of Materials and Environmental Chemistry, HUN-REN Research Centre for Natural Sciences, Budapest, Hungary

Correspondence: Balázs J. Rózsa (rozsaabal@koki.hu) | Zoltán Mucsi (zoltanmucsi@gmail.com) | Ervin Kovács (kovacs.ervin@ttk.hu)

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ABSTRACT

Novel cryptocyanine-based DNA-binding fluorescent probes were developed by introducing side chains with varying polarity to the dye scaffold. This structural modification improves water solubility, reduces aggregation in aqueous media, and enhances DNA binding affinity. Upon binding to DNA, the derivatives exhibit a high increase in fluorescence quantum yield, demonstrating their potential as fluorogenic DNA probes. The photophysical behavior of the dyes is systematically investigated using spectroscopic techniques, focusing on their environment-sensitive emission properties. These results highlight the importance of environmentally responsive dye scaffolds in the development of fluorogenic tools for nucleic acid detection and diagnostic applications.

1 | Introduction

Cryptocyanines, 1,1-dialkyl-4-[3-(quinolin-4(1H)-ylidene)prop-1-en-1-yl]quinoliniums, are cationic cyanine dyes characterized by strong absorption and emission in the near-infrared region [1, 2]. Their optical properties were investigated several decades ago, and they have found niche applications as a self-synchronizing, non-destructive, passive Q-switch in ruby lasers [3, 4]. Their photochemical behavior has been studied only more recently through theoretical calculations, focusing on fluorescence and isomerization mechanisms [5]. The electronic spectra and photophysical properties of these linearly conjugated systems are highly sensitive to conformational and environmental factors,

as revealed by low-temperature gas-phase experiments in recent years [6].

Despite these unique characteristics, their biological applications have remained unexplored and have only recently become a focus of increasing interest. Recently, mitochondria-targeting cryptocyanines have been used as photothermal agents [7]. The applicability of cryptocyanines as photosensitizers was investigated recently, and they showed promising candidates *in vitro* [6, 8]. Notably, some acridine-related derivatives were investigated as potential photosensitizers targeting DNA- or RNA G-quadruplex structures. In this case, one cryptocyanine derivative was found to exhibit a 35-fold fluorescence enhancement in the presence

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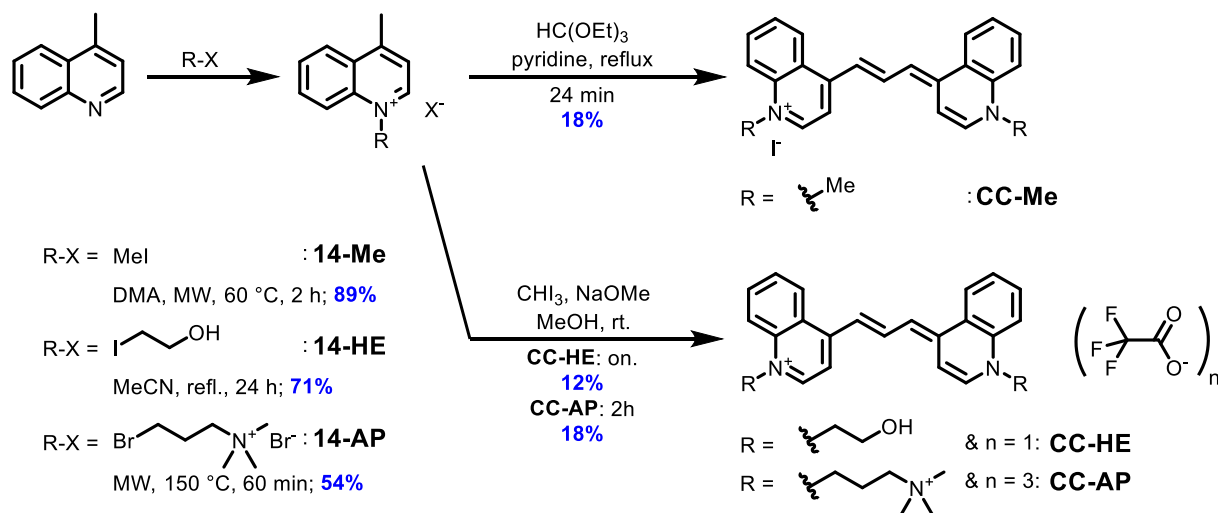


FIGURE 1 | Synthesis of the cryptocyanine dyes.

of one equivalent of RNA G-quadruplex [9]. Another application involved a cryptocyanine-based exciton-controlled hybridisation-sensitive fluorescent oligonucleotide (ECHO) probe, in which two dyes are conjugated to the seventh nucleotide of a short single-stranded DNA (ssDNA) sequence via flexible linkers. In aqueous solution, these form a non-fluorescent aggregate that dissociates upon RNA hybridization, resulting in a significant fluorescence increase [10].

However, despite their high extinction coefficients, far-red absorption, and near-infrared emission, cryptocyanines have not yet been applied in the fluorescent sensing of double-stranded DNA (dsDNA), presumably due to their tendency to aggregate unfavorably in aqueous environments [11, 12]. Their planar structure and conformational flexibility make them attractive candidates for such applications, particularly in fluorescence microscopy. In continuation of our efforts to develop novel fluorescent DNA probes [13, 14], we report the design and synthesis of cryptocyanine-based compounds with improved aqueous solubility and DNA sensing properties, enabling their use in near-infrared fluorescence biosensing.

