Modulation of intramolecular heterodimer-induced fluorescence quenching of tricarbocyanine dye for the development of fluorescent sensor†

Tomoya Hirano,*[a] Jun Akiyama,[b] Shuichi Mori[b] and Hiroyuki Kagechika*[a,b]

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Various approaches have been used to modulate the fluorescence changes of sensors in the presence of target analytes, including intramolecular interaction between fluorophores or between fluorophore and other molecular species, like resonance energy transfer (RET). Here, we focus on fluorescence quenching by intramolecular heterodimer complex formation, which can be modulated over a shorter distance range than RET. We synthesized several conjugates of tricarbocyanine, which is a near-infrared fluorophore, with several quencher candidates *via* flexible short linker structure, and examined their fluorescence properties. Of our synthesized compounds, the dabcyl group proved to be the best quencher *via* heterodimer complex formation. The fluorescence of tricarbocyanine–dabcyl conjugates in aqueous media was almost completely quenched, and there was a dramatic fluorescence enhancement when heterodimer formation was blocked. These results suggested a design approach to develop fluorescence sensors for probing proximity relationships and structural transitions.

Introduction

Fluorescent sensors, whose fluorescence properties change upon binding or reacting with an analyte, have been widely used in various fields, including cell biology and clinical diagnosis.[1,2] Various empirical or theoretical approaches have been used to obtain a sufficient fluorescence change in the presence of the analyte. For example, resonance energy transfer (RET) between two fluorophores or between a fluorophore and another molecular species has been utilized to develop fluorescent sensors for enzymatic activity or for sensing structural changes of proteins.[3,4] Its efficiency depends mainly on the distance between the donor and acceptor moieties, and when this is changed by interaction of the sensor with the analyte, there is a change of fluorescence intensity and wavelength. Photo-induced electron transfer (PeT) has also been utilized in sensors. A change of the distance between PeT donor and acceptor, or the change of the oxidation or reduction potential of these substructures in the sensor modulates the extent of PeT, resulting in a change of fluorescence intensity.[5,6] Based on this mechanism, fluorescent sensors for proton,[7,8] metal cations,[9–11] and nitric oxide[12] have been developed.

The control of intermolecular or intramolecular complex formation between two fluorophores or between a fluorophore and another molecular species has also been utilized for the development of fluorescent sensors.[13,14] For example, a carbocyanine dye-conjugated graft copolymer consisting of poly-L-lysine and methoxypolyethylene glycol succinate was reported to be useful as a fluorescent sensor for some tumors *in vivo*.[15] The fluorescence of the carbocyanine dye was quenched by homo-aggregation in the polymer, but on cleavage of the peptide chain by tumor-associated peptidase, carbocyanine is released and its fluorescence is recovered. In the case of heterodimer formation, several interactions, such as static quenching by the ground state complex and dynamic interactions like RET and PeT, can occur simultaneously. In the case of molecular beacons, which can sense specific sequences of nucleobases, two fluorophores or a fluorophore and a quencher are placed close together, where RET and/or static quenching can operate.[16–20] Upon binding to analyte oligonucleotides, the two chromophores are separated, resulting in an increase of fluorescence intensity. In recently reported fluorescent sensors for tyrosine kinase, pyrene and the hydroxyphenyl group of substrate tyrosine form a heterodimer, resulting in fluorescence quenching *via* both static and dynamic quenching.[21] Phosphate ester formation by the analyte kinase hinders the formation of this heterodimer, resulting in recovery of fluorescence.

Generally, the efficiency of RET depends on the distance between donor and acceptor, which is typically in the range of 2–10 nm.[3,4] Compared with RET, the change of fluorescence properties by the formation of homodimer or heterodimer complex requires contact formation, like van der Waals contact, which means that it can be modulated over a shorter distance range than RET, and those characteristic are useful for sensing the proximity relationship.[22–24] For example, Sauer's group reported that oxadine derivative MR121, which was used as a fluorophore, could form a non-fluorescent complex with amino acid tryptophan, and this pair could be used to study conformational dynamics of some proteins in the sub-nanometre range.[25–28] In this paper, we focused on tricarbocyanine dye, which has near-infrared fluorescence and is potentially attractive for *in vivo* fluorescence imaging,[29] as a fluorophore, and examined the structural requirements for intramolecular heterodimer formation and fluorescence quenching. Although some compounds such as NIRQ750, BHQ-3 and Cy7Q, which can quench the fluorescence of tricarbocyanine dye mainly *via* RET,[30–32] have already been reported, we tried to find the

[a]*Institutes of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan. E-mail: hiraomc@tmd.ac.jp, kage.omc@tmd.ac.jp; Fax: +81-3-5280-8127; Tel: +81-3-5280-8128*
[b]*Graduate School of Biomedical Sciences, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan*

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best quencher compound, whose absorbance has no significant spectral overlap with tricarbocyanine fluorescence. It is expected to quench the fluorescence of tricarbocyanine by heterodimer complex formation not by RET. We synthesized some conjugates of tricarbocyanine with several quencher candidates *via* various flexible short linker structures, and examined their fluorescence properties under various conditions. We anticipated that the fluorescence of compounds with appropriate quencher and linker structures would be quenched by intramolecular heterodimer formation *via* hydrophobic interaction in aqueous media, while disruption of this dimer formation, *e.g.*, by solvent polarity change or protein binding, would restore the fluorescence (Fig. 1). We considered that this might be an effective design strategy for fluorescent sensors.

