Self-quenching Mechanism: the Influence of Quencher and Spacer on Quencher-fluorescein Probes

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Fluorescent chemosensors provide an extremely sensitive optical method for the real-time monitoring of molecular recognition events. An understanding of the fluorescent on/off mechanism is essential for the rational design of fluorescent chemosensors. The majority of fluorescent chemosensors working in aqueous media operate by one of three mechanisms: (1) the suppression of photoinduced electron transfer (PET); (2) alteration of the microenvironment of a solvatochromic fluorophore; and (3) modulation of the fluorescence resonance energy transfer (FRET) between two fluorophores.[1] The first two mechanisms are suitable for small molecule sensing (*e.g.*, cations, small organic compounds, etc.), but FRET is very useful in the biopolymer state, for its distance information can be obtained in the 10 to 100 Å region.[2] In spite of the excellent applications of FRET for biomolecule sensing (c-AMP, insulin, etc.), the operations depend on a complicated biological pathway.[3]

Peptides with an FRET pair have been used as monitoring probes for a given enzyme activity. However, in various fluorophore pairs that have been examined, FRET peptide probes did not operate properly *i.e.*, they were self-quenched.[4] This self-quenching can be explained in terms of an intramolecular ground-state dimer complex between the FRET fluorophore pair in an aqueous solution.[5] The strength of the aggregation between the two dye molecules depends on the molecular structure, solvent, temperature, and the presence or absence of electrolytes.[6]

Our aim is to systematically study the self-quenching mechanism for covalently linked fluorophore pairs. For simplicity, the factors influencing the efficiency of self-quenching are disassembled into four components (Figure 1). A FRET pair was replaced by a quencher-fluorophore pair as the reporting group. This overcomes the restriction for the FRET donor-acceptor resonance condition.[7] From a practical viewpoint, water and fluorescein were chosen as the solvent and fluorophore, respectively; water is a crucial solvent for physiological systems; furthermore, fluorescein

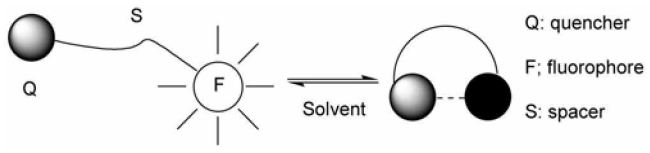

Figure 1. A fluorescent on/off mechanism induced by the intramolecular ground-state complex formation.

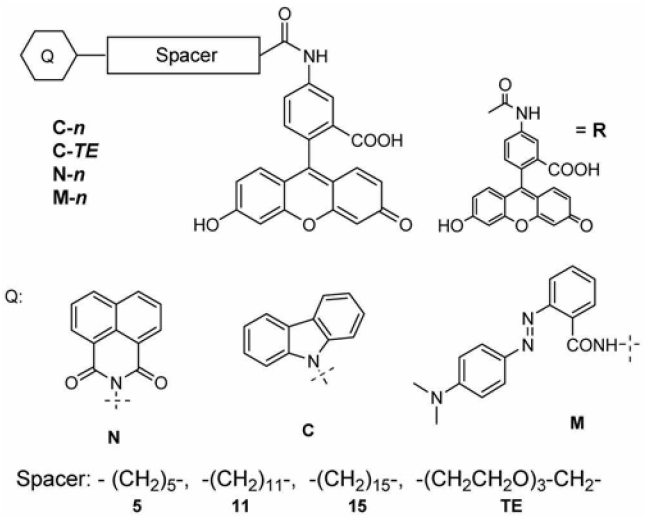

Figure 2. Nomenclature of the quencher-fluorescein probes.

has a high fluorescence quantum yield in aqueous solution and its synthetic handling has been well established.

As the quencher part, carbazole, methyl red and 1,8-naphthalimide were selected. The effect of the spacer can be controlled by varying the alkyl chain length (-$(CH_2)_n$-, n = 5, 11, 15) and a flexible tetraethylene glycol unit (Figure 2).