2 | Results and Discussion

2.1 | Synthesis of Cryptocyanine Derivatives

Two cryptocyanine derivatives containing polar auxiliary groups – 2-hydroxyethyl (CC-HE) and 3-(trimethylammonium)propyl (CC-AP) – were synthesised to increase their aqueous solubility. The simplest dimethyl-substituted derivative, CC-Me, was also synthesised as a reference compound. The two-step synthesis of cryptocyanine starts with the alkylation of 4-methylquinoline with alkyl halides (namely, methyl iodide for CC-Me, 2-iodoethanol for CC-HE, and 3-bromopropyl-trimethylammonium bromide for CC-AP) to introduce the appropriate alkyl substituents. For CC-Me, the resulting quinolinium salt was reacted with triethyl orthoformate in pyridine under microwave heating to construct the cryptocyanine core. In the case of CC-AP, the dicationic quinolinium intermediate was

not soluble in pyridine. Therefore, in this case, a modified procedure was employed in which the 4-methylquinolinium salt was deprotonated with a stronger base, NaOMe, at room temperature in methanol, followed by a reaction with a more reactive HC³⁺ synthon, iodoform, to introduce the *meso* carbon, in accordance with a literature procedure [15]. For CC-HE, both procedures were applicable, but the second one has proven more efficient (Figure 1).

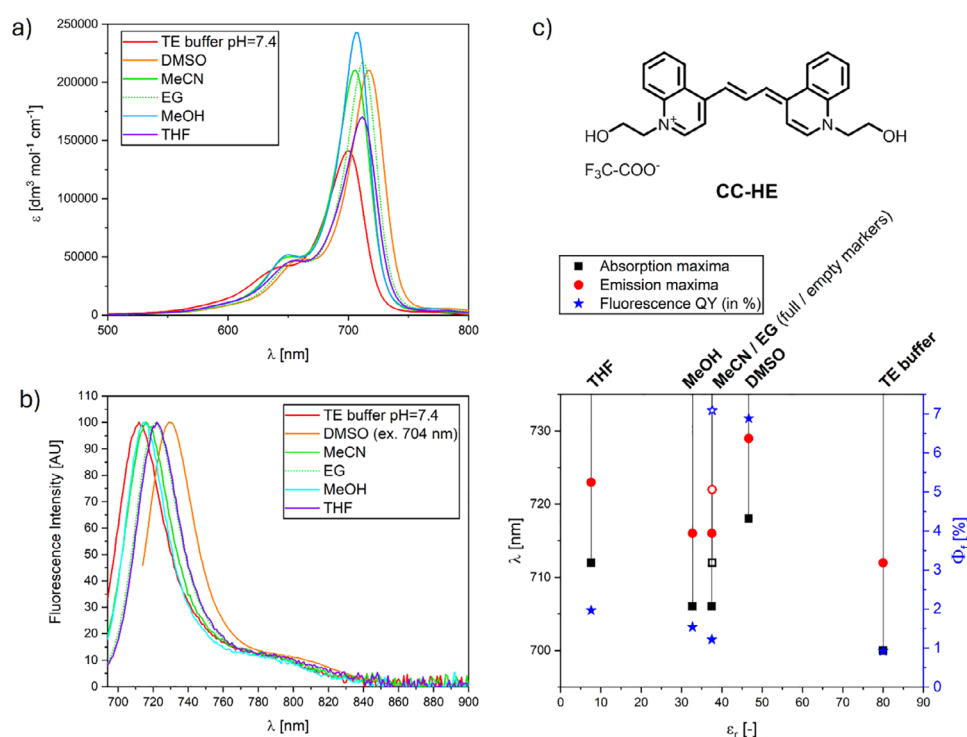
2.2 | Spectroscopic Properties of Cryptocyanine CC-Me, CC-HE, and CC-AP

In TE buffer, all cryptocyanine dyes exhibit a sharp absorption peak in the red–near-infrared region ($\lambda_{\text{abs}} = 698$ nm for CC-Me and 700 nm for CC-HE and 704 nm for CC-AP), with a high extinction coefficient (ϵ) in the range of $1\text{--}1.5 \cdot 10^5$ dm³ mol⁻¹ cm⁻¹. The ϵ of CC-AP is apparently slightly lower than that of the other two cryptocyanines; however, it should be noted that this compound is highly hygroscopic; therefore, despite careful efforts to prepare the stock solution from a dry sample, it may have contained some residual water, lowering its actual concentration (this does not affect the determination of Φ_f , nonetheless) (Table 1).

To assess the solvatochromicity, the absorption spectrum of CC-HE was recorded in various polar organic solvents. This dye exhibits exceptionally high extinction coefficient ($1.70\text{--}2.43 \cdot 10^5$ dm³ mol⁻¹ cm⁻¹) and low Stokes shift (10–12 nm; 0.025–0.027 eV) in all investigated solvents. In THF, MeOH, MeCN, and TE buffer, both absorption and extinction maxima shift in the hypsochromic direction with increasing solvent polarity, while the fluorescence quantum yield is decreased in solvents with a higher dielectric constant. However, in ethylene glycol and DMSO, the values of λ_{abs} , λ_{em} , and Φ_f are higher than expected based on the trends observed in the other four solvents. Overall, the solvatochromic effect is not very significant (all measured absorption maxima are within a 20 nm range), from which we can conclude that the excitation is accompanied by a relatively small charge shift (Figure 2).