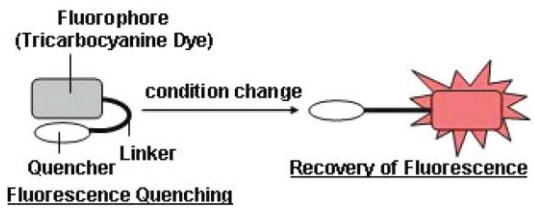


Fig. 1 Fluorescence quenching of intramolecular heterodimer formation and recovery of fluorescence in response to environmental change.

Results and discussion

Design and synthesis

Based on the strategy shown in Fig. 1, we designed tricarbocyanine derivatives that were expected not to show fluorescence until their structure was unfolded owing to disruption of intramolecular hydrophobic interaction. The designed compounds consisted of tricarbocyanine, a hydrophobic aromatic structure as the quencher candidate, and a linking group between them. As quencher candidates, benzene (**1a**), naphthalene (**1b**), biphenyl (**1c**), diphenyl ether (**1d**) and 4-(4-dimethylaminophenylazo)benzoic acid (dabcyl, **1e**), whose absorbance spectra have no significant spectral overlap with tricarbocyanine fluorescence, were selected (Fig. 2). Further, we examined the effect of linker structure by modifying compound **1e** to have a hydrophobic, longer, or shorter linking group, *i.e.*, compounds **2a**, **2b**, and **2c**, respectively.

Synthesis of compounds **1a–1e** is illustrated in Scheme 1. Condensation of monoprotected bis(2-aminoethoxy)ethane **4** with *p*-hydroxyphenylpropionic acid **3** afforded compound **5**. Tricarbocyanine **6**[33] was reacted with **5** to obtain linker–fluorophore compound **7**. After deprotection under acidic conditions, the amine **8** was reacted with succinimidyl esters of quencher candidates (**9a–9e**), yielding compounds **1a–1e**, respectively (Scheme 1). Compounds **2a–2c** were synthesized similarly to **1e** by using **10a**, **10b**, or **11c** as the starting material, respectively (Scheme 2).

Fluorescence properties in aqueous solution and methanol

Fluorescence and absorption spectra of compounds **1a–1e** in aqueous solution (10 mM sodium phosphate buffer, pH 7.4) are shown in Fig. 3. Compared with control compound **7** without a quencher moiety, whose quantum yield (Φ) was estimated to be 0.22, **1a–1d** showed partial fluorescence quenching in aqueous

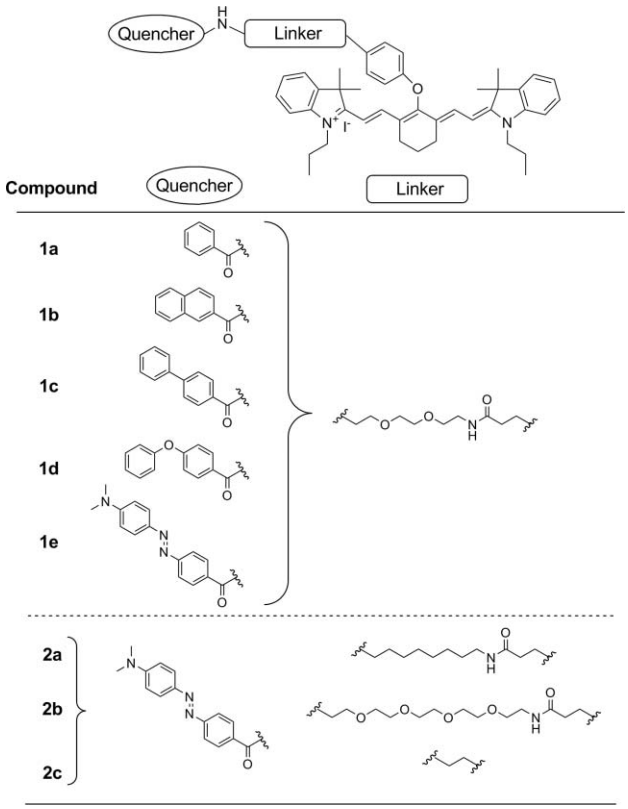
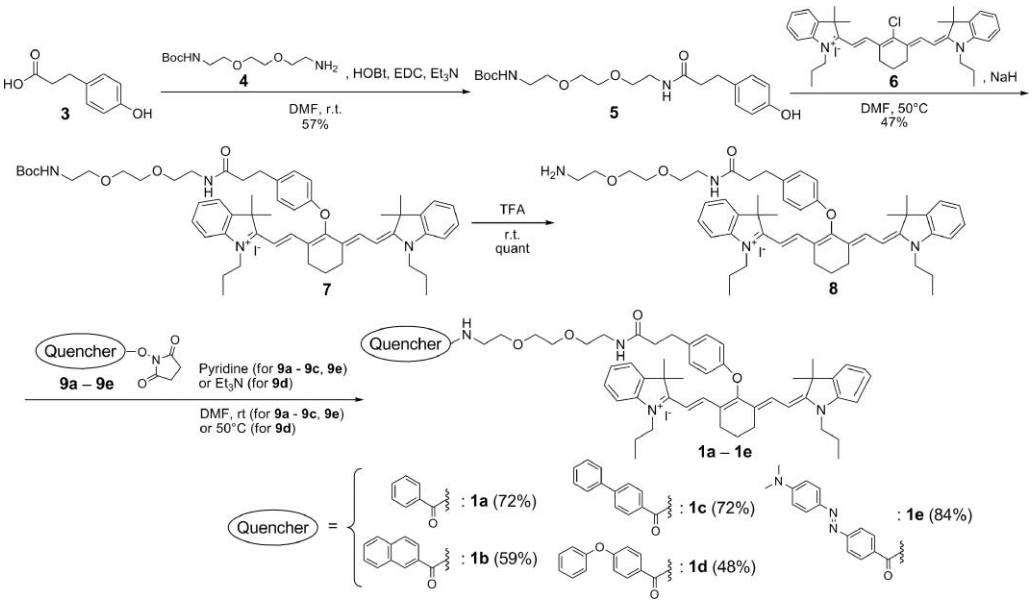