At pH 7.5 (50 mM HEPES buffer, I = 0.1 ($NaNO_3$)), the fluorescence emission of **C-*n*** (n = 5, 11, 15) and **C-*TE*** ([probes] = 1 μM) was measured (Figure 3).[8] It has been reported that there are two opposing factors determining the stability of aggregates of the derivatives containing two Rose Bengal moieties: the longer the chain, the easier it is to

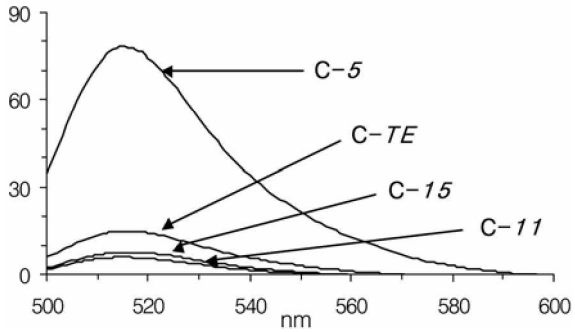

Figure 3. Fluorescene emission responses of **C-*5***, **C-*11***, **C-*15*** and **C-*TE***. [probe] = 1 μM, λ_{ex} = 490 nm.

Table 1. Fluorescence emission proprties[a]

Probe	FI[b]	QE (%)[c]
C-*5*	75.5	56
C-*11*	7.5	96
M-*5*	15.8	91
M-*11*	4.1	98
N-*5*	39.5	78
N-*11*	10.2	94

[a]Data were acquired with excitation at 490 nm in 50 mM HEPES, pH 7.5, I = 0.1 ($NaNO_3$), [Probe] = 1 μM. [b]Fluorescence intensity at λ_{max} 515 nm. [c]Quenching efficiency was defined as {[FI(**R**)-FI] × 100}/FI(**R**) (FI(**R**) = FI of a reference compound **R**, 181.8).

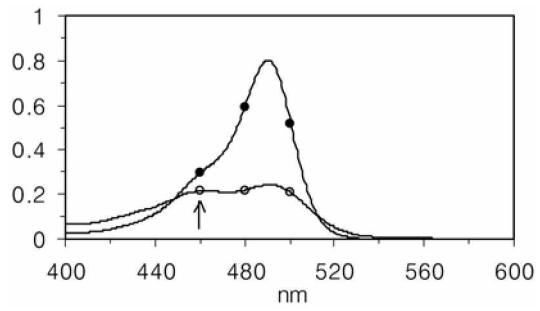

Figure 5. Absorption spectra of **R** (●) and **C-*11*** (○) ([Probe] = 10 μM, 50 mM HEPES, pH 7.5, I = 0.1 ($NaNO_3$)).

fold and bring the dye moieties together: on the other hand, the probability that the dye moieties occupy regions in space close to each other decreases as the length of the chain increases.[6] Thus, the degree of self-quenching does not decrease monotonically with the length of the methylene chain and the flexibility of the tetraethylene glycol unit. However, for effective self-quenching, the spacer should lie within a certain length above a threshold.

We investigated the influence of a quencher on the quenching efficiency by controlling the spacer length (-$(CH_2)_5$- or -$(CH_2)_{11}$-) (Table 1). Although *p*-methyl red (dabcyl or 4-(4'-dimethylaminophenylazo)benzoic acid) is known to be an optimal choice for the quencher,[9] *o*-methyl red turned out to work well as a quencher independently of spacer length. It is noteworthy that the fluorescence of carbazole responds most sensitively to the spacer length among the three quenchers. From the viewpoint of the fluorescence on/off mode, the key factor is not the absolute quenching efficiency but the relative change of quenching efficiency induced by a controllable variable.

We can confirm the intramolecular ground-state dimer structure by molecular modelling. A conformation search (1000 steps) on **C-*11*** was carried out using the Amber force field in water implemented in a Macromodel 7.0 (Figure 4). The calculated result accords with a parallel dimer structure (H-type), which is expected from a blue-shifted absorption band ($\Delta\lambda$ = 30 nm) with respect to the monomer, R (λ_{max} = 490 nm) (Figure 5). Exciton theory predicts that the excited-state levels of the monomer split in two upon complexation.

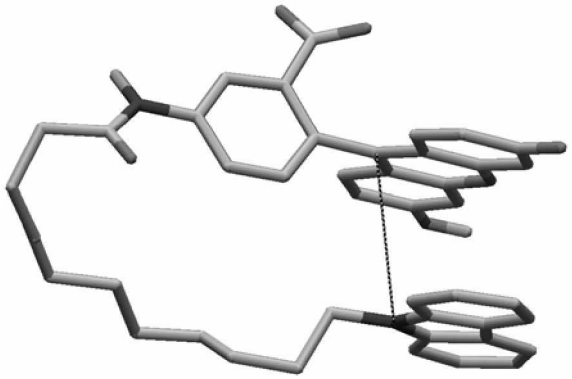

Figure 4. The lowest-energy conformation of **C-*11*** (the central distance between the two parallel planes = 3.9 Å).