TABLE 1 | Absorption and emission maxima (λ_{abs} , λ_{em}), extinction coefficient (ϵ), and fluorescence quantum yield (Φ_f) of **CC-Me**, **CC-HE**, and **CC-AP** in different media.

Dye	Solvent	λ_{abs} [nm]	λ_{em} [nm]	ϵ [$10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$]	Φ_f [%]
CC-Me	MeOH	706	717	258	0.79
CC-HE	TE buffer	700	715	141	0.45
	TE + 50 eq. DNA ^a	720	732	139	14.3
	TE + 50 eq. BSA ^b	704	722	105	4.31
	DMSO	718	729	210	6.89
	MeCN	706	716	211	1.22
	ethylene glycol	712	722	218	7.09
	MeOH	706	716	243	1.54
	THF	712	723	170	1.96
CC-AP	TE buffer	704	716	109	0.60
	TE + 50 eq. DNA ^(a)	721	733	97.7	17.9
	TE + 50 eq. BSA ^(b)	710	725	87.5	7.13
	MeOH	710	719	102	0.86

^aThe concentration of DNA is expressed as the concentration of base pairs.^bThe concentration of BSA is expressed as the concentration of macromolecules.**FIGURE 2** | Spectroscopic characteristics of **CC-HE** in various solvents (EG = ethylene glycol). a) Absorption spectra (measured in the 1–4 μM range). b) Normalised emission spectra (measured from a 1 μM solution; λ_{ex} = 680 nm, unless stated otherwise). c) Structural formula and a summary of spectroscopical properties, here graphed as a function of the relative permittivity (ϵ_r) of the solvents.

The methyl-substituted derivative, **CC-Me**, is only sparingly soluble in aqueous buffer, which renders its use unfavorable in most biological applications. The shoulder ($\sim 622 \text{ nm}$) of its main absorption peak increases with concentration in the 1–6 μM range, suggesting the presence of H-aggregates. This observation is consistent with the ECHO probes reported

by Ikeda et al., which contain two cryptochrysin units and in which a dimeric H-aggregate provides the most intensive absorption peak [10]. This change in the shape of absorption spectra is lesser for **CC-HE** in the same concentration range, and for **CC-AP**, it is practically non-existent, which confirms that the introduction of polar or cationic auxiliary

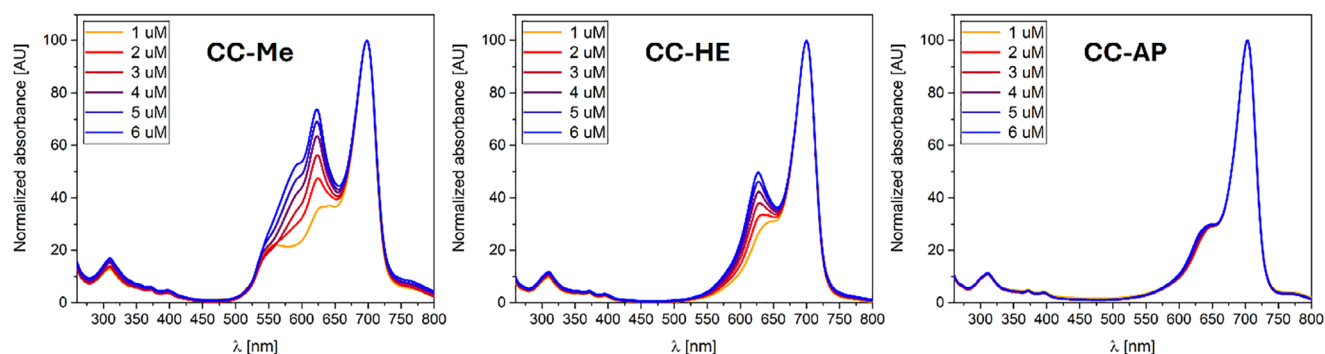


FIGURE 3 | Concentration dependence of the normalised absorption spectra of cryptocyanine dyes **CC-Me**, **CC-HE**, and **CC-AP** in pH = 7.4 TE buffer.

groups increases aqueous solubility and mitigates dye aggregation (Figure 3).

Another attribute of cryptocyanines observed in water is that in a more acidic environment, the molecule can be protonated on one of the benzylic carbons, which breaks the conjugation between the two quinoline units. This was demonstrated for **CC-AP** by an NMR study and for **CC-HE** by dissolving the dye in acidified D₂O and detecting the H–D exchange by HPLC-MS (Figure 4).

The changes in the absorption and emission spectra of cryptocyanines suggest a complex DNA-binding behavior. When titrating a 1 μM solution of the dyes with dsDNA, the absorbance spectrum initially exhibits a hypochromic behavior, while the shape of the spectra remains roughly the same, with a slight increase in the red-shifted shoulder of the main absorption peak (Figure 5a,b). In this low DNA concentration range, **CC-HE** exhibits close to zero fluorescence enhancement, while the effective quantum yield of **CC-AP** decreases with DNA concentration. However, at a base-pair-to-dye ratio of approximately 1–1.5, both the absorption and the emission maxima start to shift toward the bathochromic direction while Φ_f increases sharply. While **CC-HE** and **CC-AP**

exhibited similar Φ_f , above 1.5 μM, **CC-AP** displays notably higher fluorescence quantum yields (Figure 5c). Repeating the DNA titration experiment using a 0.1 μM solution of **CC-HE** resulted in similar Φ_f values for identical $C_{\text{DNA}}/C_{\text{dye}}$ ratios (Figure 5d).