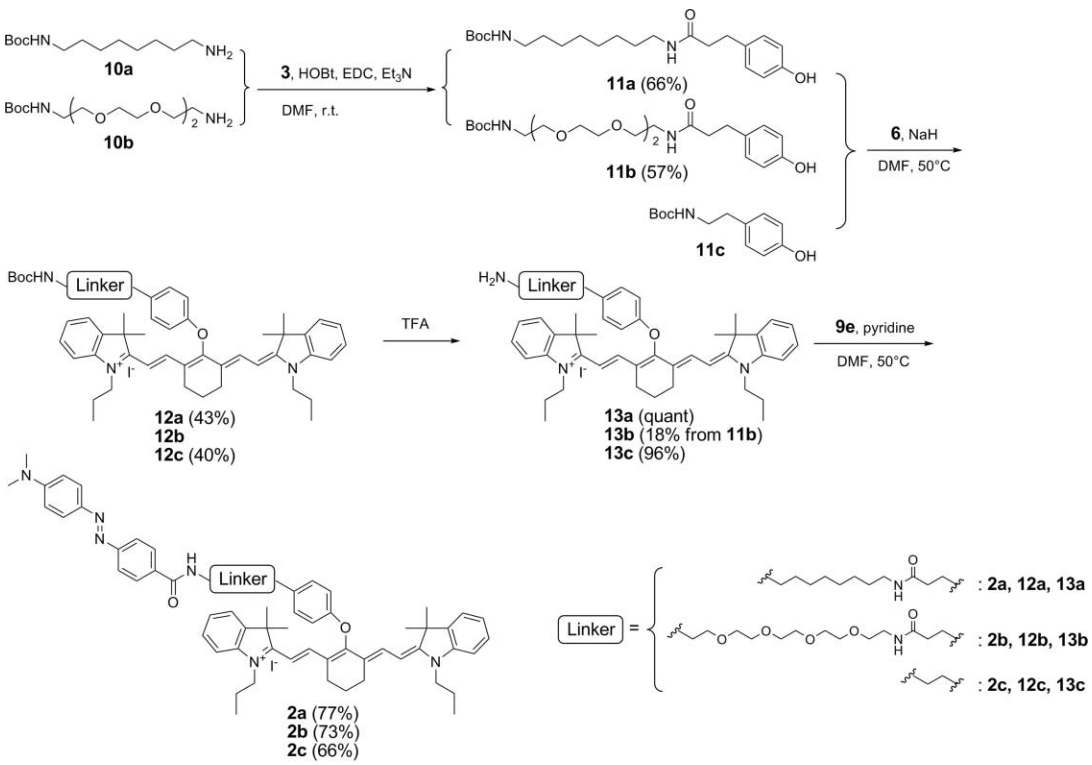
Fig. 2 Structures of quencher–linker–fluorophore (tricarbocyanine) conjugated compounds.

solution, and their quantum yields were 0.19, 0.15, 0.13 and 0.13 respectively (Table 1, Fig. 3(a)). Compound **1e** showed strong fluorescence quenching in aqueous solution, and its quantum yield was less than 0.01 (Table 1, Fig. 3(a)). In the absorption spectra (Fig. 3(b)), compounds **1a–1d** showed almost no significant shift in the absorption maximum from that of the control compound **7** and decreased absorption around 770 nm was observed, except for **1a**. However, in the case of compound **1e**, its absorption maximum (663 nm) was blue-shifted from that of compound **7** (766 nm). Absorption maximum shift was also observed around the region of 350 nm–550 nm, where the dabcyl group, the quencher moiety of **1e**, has absorption. **1e** showed blue shift, compared with the control dabcyl derivative, dabcyl *N*-(2-hydroxyethyl)amide (Dabcyl) (Fig. 3(c)). Those spectral shapes did not change in the concentration range from 0.2 μM to 4 μM (data not shown). This spectral change indicates the presence of intramolecular heterodimeric folding structures involving tricarbocyanine and the dabcyl group. In methanol solution, the partially or completed quenched fluorescence was almost completely restored, becoming similar to that of **7** (Fig. 4(a), Table 1). The absorption spectra also became similar (Fig. 4(b)) even in the case of the largely blue-shifted spectra of **1e**, which also gave a similar absorption spectrum as the control dabcyl derivative, "Dabcyl" (Fig. 4(c)). Our results indicate that the dabcyl group is the best quencher moiety of our quencher candidates for linking to tricarbocyanine dye, affording the largest enhancement of fluorescence in methanol compared with aqueous solution.

