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Modifications of DCDHF single molecule fluorophores to impart water solubility

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Abstract

A series of dicyanomethylenedihydrofuran (DCDHF) fluorophores with different hydrophilic groups were synthesized and their photophysical properties and water solubilities were measured. Significant water solubility was achieved without compromising desirable photophysical properties, permitting applications of these fluorophores in biological systems.

Dicyanomethylenedihydrofuran (DCDHF) chromophores are useful not only as photorefractive materials^{1–4} but also can be imaged at the single-molecule level with additional beneficial properties such as viscosity-dependent fluorescence, strong solvatochromism, significant ground-state dipole moment and moderate hyperpolarizability.^{5–11} From a synthetic point of view, this family of fluorophores also has a high degree of flexibility in all of the donor, acceptor and conjugated core substructures (Fig. 1).

Most of the previously described DCDHF fluorophores are soluble in organic solvents but are not soluble in aqueous media. For example, DCDHF-6¹ (**1**: R₁=R₂=*n*-hexyl, R₃=R₄=methyl; *n*=0 and Ar=phenyl) and DCDHF-2-V¹⁰ (**2**: R₁=R₂=ethyl; R₃=R₄=methyl; *n*=1 and Ar=phenyl) have less than 0.001 ppm water solubility. With alkyl substituents at the various R positions, such molecules have an amphiphilic motif and label cell membranes easily.¹¹ In order to prevent membrane binding and enable applications in the cellular cytosol, it is useful to develop water soluble forms.¹² To explore the water solubility potential of these fluorophores, we modified the donor component with a variety of hydrophilic groups (alcohol, carboxylic acid, and sulfonic acid), and their water solubility and photophysics in aqueous environments were determined.

Fluorophores **6** and **9** (alcohols) were first synthesized as shown in Scheme 1. Intermediate **5** was made by condensation of 3-hydroxy-3-methyl-2-butanone with malononitrile.¹³ Alkylation of 2-(anilino)ethanol, protection of the alcohol group by esterification (**3**), Vilsmeier reaction, and deprotection gave the key aldehyde precursor **4**, which was subsequently condensed with intermediate **5** to give fluorophore **6** with one free alcohol group terminating the short alkyl chain. Alternatively, alkylation of *N*-ethylaniline with 6-chloro-1-hexanol, protection of the alcohol group by esterification (**7**), Vilsmeier reaction and deprotection gives the key aldehyde precursor **8**, which was also condensed with intermediate **5** to give the desired fluorophore **9** with one free alcohol group terminating the longer chain. Measurements (Table 1) show that both of these two mono-alcohol terminated fluorophores still have very low water

solubility (around 0.01 ppm) with fluorophore **6** slightly higher than fluorophore **9** (Scheme 1).

Although these two fluorophores show very low water solubility, it is improved compared with fluorophores without any alcohol group at all. Therefore, we designed fluorophore **12** to increase water solubility by removing the vinyl linkage. The synthesis of the key intermediate **10** has been reported before.¹ Commercially available 2-(*n*-butylamino)ethanol **11** was chosen as a nucleophile to prepare fluorophore **12**.¹⁵ Measurements show a solubility of 0.40 ppm for **12**, which is better than the dyes with the vinyl linkage but still insufficient for our applications. Next, we decided to increase the number of the free alcohols to improve water solubility and so fluorophores **14** and **15** were subsequently synthesized. Glycidol undergoes nucleophilic attack with *n*-butylamine to give secondary amine **13**, which then undergoes the same nucleophilic aromatic substitution on **10** to give fluorophore **14**.¹⁵ The same reaction occurs between *N*-methyl-D-glutamine and intermediate **10** to give multiply hydroxylated fluorophore **15**.¹⁵ Measurements (Table 1) show that the solubility increases almost 10 times with an increase of one free alcohol group, and increases another 30 times with an increase of another three free alcohol groups (two and five total alcohol groups, respectively, in **14** and **15**) (Scheme 2).

Thus far, we had been able to increase the water solubility of the DCDHF fluorophores from less than 0.001 ppm to 150 ppm. Clearly, addition of hydroxyl groups alone is not a particularly effective means to enhance solubility so we switched our attention to other functional groups beginning with carboxylic acid groups. *N*-Methylbutyric acid and iminodiacetic acid reacted with **10** to give fluorophores **16** and **17**, respectively.¹⁵ Fluorophore **16** with a single carboxylic acid has only 3.80 ppm water solubility, which is comparable to fluorophore **14** with two free alcohol groups. However, and to our surprise, fluorophore **17** with two carboxylic acids was found to have 2830 ppm water solubility, which is at least 20 times higher than that compared with fluorophore **15**, the one with five free alcohol groups (Scheme 3).