In theory, the lack of fluorescence enhancement could be attributed to Förster resonance energy transfer (FRET) between the adjacent fluorophores. However, this mechanism does not account for the abrupt shift in absorption and emission maxima observed around $C_{\text{DNA}}/C_{\text{dye}} = 1.2$. Therefore, the most plausible explanation for this unexpected fluorescence response is that the dye binds to DNA in (at least) two distinct ways, as a weakly fluorescent and as a strongly fluorescent form. The weakly fluorescent complex is probably a bound aggregate, and the strongly fluorescent complex is likely to be a bound monomer (Figure 5e). In this context, the binding and fluorescence behavior of these cryptocyanine dyes is hypothesized to resemble the complexation-induced sequential aggregation–deaggregation process observed for acridine orange upon binding to sulfobutylether-β-cyclodextrin, which similarly results in characteristic changes in the fluorophore’s absorption and emission

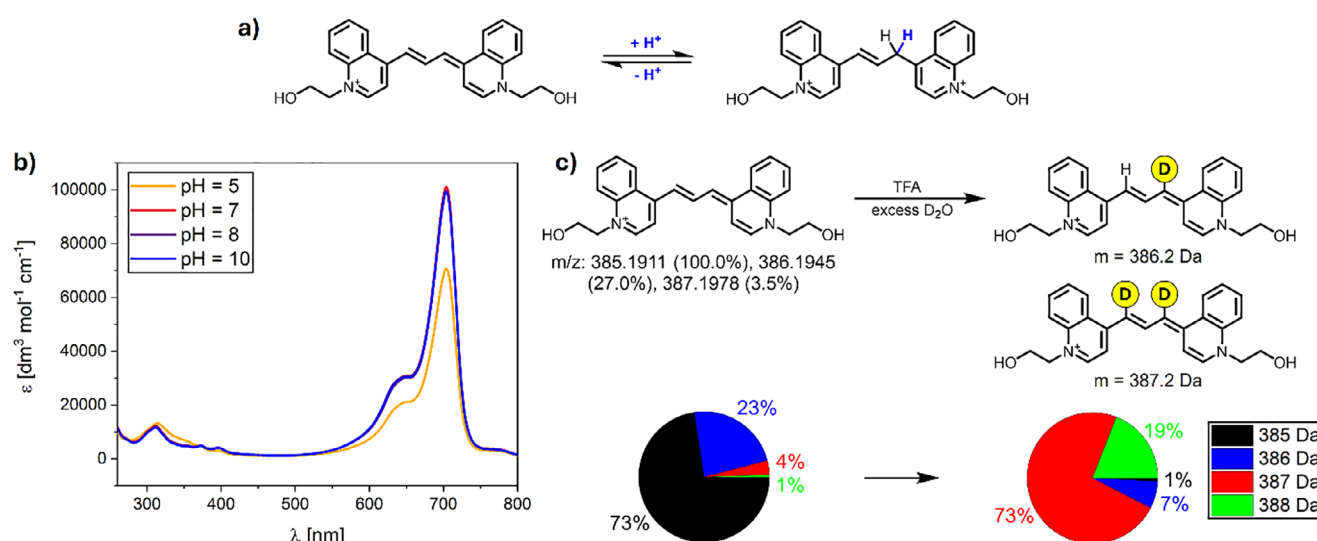


FIGURE 4 | Detection of benzylic protonation of cryptocyanines. a) Benzylic protonation equilibrium of **CC-HE**. b) Dependence of the absorption spectrum of **CC-HE** on pH. c) Deuteration of **CC-HE** in acidic D₂O. The pie chart represents the ratio of MS signals (belonging to different isotopologues) detected in HPLC-MS.

spectra [16]. At a low $C_{\text{DNA}}/C_{\text{dye}}$ ratio, the binding sites of all DNA chains are saturated with bound aggregates that have similar absorption and emission maxima to those of the solvated monomer, but their extinction coefficient is lower, while their Φ_f is similar. The lack of change in absorption and emission maxima below $C_{\text{DNA}}/C_{\text{dye}} = 1.2$ for **CC-HE** and $C_{\text{DNA}}/C_{\text{dye}} = 0.6$ for **CC-AP** suggests that in this $C_{\text{DNA}}/C_{\text{dye}}$ range, the prevalence of bound monomers is negligible, while the ~21% change in extinction coefficient can be only explained if a significant portion of dye molecules is in a different chemical environment. At higher $C_{\text{DNA}}/C_{\text{dye}}$ ratios, however, the red-shifted, strongly fluorescent, tightly bound form starts to emerge, and from $C_{\text{DNA}}/C_{\text{dye}} = 1.2$ its ratio steadily increases according to the sharp increase in Φ_f , λ_{abs} , and λ_{em} (Figure 5b,c).