One level is of lower and the other of higher energy with respect to the monomer excited state. For parallel dimers (H-type), the transition to the lower energy excited state is forbidden.[6] The blue-shifted band indicates a bichromophoric interaction in the probe, suggesting the formation of intramolecular ground-state dimers between the quencher and fluorescein.[4a] In the cases where there is sufficient spacer length (**C-*11***, **C-*15***, **M-*11***, **N-*11***), a coupling between the ground electronic states of the dimeric chromophores will disperse the excited state of fluorescein nonradiatively. The spacer of **C-*5***, **M-*5***, and **N-*5*** should be short enough to keep the two chromophores from close contact and thus intramolecular electronic energy transfer (EET) will predominate.[10] Since the quencher-fluorophore distance is constant (n = 5), the main factor of intra-EET will be the overlap integral, *J*. The shortest absorption λ_{max} and the lowest ε of carbazole imply that the carbazole-fluorescein pair will minimize the overlap integral, *J*, and suppress the self-quenching most effectively.[11] This quencher-fluorescein system could be utilized to develop a novel sensor in which an analyte disturbs the intramolecular quencher-fluorescein dimer state and triggers a fluorescent enhancement.[12]

In conclusion, we prepared a series of probes for the systematic study of the self-quenching mechanism and examined the influence of quencher and spacer on the self-quenching of quencher-fluorescein pairs. The molecular modelling and the blue-shifted band in UV reveal that this self-quenching is due to intramolecular ground-state dimers. Compared to the other quenchers, carbazole responds most sensitively to changes in distance.

Experimental section

A typical procedure for the synthesis of Quencher-Fluorescein probes. To a slurry of **N-*5*-COOH** (100 mg, 0.32 mmol) in toluene (5 mL) was injected $(COCl)_2$ (2 M in CH_2Cl_2, 0.3 mL) and the gas evolution was observed immediately. After stirring for 1 hr at rt, all volatiles were evaporated and the residual dried in vacuum for 1 hr. To the acid chloride were injected the **FPv** solution (0.08 M in THF, 4 mL) and TEA (0.1 mL). After overnight stirring, the reaction mixture was partitioned into ethyl acetate/water and the organic phase washed with water (× 3), then dried in $MgSO_4$. Flash chromatographic purification (hexane/ethyl

acetate = 2:3) afforded **N-5-FPv** (190 mg, 74% yield).

To a solution of **N-5-FPv** (190 mg, 0.23 mmol) in THF and MeOH (3 mL/3 mL) was added 1 N NaOH (1 mL, 4 eq) and the reaction mixture was stirred at rt for 12 hr. The mixture was concentrated to 1/4 of the original volume, and then acidified with 1 N HCl (1 mL). The precipitate was collected and dried (quantitative yield). Without further purification, **N-5** was characterized and used in fluorescence measurement.

C-5: ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 1.39 (br, 2H, CH_2), 1.67 (br, 2H, CH_2), 1.82 (br, 2H, CH_2), 2.33 (t, J 7.3 Hz, 2H, COCH_2), 4.42 (t, J 7.0 Hz, 2H, NCH_2), 6.53-6.60 (m, 4H, F), 6.67 (s, 2H, F), 7.16-7.21 (m, 2+1H, C+F), 7.44 (dd, 2H, C), 7.61 (d, J 8.2 Hz, 2H, C), 7.80 (d, J 8.4 Hz, 1H, F), 8.15 (d, J 7.7 Hz, 2H, C), 8.30 (s, 1H, F), 10.12 (br, 2H, *OH*), 10.31 (s, 1H, CO*NH*); FAB-MS (*m*-NBA): calcd. for [$C_{38}H_{30}N_2O_6$] = 610.21, found [M+H] = 611.

C-11: ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 1.06-1.25 (m, 14H, CH_2, pivaloyl *tert*-Bu singlet completely disappeared), 1.60 (br, 2H, CH_2), 1.75 (br, 2H, CH_2), 2.35 (t, J 7.3 Hz, 2H, COCH_2), 4.38 (t, J 7.0 Hz, 2H, NCH_2), 6.52-6.60 (m, 4H, F), 6.67 (s, 2H, F), 7.16-7.20 (m, 2+1H, C+F), 7.44 (dd, 2H, C), 7.59 (d, J 8.2 Hz, 2H,C), 7.82 (d, J 8.4 Hz, 1H, F), 8.14 (d, J 7.6 Hz, 2H, C), 8.33 (s, 1H, F), 10.13 (br, 2H, *OH*), 10.34 (s, 1H, CO*NH*); FAB-MS (*m*-NBA): calcd. for [$C_{44}H_{42}N_2O_6$] = 694.30, found [M+H] = 695.