Although fluorophore **17** already has enhanced water solubility, we still wanted to push further, and so a sulfonic acid group was introduced next into the system. In this synthesis, 1,3-propanesultone underwent attack from either *n*-butylamine or dodecylamine to give precursors **18** and **20**, respectively, which underwent the same reaction with aryl fluoride intermediate **10** to give fluorophores **19** and **21**.¹⁶ Measurements show that these sulfonic acid substituted fluorophores have significant water solubilities (larger than 2×10^4 ppm). The influence of this single sulfonic acid is also so large that we decided to add the vinyl linkage back again so that we could also assay the group of DCDHF-V fluorophores containing the vinyl linkage. Fluorophore **24** was synthesized by nucleophilic aromatic substitution of 4-fluorobenzaldehyde with 2-(methylamino)ethanol. The alcohol was then deprotonated and reacted with a sultone to attach a sulfonic acid to the benzylaldehyde substrate. Finally, the aldehyde was condensed with intermediate **5** to provide the desired vinyl DCDHF-V fluorophores functionalized with sulfonic acid (Scheme 4). Here again measurements indicate water solubility larger than 2×10^4 .

With these highly water soluble fluorophores available, our concerns about whether or not the presence of these hydrophilic groups will affect the photophysical properties of these fluorophores could be evaluated. To this end, we compared the fluorophores bearing different hydrophilic groups with their original low polarity analogs **1** and **2**. Table 1 shows that within groups **I** (DCDHF) and **II** (DCDHF-Vs) the absorption and emission wavelengths are quite similar. The differences for fluorophores **1**, **2**, **6** from their counterparts can be attributed to the addition of ethanol to the aqueous solution. A few other fluorophores in Table 1 (i.e., **17**, **19**, and **27**) exhibit somewhat blue-shifted absorption or emission relative to their counterparts. Given the amphiphilic character of these molecules, the anomalous photophysics may be due

to the formation of aggregates or micelles in water at the relatively high concentrations required for bulk measurements. However, at the nano-molar concentrations actually used for single-molecule experiments, such effects are unlikely. For instance, although the fluorophores used in our earlier studies were not soluble in water at bulk concentrations, single molecules were observed diffusing in the cell membrane at the nanomolar regime.¹¹ As such, this means that while we increased the water solubility of this family of fluorophores, the desirable spectroscopic properties were essentially unchanged.

This outcome is also confirmed by independent viscosity dependence experiments. Previously, in a mixed solvent of glycerol and methanol, the quantum yield of fluorophore **1** was found to grow with the increase of the glycerol content.⁷ Here, two identical solutions, each of **17** and **24** in water, were prepared. For each pair, the sample on the right was frozen while the other sample remained at room temperature (Fig. 2). Under illumination with a UV lamp, the liquid sample (left of each pair) shows limited fluorescence while the frozen sample (right of each pair) is strongly emissive. Therefore, the viscosity dependence of the emission is maintained after the addition of dicarboxylic acid and sulfonic acid. This suggests that our DCDHF fluorophores have the potential for application in a cellular environment beyond lipid-analog membrane probes.

All the examples of solubilized DCDHF fluorophores described thus far are anionic. We have also briefly examined some cationic derivatives. The secondary amine in *N,N,N'*-trimethylethylenediamine reacts with **10** to give fluorophore **25**, which next underwent alkylation at the trialkylamine end with methyl iodide to give monocationic fluorophore **26**. In a similar fashion, alkylation of **25** with 1,3-diiodopropane gives dicationic dimer fluorophore **27** (Scheme 5). Although **26** and **27** are not as water soluble (730 ppm, 470 ppm respectively) as fluorophores **19**, **21** and **24**, they demonstrate still another option to functionalize and solubilize this family of DCDHF fluorophores.