3 | Conclusions

In this study, we successfully synthesized novel cryptocyanine derivatives bearing polar side chains, alongside the known dimethyl analogue. The introduction of polar substituents effectively reduced dye aggregation in aqueous media, and the new compounds were comprehensively characterized by spectroscopic methods. The prepared cryptocyanines exhibited a significant increase in fluorescence quantum yield upon binding to double-stranded DNA, highlighting their strong potential as fluorogenic probes. These properties make them particularly attractive for fluorescence microscopy applications, where low dye concentrations and nucleic acid-rich microenvironments can be leveraged for selective signal enhancement. While their use in high dye-to-DNA ratio systems – such as spectroscopic quantification or PCR assays – may be limited due to non-classical binding behavior, their promising photophysical characteristics ($\lambda_{\text{em}} = 730$ nm) open up new avenues for nucleic acid detection and imaging in complex biological settings.

4 | Experimental Section

4.1 | General Remarks

All reagents and solvents were purchased from Fluorochem, Merck, or VWR. Deionized water (>10 MΩ cm) was prepared using a Milli-Q RiOs-DI-3UV system.

Microwave-assisted reactions were carried out in an Anton-Paar Monowave 450 microwave reactor.

Gradient elution preparative RP-HPLC purification processes were conducted on a Teledyne ISCO ACCQPrep HP150 chromatography system equipped with a Phenomenex Gemini 10 μm NX-C18 110 Å column.

For reaction monitoring, a Shimadzu LC-40D XR UPLC-MS system was used, equipped with a SIL-40C XR autosampler, SPD-M40 photodiode array detector, and an LCMS-2020 DUIS Mass Spectrometer operated in alternating negative and positive modes. An Ascentis Express C18, 2 μm UHPLC column (L × I.D. 5 cm × 2.1 mm) was used at 40°C, provided by a CTO-40s column oven. Gradient elution was applied using 0.1% v/v TFA in water and MeCN.

NMR experiments were carried out on a 600, 500, 400 MHz Varian NMR System spectrometers equipped with inverse detection probes at 30°C. Notation for the splitting patterns in the ^1H NMR spectra include singlet (s), doublet (d), triplet (t), broad (br), and multiplet or overlapping peaks (m). Chemical shifts (δ values) are reported in ppm, and coupling constants (J) are expressed in Hertz (Hz). ^1H and ^{13}C assignments were obtained using the combination of 2D ^1H - ^1H COSY, ^1H - ^1H TOCSY, ^1H - ^1H ROESY or ^1H - ^1H NOESY, ^1H - ^{13}C HSQC and ^1H - ^{13}C HMBC measurements. In the NMR spectra, ^1H assignments are marked in blue, while ^{13}C assignments are marked in red on the corresponding compound schemes.

Absorption spectra were recorded on a Shimadzu UV-1900i UV-vis spectrophotometer in 1 × 1 cm polymethacrylate cuvettes. Emission spectra were recorded on a Shimadzu RF-6000 spectrofluorimeter. UVASol. DMSO (Supelco) was used for the preparation of stock solutions.

Photostability was studied in a ThalesNano PhotoCube photochemical reactor.

4.2 | Synthetic Procedures

4.2.1 | Synthesis of

1-methyl-4-((E)-3-((E)-1-methylquinolin-4(1H)-ylidene)prop-1-en-1-yl)quinolin-1-ium iodide, CC-Me

191 mg (0.67 mmol) 1,4-dimethylquinolinium iodide was suspended in a solution of 233 μL (207 mg, 1.4 mmol, 2.1 equiv.) triethyl orthoformate in 1 mL pyridine under an inert atmosphere and refluxed at 115°C for 24 min. The reaction mixture was cooled down to room temperature and diluted with DCM. The addition of aqueous saturated NaHCO_3 solution resulted in the formation of a dark precipitate. The solid was filtered, washed with distilled water and DCM, and dried in a vacuum oven. The desired compound, **CC-Me** was obtained as a dark green crystalline solid (27 mg, 18%).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.66 (t, $J = 13.0$ Hz, 1H), 8.36 (d, $J = 8.4$ Hz, 2H), 8.10 (d, $J = 7.3$ Hz, 2H), 7.86 (t, $J = 7.7$ Hz, 2H), 7.81 (d, $J = 8.6$ Hz, 2H), 7.73 (d, $J = 7.3$ Hz, 2H), 7.61 (t, $J = 7.6$ Hz, 2H), 7.09 (d, $J = 12.9$ Hz, 2H), 3.98 (s, 6H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 148.58, 142.62, 141.55, 139.16, 132.78, 126.12, 124.70, 124.10, 117.59, 110.42, 108.41, 41.37. MS (ESI) m/z 325 (M^+).

4.2.2 | Synthesis of 1-(2-hydroxyethyl)-4-((E)-3-((E)-1-(2-hydroxyethyl)quinolin-4(1H)-ylidene)prop-1-en-1-yl)quinolin-1-ium trifluoroacetate, CC-HE

In a dry Schlenk flash, 250 mg (0.793 mmol) 1-(2-hydroxyethyl)-4-methylquinolinium iodide and 156 mg (0.397 mmol, 0.5 equiv.) iodoform were dissolved in 20 mL anhydrous methanol under an inert atmosphere. 907 μL (3.97 mmol, 10 equiv.) 25% NaOMe solution in methanol was added dropwise. The reaction mixture was stirred at room temperature for 2 h. The reaction was quenched by the dropwise addition of 341 μL (5.95 mmol, 15 equiv.) acetic acid, then the solvents were removed under reduced pressure.