M-5: ^{1}H NMR (300 MHz, CD_3OD, 25 °C): δ 0.94 (br, 2H, CH_2), 1.3-1.7 (m, 6H, CH_2), 2.36 (t, J 7.5 Hz, 2H, COCH_2), 3.06 (s, 6H, $(CH_3)_2$N), 6.49-6.57 (m, 6H, F), 6.91 (d, J 9.2 Hz, 2H, M), 7.05-7.18 (m, 6H), 7.30 (dd, J_1 9.1 Hz, J_2 1.9 Hz, 1H), 7.62-7.76 (m, 5H), 7.97 (d, J 10.4 Hz, 1H), 8.04 (dd, J_1 15.4 Hz, J_2 2.2 Hz, 2H); FAB-MS (*m*-NBA): calcd. for [$C_{41}H_{37}N_3O_7$] = 711.27, found [M+H] = 712.

M-11: ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 1.1-1.3 (m, 16H, CH_2), 1.45-1.6 (m, 4H, CH_2), 2.36 (t, J 7.3 Hz, 2H, COCH_2), 3.07 (s, 6H, $(CH_3)_2$N), 6.52-6.61 (m, 4H, F), 6.66 (s, 2H, F), 6.84 (d, J 9.2 Hz, 2H, M), 7.19 (d, J 8.4 Hz, 1H, F), 7.4-7.5 (m, 2H, M), 7.63 (d, J 7 Hz, 1H, M), 7.70-7.80 (m, 3+1H, M+F), 8.33 (s, 1H, F), 8.53 (br, 1H, MCO*NH*), 10.1 (br, 2H, *OH*), 10.35 (s, 1H, CO*NH*F); FAB-MS (in DMSO with Gly): calcd. for [$C_{47}H_{49}N_3O_7$] = 795.36, found [M+H] = 796.5.

N-5: ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 1.42 (br, 2H, CH_2), 1.6-1.7 (m, 4H, CH_2), 2.40 (t, J 7.2 Hz, 2H, COCH_2), 4.08 (t, J 7.3 Hz, 2H, NCH_2), 6.52-6.61 (m, 4H, F), 6.66 (s, 2H, F), 6.84 (d, J 9.2 Hz, 2H, M), 7.19 (d, J 8.4 Hz, 1H,F), 7.80 (d, J 8.4 Hz, 1H, F), 7.88 (dd, 2H, N), 8.31 (s, 1H, F), 8.45-8.53 (m, 4H, N), 10.13 (br, 2H, *OH*), 10.36 (s, 1H, CO*NH*); FAB-MS (*m*-NBA): calcd. for [$C_{38}H_{28}N_2O_8$] = 640.18, found [M+H] = 641.

N-11: ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 1.2-1.4 (m, 14H, CH_2), 1.6-1.7 (m, 4H, CH_2), 2.36 (t, J 7.3 Hz, 2H, COCH_2), 4.04 (t, J 7.4 Hz, 2H, NCH_2), 6.52-6.61 (m, 4H, F), 6.66 (s, 2H, F), 7.19 (d, J 8.4 Hz, 1H, F), 7.82 (d, J 8.4 Hz, 1H, F), 7.88 (dd, 2H, N), 8.32 (s, 1H, F), 8.45-8.53 (m, 4H, N), 10.1 (br, 2H, *OH*), 10.34 (s, 1H, CO*NH*); FAB-MS (*m*-NBA): calcd. for [$C_{44}H_{40}N_2O_8$] = 724.28, found [M+H] = 725.

Spectroscopic Analysis. UV (Beckman DU® 650) and fluorescence (JASCO FP 750) spectrometers were used. Probes were dissolved in DMSO to obtain 10 mM stock solution. Samples were prepared by dilution of the stock solution with 50 mM HEPES buffer, I = 0.1 ($NaNO_3$). The slit width of fluorescence spectrometers was 5 nm for both excitation and emission.

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Supporting Information Available: Synthetic schemes and selected spectral data are available on request from the correspondence author (jihong@snu.ac.kr).

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