Among the compounds with various linker structures, compound **2b** (absorption maximum at 776 nm) with a longer linker



Scheme 1 Synthesis of compounds **1a–1e**



Scheme 2 Synthesis of compounds **2a–2c**

group showed an absorption spectrum slightly red-shifted from that of **7** (absorption maximum at 766 nm) in aqueous solution. Compound **2a** with a hydrophobic linker and **2c** with a shorter linker group showed broad spectra, with maxima at 710 nm and 693 nm, respectively, so those spectra seemed intermediates between those of **2b** and **1c** (Fig. 5(a), Table 1). Every compound showed sufficient fluorescence quenching in aqueous media, with quantum yields of 0.02 for **2b** and less than 0.01 for **2a** and **2c**, compared with control compound **12c** without a quencher moiety, whose quantum yield is 0.17 (Table 1, Fig. 5(b)). This quenched fluorescence could be restored by increasing the percentage of methanol in the solvent (aqueous methanol). Compound **2c** with a shorter linking group showed almost similar values to **12c** in 50% aqueous methanol solution, whereas **1e**, **2a** and **2b** showed 56%, 74%, and 61% as a percentage of the value of **12c** (taken as 100%) under same condition (Fig. 5(b)). In methanol solution, every compound showed a similar fluorescence spectrum to the control compound **12c.**

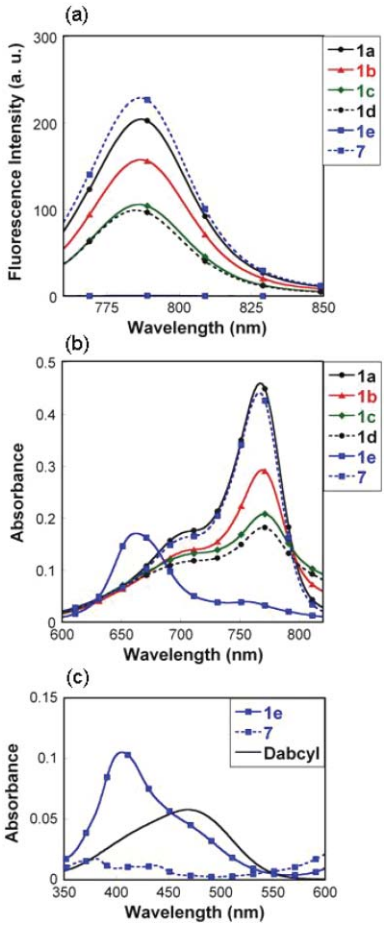


Fig. 3 Absorption and fluorescence spectra of compounds **1a–1e** and **7** (2 μM) in 10 mM sodium phosphate buffer (pH 7.4). (a) Fluorescence spectra (excitation at 745 nm), (b) and (c) absorption spectra are shown. In (c), the absorption spectrum of the control dabcyl compound (dabcyl *N*-(2-hydroxyethyl)amide, designated as "Dabcyl") is also shown. Each solution contained 0.2% DMSO as a cosolvent.

Since the spectral overlap between the absorption of the dabcyl group and the fluorescence of the tricarbocyanine group was estimated to be almost zero, the fluorescence quenching would not arise from RET. Among possible mechanisms, contact-mediated quenching was thought to be most likely. From absorption spectra, **1e** showed blue-shifted spectra, **2a** showed spectra a little red-shifted, and **2b** and **2c** showed those intermediate. Such differences in absorption spectra would be derived from a difference in the quenching mechanism. One is static quenching *via* ground state complex formation between tricarbocyanine and dabcyl group, and similar spectral changes have been reported for heterodimer pairs such as Cy5-BHQ1.[18–20] Another one is dynamic quenching, like photo-induced electron transfer (PeT). The length and constituents of the linker structure were thought to cause these differences. For elucidation of such mechanisms, time-resolved fluorescence measurement, fluorescence correlation spectroscopy and molecular dynamics simulation would be necessary, as for the previously reported case of oxadine dyes MR121, MR113 or Rhodamine 6G in formations with amino acid tryptophan, and such work is in progress.

Table 1 Fluorescent properties of the synthesized compounds

Compound	Solvent[a]	Absorption max/nm	Emission max/nm	Quantum yield[c]
1a	Water	768	783	0.19
	Methanol	767	783	0.44
1b	Water	769	784	0.15
	Methanol	767	782	0.44
1c	Water	770	784	0.13
	Methanol	768	782	0.44
1d	Water	768	784	0.13
	Methanol	767	782	0.45
1e	Water	663	n.d.[b]	<0.01
	Methanol	768	783	0.41
7	Water	766	783	0.22
	Methanol	767	782	0.46
2a	Water	710	n.d.[b]	<0.01
	Methanol	767	782	0.44
2b	Water	776	784	0.02
	Methanol	769	781	0.42
2c	Water	693	n.d.[b]	<0.01
	Methanol	767	782	0.45

[a] All data were measured in 10 mM sodium phosphate buffer (pH 7.4) or methanol. [b] Not determined. [c] Quantum yields of fluorescence were determined using that of indocyanine green (0.13) in DMSO as a standard.[34]