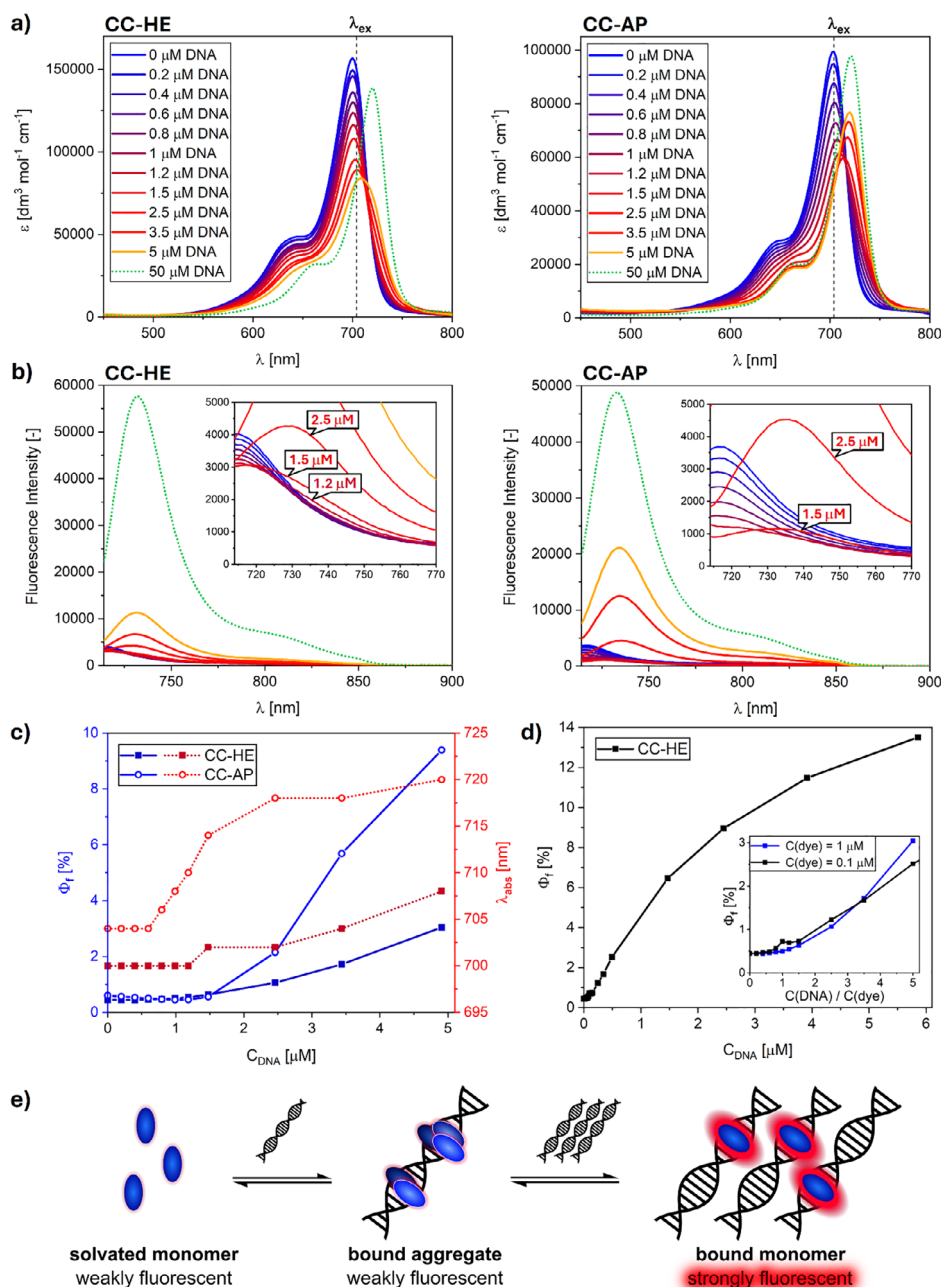


FIGURE 5 | a) Absorption and b) emission spectra of **CC-HE** and **CC-AP** in the presence of different concentrations of dsDNA. c) Effective fluorescence quantum yield (Φ_f) and absorption maximum of **CC-HE** and **CC-AP** as a function of DNA concentration (measured from a 1 μM dye solution in pH = 7.4 TE buffer). d) Effective fluorescence quantum yield (Φ_f) of **CC-HE** as a function of DNA concentration (measured from a 0.1 μM dye solution, $\lambda_{\text{ex}} = 704 \text{ nm}$). Insert: comparison of DNA titrations from 1 and 0.1 μM dye solutions, as a function of DNA/dye concentration ratio. e) Postulated sequential aggregation–deaggregation equilibrium of cryptochanines, underlying the unusual fluorescence response.