In conclusion, we have synthesized a series of DCDHF fluorophores with a range of functional groups and modified their water solubility without compromising their photophysical properties. These results suggest that this class of fluorophores possess strong potential for a broad range of bio-labeling applications. The methods applied here to solubilize the DCDHF fluorophores should also be applicable in other cases, where water solubilization is required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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14. The water solubilities of the DCDHF chromophores were determined from UV-vis data. A standard solution of known concentration was prepared by dissolving about 1 mg of the compound in methanol/H₂O (3/7, 10 ml) for fluorophores **6**, **9**, **12**, **14–16**, or in methanol/H₂O (2/8, 10 ml) for fluorophores **17**, **19**, **21**, **24**, **26**, **27**. The saturated solutions were prepared by suspending about 2 mg or more fluorophores in H₂O (10 ml) and then stirring at 60 °C for 2 h, followed by equilibration at room temperature for 12 h. These samples were passed through a syringe filter (2 μm). The absorption could be measured from the UV-vis spectra and the absorption coefficients could be calculated from standard solutions, which were used to determine the concentration of the substrate in the pure H₂O sample. The results reported are averages of duplicate runs. Benbow JW, Bernberg EL, Korda A, Mead JR. Antimicrob. Agents Chemother 1998;42:339–343.343 [PubMed: 9527782]
15. General methods to obtain fluorophores **12**, **14–17**, **25**: the starting disubstituted amine was mixed with precursor **10** in dry pyridine. The reaction mixture was then stirred at room temperature or heated to 40 °C overnight depending on the substrate. Pyridine was then distilled out under vacuum and the residue was purified via flash chromatography.
16. General methods to obtain fluorophores **19**, **21**: the starting disubstituted amine was mixed with precursor **10** in anhydrous DMF with 2 equiv *i*-Pr₂NEt. The reaction mixture was then stirred at 40 °C overnight. Hydrochloric acid (10%) was added and the suspension was filtered to give the product as a yellow solid.

Figure 1.
Generic structure of the DCDHF fluorophores.

Figure 2. Fluorophores **17** and **24** dissolved in liquid water (vials *l*) and in solid ice (vials *s*) under irradiation with a UV lamp (a filter was used to block the scattered light). The fluorescence is significantly enhanced in the ice samples responding to the dramatic enhancement in viscosity.

Scheme 1.

Synthesis of DCDHF-V dyes with a single primary alcohol group in the amine donor tail. Reagents and conditions: (a) 1-iodohexane, K_2CO_3 , DMF and then Ac_2O , Py; (b) POCl_3 , DMF and HCl/methanol reflux; (c) Py, acetic acid; (d) 6-chloro-1-hexanol; NaOH, KI, DMF and Ac_2O , Py.

Scheme 2.

Synthesis of DCDHF dyes with alcohol functionalization in the amine donor tail. (a) *n*-Butylamine, reflux; (b) pyridine.

Scheme 3.
Synthesis of DCDHF dyes with carboxylic acid functionalization in the amine donor tail. (a) Pyridine.

Scheme 4.

DCDHF and DCDHF-V dyes functionalized with sulfonic acids. Reagents and conditions: (a) 1,3-propanesultone, reflux; (b) pyridine; (c) DMF, K_2CO_3 , 2-(methylamino)ethanol, 130 °C; (d) NaH, DMF, 1,3-propanesultone; (e) pyridine, acetic acid.

Scheme 5.

The preparation of water solubilized DCDHF dyes, which are functionalized with quaternary amines. Reagents and conditions: (a) pyridine; (b) 1-iodomethane, CH₂Cl₂; (c) 1,3-diiodopropane, CH₂Cl₂.

Table 1
Photophysical properties and water solubility measurements of different DCDHF (I) and DCDHF-V (II) chromophores

	$\lambda_{\max}$	λ_{em}	Φ_F (water)	$\epsilon_{\max}$ (Lcm ⁻¹ mol ⁻¹)	Solubility in water 14 (ppm)
I	1	470 ^a	0.12	89,900	<0.001
	12	498	0.001	15,100	0.40
	14	496 ^a	0.002	44,200	1.40
	15	493	0.001	64,700	1.50 × 10 ⁻²
	16	500	0.001	37,300	3.80
	17	489	0.002	31,500	2.80 × 10 ⁻³
	19	500	0.001	79,300	>2 × 10 ⁻⁴
	21	496 ^b	—	65,800	>2 × 10 ⁻⁴
	26	477	0.001	57,300	7.30 × 10 ⁻²
	27	480	0.004	42,700	4.70 × 10 ⁻²
	2	560 ^a	0.002	13,500	<0.001
II	6	535 ^a	0.002	77,900	~0.05
	9	587 ^b	—	87,200	~0.01
	24	585	0.01	37,000	>2 × 10 ⁻⁴

^a A small volume of ethanol stock solution added to the cuvette of water.

^b Aggregation was observed in water.