Fluorescence change in the presence of cyclodextrins or various proteins

Cyclodextrins (CDs) are torus-shaped cyclic oligosaccharides composed of D-glucopyranose units, and can include a variety of organic compounds in their cavities.[35] Fluorescence quenching by heterodimer formation, *e.g.*, in the case of the coumarin–fluorescein pair, is reported to be hindered by the inclusion of substructure within cyclodextrin.[36] Therefore, the fluorescence intensity of our compounds was examined in the presence of cyclodextrins of various sizes. Whereas the fluorescence intensity of **7** without a quencher part almost did not change by the addition of cyclodextrins, the quenched fluorescence of compound **1e** in aqueous solution was restored by the addition of 10 mM γ-cyclodextrin (γ-CD) (Fig. 6(a)). The recovery of the fluorescence depended on the cavity size of CD, and the addition of β-CD or α-CD with a smaller cavity gave less recovery. In the case of compounds **1b–1d**, whose fluorescence was partially quenched in aqueous solution, similar recovery was observed by the addition of γ-CD. β-CD also induced a relatively large recovery of fluorescence for compounds **1c** and **1d**, compared with **1e**. Thus, the size of the quencher part is significant for the efficiency of fluorescence recovery by the addition of β-CD. The absorption spectra of compound **1e** at various concentrations of γ-CD showed concentration-dependent change (Fig. 6(b)). As the concentration of γ-CD increased (0–10 mM), the absorption at 663 nm was decreased, while that at 772 nm was increased. The absorption of dabcyl moiety around 350 nm ~ 550 nm was also changed. The absorption at 407 nm was decreased and that around 440 nm was increased, and the spectral shape became similar to that of control compound, dabcyl *N*-(2-hydroxyethyl)amide (Fig. 6(c)). These data suggested that the fluorescence recovery in the presence of cyclodextrins is presumably a consequence of the inclusion of the substructure of each compound, probably the quencher, into the cyclodextrins. Thus, the hindrance to heterodimer formation in aqueous media could recover the quenched fluorescence as

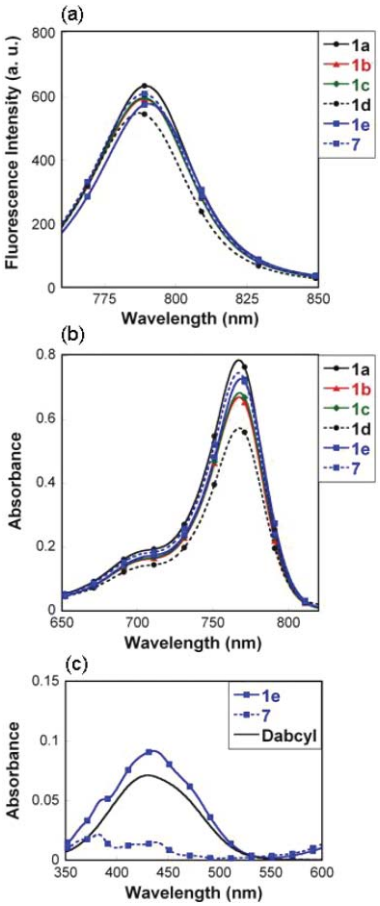


Fig. 4 Absorption and fluorescence spectra of compounds **1a–1e** and **7** (2 μM) in methanol. (a) Fluorescence spectra (excitation at 745 nm), (b) and (c) absorption spectra are shown. In (c), the absorption spectrum of the control dabcyl compound (dabcyl *N*-(2-hydroxyethyl)amide, designated as "Dabcyl") is also shown. Each solution contained 0.2% DMSO as a cosolvent.

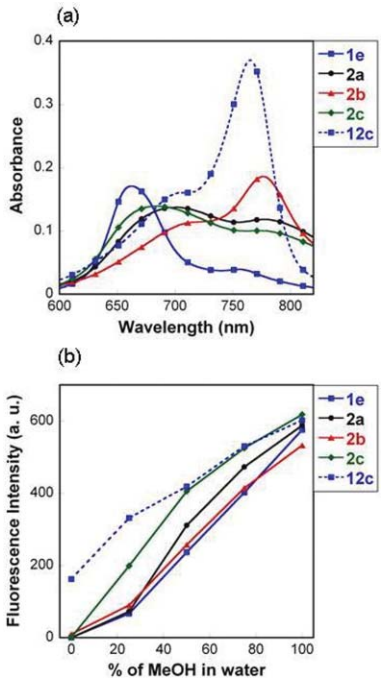


Fig. 5 (a) Absorption spectra of **1e**, **2a**, **2b**, **2c** and **12c** in 10 mM sodium phosphate buffer (pH 7.4). (b) Changes of fluorescence intensity (excitation at 745 nm, emission at 790 nm) of **1e**, **2a**, **2b**, **2c** and **12c** in various percentages of methanol in 10 mM sodium phosphate buffer (pH 7.4). All spectra were measured at a concentration of 2 μM in the presence of 0.2% DMSO as a cosolvent.

well as change of solvent polarity as shown in Fig. 3–5. Even in the extended form of these compounds, the distance between fluorophore and quencher could be relatively short, and therefore this pair could be utilized for sensing proximity relationships.