The crude product was purified by preparative HPLC (Eluent: 0.2% TFA water–MeCN, gradient). The **CC-HE** product was obtained as a greenish amorphous solid with a golden luster (23 mg, 12%).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.68 (t, $J = 12.9 \text{ Hz}$, 1H), 8.38 (dd, $J = 8.7, 1.4 \text{ Hz}$, 2H), 8.02 (d, $J = 7.3 \text{ Hz}$, 2H), 7.94 (d, $J = 8.7 \text{ Hz}$, 2H), 7.82 (ddd, $J = 8.5, 6.8, 1.3 \text{ Hz}$, 2H), 7.73 (d, $J = 7.4 \text{ Hz}$, 2H), 7.60 (t, $J = 7.6 \text{ Hz}$, 2H), 7.10 (d, $J = 12.9 \text{ Hz}$, 2H), 4.49 (t, $J = 5.1 \text{ Hz}$, 4H), 3.79 (t, $J = 5.1 \text{ Hz}$, 4H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 148.48, 142.50, 141.69, 138.27, 132.40, 125.74, 124.77, 124.21, 117.49, 110.24, 107.96, 58.81, 55.46. MS (ESI) m/z 385 (M^+).

4.2.3 | Synthesis of 1-(3-(trimethylammonio)propyl)-4-((E)-3-((E)-1-(3-(trimethylammonio)propyl)quinolin-4(1H)-ylidene)prop-1-en-1-yl)quinolin-1-ium tris(trifluoroacetate), **CC-AP**

In a pre-dried Schlenk flask, 73.3 mg (0.186 mmol, 0.5 equiv.) iodoform and 151 mg (0.372 mmol) 4-methyl-1-(3-(trimethylammonio)propyl)quinolin-1-ium dibromide were dissolved in 8 mL anhydrous methanol under an inert atmosphere. 150 μL (0.656 mmol, 1.7 equiv.) 25% NaOMe solution in methanol was added dropwise. The reaction mixture was stirred at room temperature overnight. The reaction was

quenched by the dropwise addition of excess acetic acid, after which the solvents were removed under reduced pressure. The crude product was purified by preparative HPLC (Eluent: 0.2% TFA water—MeCN, gradient). The desired **CC-AP** product (28 mg, 18%) was obtained as a blue amorphous solid.

^1H NMR (600 MHz, DMSO- d_6) δ 8.74 (t, J = 12.9 Hz, 1H), 8.41 (dd, J = 8.8, 1.4 Hz, 2H), 8.13 (d, J = 7.3 Hz, 2H), 7.98 (d, J = 8.7 Hz, 2H), 7.88 (ddd, J = 8.5, 6.9, 1.3 Hz, 2H), 7.82 (d, J = 7.4 Hz, 2H), 7.64 (dd, J = 8.3, 7.0 Hz, 2H), 7.17 (d, J = 13.0 Hz, 2H), 4.46 (t, J = 7.5 Hz, 4H), 3.47 (m, 4H), 3.08 (s, 18H), 2.27 (tq, J = 11.9, 7.8, 6.2 Hz, 4H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 148.59, 142.99, 140.74, 137.97, 132.89, 126.03, 124.97, 124.22, 117.23, 111.10, 108.77, 62.36, 52.46, 50.07, 22.55. MS (ESI) m/z 166 (M/3) $^+$.

4.3 | Deuteration of CC-HE for NMR Analysis

2.5 mg of **CC-HE** was dissolved in 0.6 mL of D_2O and transferred to an NMR tube. 10 equiv. trifluoroacetic acid was then added. ^1H -NMR, ^{13}C -NMR, COSY, HSQC, HMBC, NOESY, and TOCSY spectra were recorded. LC-MS measurements were carried out from the NMR sample.

^1H NMR (600 MHz, D_2O) δ 9.18 (d, J = 6.1 Hz, 1H), 9.03 (d, J = 6.4 Hz, 1H), 8.64 (dd, J = 8.5, 1.3 Hz, 1H), 8.45 (dd, J = 8.4, 1.2 Hz, 1H), 8.42 (d, J = 9.1 Hz, 1H), 8.32 (d, J = 8.9 Hz, 1H), 8.28 – 8.22 (m, 1H), 8.19 (m, 1H), 8.05 (m, 1H), 8.04 (m, 2H), 7.93 (dd, J = 8.5, 7.1 Hz, 1H), 7.23 (s, 1H), 5.09 (t, J = 7.8 Hz, 2H), 5.01 (t, J = 7.7 Hz, 2H), 3.60 (m, 2H), 3.53 (m, 2H), 3.10 (s, 9H), 3.08 (s, 9H), 2.60 (m, 4H); ^{13}C NMR (151 MHz, D_2O) δ 159.35, 154.32, 147.99, 147.24, 140.39, 137.94, 137.62, 136.10, 135.96, 130.35, 129.81, 129.07, 127.39, 126.95, 126.62, 122.31, 118.98, 118.30, 117.89, 62.61, 53.98, 53.67, 53.03, 35.89 (m), 23.13, 23.00.

The carbon signal corresponding to the dideuterated carbon at 35.89 ppm was detected in the HMBC measurement. MS (ESI) m/z 387, (M(D_2)) $^+$, 388, (M-(D_3)) $^+$, 386, (M-(D)) $^+$.

4.4 | Spectroscopic Characterization

Absorption spectra were recorded on a Shimadzu UV-1900i UV-vis spectrophotometer in 1 cm quartz cuvettes. During the measurements, a 2 mM stock solution of the dyes was added to 2 mL TE buffer (Tris-EDTA, pH = 7.4, 10 mM Tris-HCl, 1 mM EDTA) in 2 μL increments. The extinction coefficient was calculated from the linear regression of 6 measurements.