In order to explore the suitability of our compounds for biological applications, the fluorescence change of **1e** was measured in the presence of various proteins such as bovine serum albumin (BSA), Ovalbumin (Oval), Chicken egg lysozyme (Lyso) or Ribonuclease A (RNase), and these results are shown in Fig. 7 along with that of 1-(anilino)naphthalene-8-sulfonate (ANS), that is reported as a fluorescent sensor of protein surface hydrophobicity.[37,38] The fluorescence of **1e** increased in the presence of BSA or high concentration of Oval, and showed no significant enhancement by Lyso or RNase. In the case of ANS, BSA also induced fluorescence enhancement, whereas Oval, Lyso or RNase induced little increase of fluorescence, which is coincident with the reported fluorescence quantum yields of ANS in binding to those proteins, 0.12 for BSA, 0.008 for Oval, 0.008 for Lyso and 0.005 for RNase, respectively.[37] The difference between the fluorescence response of **1e** and ANS would be partly derived from the sensitivity to the protein surface hydrophobicity, (the calculated average hydrophobicity values based on the amino acid composition[39] is BSA > Oval > Lyso > RNase), and compound **1e** might be more sensitive than ANS. In addition, specific interaction of substructure of **1e** also might cause such difference, so the optimization of the substructure or conjugation with other molecular species, such as ligand molecules for receptor proteins, should enable us to develop fluorescent sensors with selectivity for particular proteins.

From the fluorescence intensity of compound **7**, which does not have dabcyl group, with various concentrations of BSA (Fig. 7(c)), the fluorescence of tricarbocyanine was also increased by the binding with a relatively high concentration of BSA. So for the fluorescence enhancement of **1e** by binding with BSA, various mechanisms including the hindrance of intramolecular heterodimer formation and the change of the environment around tricarbocyanine, both of which could induce the fluorescence enhancement, were thought to simultaneously operate, whereas by introduction of dabcyl group, the detection limit of **1e** for sensing BSA was greatly improved.

Conclusions

Our data indicate that molecules consisting of tricarbocyanine dye and dabcyl group linked *via* short and flexible linker form intramolecular heterodimers, resulting in almost complete quenching of fluorescence in aqueous media. The fluorescence is restored by disruption of the heterodimer under various conditions, *e.g.*, change of solvent polarity, inclusion of substructure within cyclodextrin or protein binding. Even in the extended form of our compounds, the distance between tricarbocyanine and the

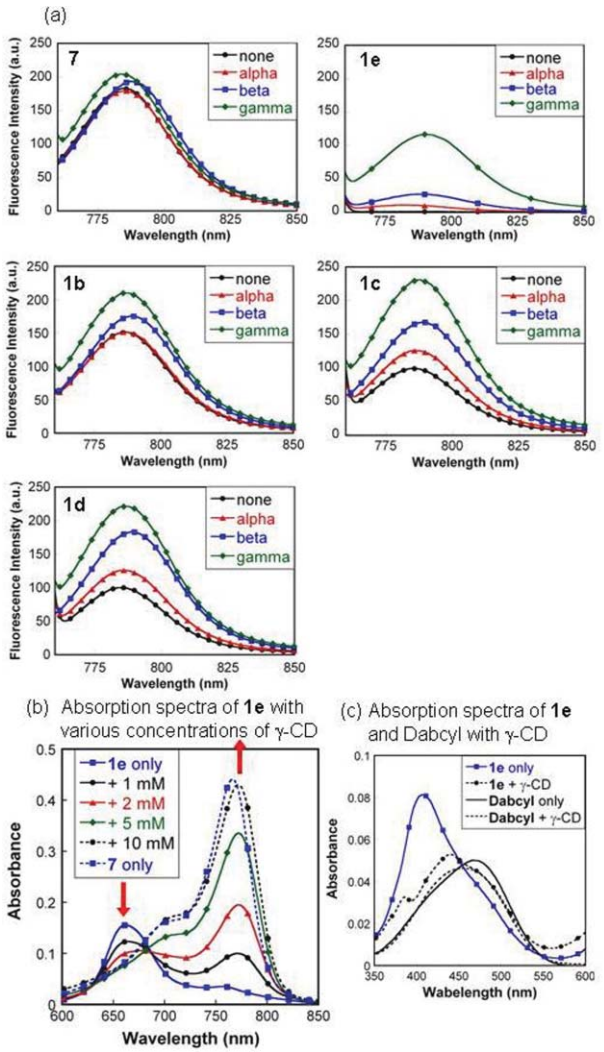


Fig. 6 (a) Fluorescence spectra (excitation at 745 nm) of **7** and **1b–1e** on the addition of 10 mM of cyclodextrin (α-CD (alpha), β-CD (beta) or γ-CD (gamma)). (b) Absorption spectra of **1e** at various concentrations of γ-CD. The absorption spectrum of **7** is also shown. (c) Absorption spectrum of **1e** and the control dabcyl compound (dabcyl *N*-(2-hydroxyethyl)amide, designated as "Dabcyl") on the addition of 10 mM γ-CD. All spectra were measured at 2 μM of sensor in the presence of 0.2% DMSO as a cosolvent in 10 mM sodium phosphate buffer (pH 7.4).

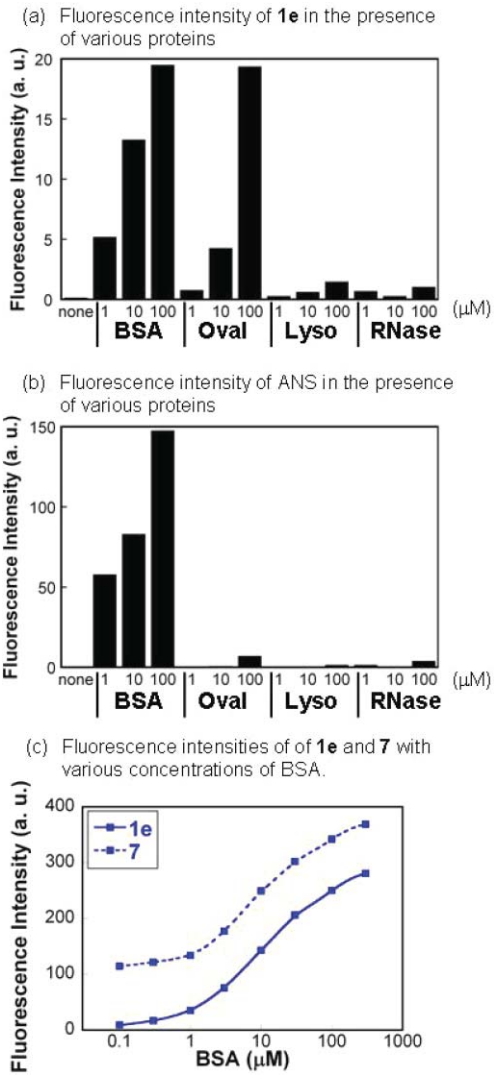


Fig. 7 Fluorescence intensity of (a) **1e** (0.5 μM, excitation at 745 nm, emission at 790 nm) or (b) ANS (0.5 μM, excitation at 370 nm, emission at 460 nm) in 10 mM sodium phosphate buffer (pH 7.4) in the presence of 1–100 μM of Bovine serum albumin (BSA), Ovalbumin (Oval), Chicken egg lysozyme (Lyso) or Ribonuclease A (RNase). (c) Fluorescence intensities of **1e** and **7** (0.5 μM, excitation at 745 nm, emission at 790 nm) with various concentrations of BSA.

dabcyl group could be short enough for RET to generally occur. However, such interactions would not occur efficiently for the tricarbocyanine–dabcyl pair, partly because there is no significant spectral overlap between tricarbocyanine fluorescence and dabcyl absorption. This characteristic should make these molecules useful for sensing changes of distance in the nm range, as well as recently reported "short-distance" RET pairs.[40,41] These insights should be useful in developing new fluorescent sensors as tools to probe relative proximity relationships and structural transitions.

Experimental

See the ESI† for the synthesis and characterization data for compounds **1a–1d**, **2a–2c** and their intermediates.

Materials and Reagents

All reagents were purchased from Sigma-Aldrich Chemical Co., Tokyo Kasei Kogyo Co, Wako Pure Chemical Industries, and Kanto Kagaku Co., Inc. Silica gel for column chromatography was purchased from Kanto Kagaku Co., Inc. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 500 spectrometer. Mass spectral data was obtained on Bruker Daltonics microTOF-2focus in the positive and negative ion detection modes. Melting points were taken on a Yanagimoto micro melting point apparatus and are uncorrected. Elemental analyses were carried out by Yanaco MT-6 CHN CORDER spectrometer. UV spectra were recorded with JASCO V-550, and fluorescence spectra were recorded with JASCO FP-6600.

Preparation of 5

3-(*p*-Hydroxyphenyl)propionic acid (**3**, 0.12 g, 1.0 mmol), triethylamine (0.21 ml, 1.5 mmol), 1-hydroxybenzotriazole monohydrate (0.18 g, 1.2 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.23 g, 1.2 mmol) were added to a solution of **4** (0.30 g, 1.2 mmol) in DMF (10 ml). After 15 h, the reaction mixture was evaporated. The residue was diluted with dichloromethane, and the organic layer was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated. The crude was purified by silica gel column chromatography (5% MeOH–CH_2Cl_2) and 0.22 g of **5** (0.58 mmol, 57%) was obtained as colorless liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.03 (2 H, d, J = 8.3 Hz), 6.75 (2 H, d, J = 8.3 Hz), 6.59 (1 H, br s), 5.89 (1 H, br s), 5.00 (1 H, br s), 3.60–3.49 (6 H, m), 3.45 (2 H, t, J = 4.6 Hz), 3.41 (2 H, m), 3.30 (2 H, m), 2.87 (2 H, t, J = 7.2 Hz), 2.43 (2 H, t, J = 7.2 Hz), 1.45 (9 H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.8, 156.2, 154.86, 131.9, 129.3, 115.4, 79.6, 70.3, 70.1, 70.0, 69.9, 69.9, 40.2, 39.2, 38.6, 30.9, 28.4; HRMS (ESI$^+$) Calcd for $C_{20}H_{32}N_2NaO_6$ (M + Na^+): 419.2153; Found: 419.2147.