Emission spectra were recorded on a Shimadzu RF-6000 spectrofluorimeter. Emission of the dyes in the absence of DNA was measured during the DNA titration experiments (*vide infra*), using 1.0 μM solutions of the dyes in TE buffer (pH = 7.4). Emission of the dye-DNA complex was recorded from a 1 μM solution of the dyes in a 50 μM solution of plasmid DNA in TE buffer (pH = 7.4).

4.5 | DNA Titration Experiments

Fluorescence enhancement of the dyes was measured using pAAV-hSyn-DIO-ChrimsonR-mRuby3-ST plasmid dsDNA containing 6882 base pairs with a 53.8% GC content. The plasmid DNA was purified by EndoFree Plasmid Maxi Kit (Qiagen),

following the manufacturer's instructions. Concentration of the purified plasmid was measured by NanoDrop 2000/2000c Spectrophotometers (Thermo Scientific). The exact sequence of the plasmid used in DNA titration experiments is available in the [Supporting Information](#).

To perform the DNA titration experiments from a 1 μM solution of the dyes, the following stock solutions were prepared:

1. 1 mM solution of the dyes in DMSO
2. 1 and 0.1 mM solutions of dsDNA (these values refer to the concentration of base pairs) in TE buffer

Each measurement started with recording an emission spectrum from 2.0 mL blank TE buffer. To this buffer was added:

1. 2 μL 1 mM (or 0.1 mM) solution of the given dye
2. $6 \times 4 \mu\text{L} + 1 \times 6 \mu\text{L}$ 0.1 mM dsDNA solution
3. $2 + 2 + 3 \mu\text{L}$ 1 mM dsDNA solution

After each addition, the solution was homogenized, and an emission spectrum was recorded using the absorption maximum of the given free dye as excitation wavelength (1 background + 10 data points).

To perform the DNA titration experiments from a 0.1 μM solution of the dyes, the following stock solutions were prepared:

1. 0.1 mM solution of the dyes in DMSO
2. 1, 0.1, and 0.01 mM solution of dsDNA (these values refer to the concentration of base pairs) in TE buffer

Each measurement started with recording an emission spectrum from 2.0 mL blank TE buffer. To this buffer was added:

1. 2 μL 0.1 mM (or 0.1 mM) solution of the given dye
2. $6 \times 4 \mu\text{L} + 1 \times 6 \mu\text{L}$ 0.01 mM dsDNA solution
3. $2 \times 2 \mu\text{L} + 1 \times 3 \mu\text{L}$ 0.1 mM dsDNA solution
4. $2 + 2 + 3 + 4 \mu\text{L}$ 1 mM dsDNA solution

After each addition, the solution was homogenized, and an emission spectrum was recorded using the absorption maximum of the given free dye as excitation wavelength (1 background + 14 data points).

For each spectrum, an effective quantum yield was calculated using the relative quantum yield method [2]. The word *effective* stands for the fact that the measured fluorescence quantum yield is the weighted average of the Φ_f of the free and the various DNA-bound molecules (Φ_f^0 and Φ_f^{DNA}) present in dynamic equilibrium in solution.

4.6 | Study of Selectivity Over BSA

The specificity of DNA binding over binding to BSA (bovine serum albumin) was investigated by titrating the dye solutions

(**CC-HE** and **CC-AP**) with a BSA stock solution. The concentration of the BSA solution was determined spectrophotometrically previously (ϵ_{280} value was taken from: “*TECH TIP #6 Extinction Coefficients*” (PDF). Thermo Scientific. Retrieved 1 December 2025).

For both cryptocyanines, the effective fluorescence quantum yield was found to slowly increase over the $C_{\text{BSA}}/C_{\text{dye}} = 0\text{--}5$ range, without any sudden changes in the shape of the titration curve. This change in Φ_f is accompanied by no significant change in absorption maxima and a small (7–9 nm) bathochromic shift in emission maxima (Figure S9).

4.7 | Photostability Tests

1 μM solutions of **CC-HE**, **CC-AP**, and **Rhodamine-800** (reference) were irradiated with 690 nm red light in a Shimadzu RF-6000 spectrofluorometer; after each irradiation period, the solution was homogenized, and an emission spectrum was recorded ($\lambda_{\text{ex}} = 690$ nm; excitation bandwidth: 5 nm; emission bandwidth: 5 nm). The change of concentration was determined fluorimetrically, directly from the irradiated solutions ($\lambda_{\text{ex}} = 690$ nm).

Since the investigated dyes have different absorptions at the photolysis wavelength, we have set the concentrations to match the absorbance of a 1 μM Rhodamine-800 solution.

1. **CC-HE**: $\epsilon_{690} = 29400 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$; $C = 0.696 \mu\text{M}$
2. **CC-AP**: $\epsilon_{690} = 23200 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$; $C = 1.01 \mu\text{M}$
3. **Rhodamine-800**: $\epsilon_{690} = 44200 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$; $C = 1.00 \mu\text{M}$

Under these circumstances, both cryptocyanines exhibit somewhat higher photobleaching than **Rhodamine-800**, with a twofold difference between the initial reaction rates of the two extremes, **Rhodamine-800** (most photostable) and **CC-HE** (least photostable) (Figure S10).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: adsr70114-sup-0001-SuppMat.docx.