Preparation of 7

Sodium hydride (2.7 mg, 0.11 mmol) was added to a solution of **5** (44 mg, 0.11 mmol) in anhydrous DMF (5 ml) at 0 °C under an argon atmosphere. After 30 min, **6** (67 mg 0.10 mmol) was added to the reaction mixture, and the whole was stirred overnight at 50 °C. The mixture was quenched with saturated aqueous ammonium chloride, and the solvent was evaporated. The residue was diluted with dichloromethane, and the organic layer was washed with water and brine, dried over Na_2SO_4, filtered and evaporated. The crude was purified by silica gel column chromatography (3% MeOH–CH_2Cl_2 then 8% MeOH–CH_2Cl_2) and 49 mg of **7** (48 μmol, 47%) was obtained as a green solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.91 (2 H, d, J = 14.1 Hz), 7.85 (1 H, br s), 7.31 (2 H, dd, J = 7.9, 7.5 Hz), 7.29 (2 H, d, J = 7.4 Hz), 7.24 (2 H, d, J = 8.6 Hz), 7.17 (2 H, dd, J = 7.5, 7.4 Hz), 7.04 (2 H, d, J = 7.9 Hz), 6.91 (2 H, d, J = 8.6 Hz), 5.97 (2 H, d, J = 14.1 Hz), 5.14 (1 H, br s), 3.97 (4 H, t, J = 7.3 Hz), 3.56 (6 H, m), 3.50 (2 H, t, J = 5.2 Hz), 3.39 (2 H, m), 3.26 (2 H, m), 2.90 (2 H, t, J = 8.0 Hz), 2.67 (4 H, t, J = 5.8 Hz), 2.55 (2 H, t, J = 8.0 Hz), 2.02 (2 H, quintet, J = 5.8 Hz), 1.83 (4 H, sextet, J = 7.4 Hz), 1.40 (9 H, s), 1.31 (12 H, s), 1.02 (6 H, t, J = 7.4 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.8, 172.2, 164.9, 158.1, 156.1, 142.4, 142.1, 140.9, 136.0, 130.3, 128.5, 125.1, 122.3, 121.9, 114.2, 110.3, 99.5, 78.9, 70.3, 70.2, 70.0, 69.6, 49.0, 45.8, 40.3, 38.9, 38.1, 30.9, 28.4, 27.8, 24.2, 21.0, 20.7, 11.6; HRMS (ESI$^+$) Calcd for $C_{56}H_{75}N_4O_6$ (M – I^-): 899.5681; Found: 899.5658.

Preparation of 8

A solution of **7** (20 mg, 0.020 mmol) in trifluoroacetic acid (1 ml) was stirred at room temperature for 1 h, and was evaporated. The residue was purified by silica gel column chromatography (eluent: 10% MeOH–CH_2Cl_2 then 20% MeOH–CH_2Cl_2) and 22 mg of **8** was obtained as green solid, which was used in next reaction without further purification. ^{1}H NMR (500 MHz, CD_3CD) δ 7.98 (2 H, d, J = 14.2 Hz), 7.38 (2 H, d, J = 7.3 Hz), 7.36 (2 H, dd, J = 8.0, 7.5 Hz), 7.25 (2 H, d, J = 8.7 Hz), 7.24 (2 H, d, J = 8.0 Hz), 7.20 (2 H, dd, J = 7.5, 7.3 Hz), 7.03 (2 H, d, J = 8.7 Hz), 6.14 (2 H, d, J = 14.2 Hz), 4.06 (4 H, t, J = 7.4 Hz), 3.67 (2 H, t, J = 5.0 Hz), 3.64–3.58 (4 H, m), 3.48 (2 H, t, J = 5.8 Hz), 3.34 (2 H, m), 3.01 (2 H, t, J = 5.0 Hz), 2.84 (2 H, t, J = 7.9 Hz), 2.72 (4 H, t, J = 6.0 Hz), 2.43 (2 H, t, J = 7.9 Hz), 2.03 (2 H, quintet, J = 6.0 Hz), 1.82 (4 H, sextet, J = 7.4 Hz), 1.33 (12 H, s) 1.00 (6 H, t, J = 7.4 Hz).

Preparation of 1e

Compound **9e** (8.8 mg, 24 μmol) was added to a solution of **8** (20 mg, 19 μmol) in DMF/pyridine (3 : 1, 4 ml), and the mixture was stirred overnight. The reaction mixture was evaporated, and the residue was diluted with dichloromethane. The organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated. The crude was purified by silica gel column chromatography (10% MeOH–CH_2Cl_2) and 12 mg of **1e** (16 μmol, 84%) was obtained as a green powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.16 (1 H, br s), 8.10 (2 H, d, J = 8.5 Hz), 7.91 (2 H, d, J = 14.2 Hz), 8.09 (1 H, br s), 7.88 (2 H, d, J = 9.2 Hz), 7.84 (2 H, d, J = 8.5 Hz), 7.32–7.29 (6 H, m), 7.17 (2 H, dd, J = 7.6, 7.2 Hz), 7.00 (2 H, d, J = 7.9 Hz), 6.90 (2 H, d, J = 8.6 Hz), 6.74 (2 H, d, J = 9.2 Hz), 5.90 (2 H, d, J = 14.2 Hz), 3.91 (4 H, t, J = 7.3 Hz), 3.71 (2 H, t, J = 5.3 Hz), 3.64–3.55 (8 H, m), 3.46 (2 H, m), 3.09 (6 H, s), 2.92 (2 H, t, J = 7.8 Hz), 2.63 (4 H, t, J = 5.8 Hz), 2.58 (2 H, t, J = 7.8 Hz), 2.00 (2 H, quintet, J = 5.8 Hz), 1.82 (4 H, sextet, J = 7.4 Hz), 1.30 (12 H, s), 1.01 (6 H, t, J = 7.4 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1, 172.3, 167.1, 165.1, 158.2, 154.6, 152.6, 143.8, 142.5, 142.0, 141.0, 136.3, 134.8, 130.5, 128.5, 128.5, 125.2, 122.5, 122.0, 121.9, 114.3, 111.5, 110.3, 99.4, 70.3, 70.2, 69.9, 69.8, 49.2, 45.8, 40.3, 39.9, 39.2, 38.3, 31.1, 27.9, 24.2, 21.1, 20.7, 11.6; HRMS (ESI$^+$) Calcd for $C_{66}H_{80}N_7O_5$ (M – I^-): 1050.6215; Found: 1050.6229.

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