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Development of a Time-Resolved Fluorometric Detection System Using Diffusion-Enhanced Energy Transfer

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A novel detection system using both emission energy transfer and time-resolved fluorometry (TRF) was developed, with a europium chelate as the energy donor and a novel fluorophore SNR1, excitable with long-wavelength light corresponding to europium emission, as the energy acceptor. When the donor and acceptor molecules were mixed in solution, energy transfer was observed without direct attachment of the donor and the acceptor, via a diffusion-enhanced energy-transfer mechanism. Thus, the acceptor emission can be detected as a long-lifetime fluorescence in TRF. When the fluorescence properties of the acceptor molecule are changed by interaction with an enzyme or other bioactive molecule, the change can be detected as a long-lived sensitized emission. If we develop or select suitable acceptor molecules, this simple and convenient system should be applicable to a wide variety of bioactive molecules. Since it is based on TRF, it can be used for high-resolution assay.

Autofluorescence or other background fluorescence can be a hindrance in fluorescence measurements. Time-resolved fluorometry (TRF) is an effective means to minimize background fluorescence; a pulse of excitation light is applied and the emitted light is collected for a short period after a delay time, during which short-lived background fluorescence originated from organic compounds decays. Emission energy transfer is an energy transmission phenomenon from a donor to an acceptor.[1,2] The relative changes of donor−acceptor arrangement or separation distance are employed as a detection principle in most emission energy-transfer assays.[3−7] Homogeneous time-resolved fluorescence[8−10] (HTRF) has been reported to have the advantages of a lower detection limit and higher specificity of detection as compared with conventional emission energy-transfer assays. It is also reported that luminescence resonance energy transfer[11] (LRET), in which emission energy transfer and TRF are combined, can greatly reduce experimental background noise. In most studies using the HTRF system, europium chelates, which possess a long-lived fluorescence, are used as the donor. A fluorescent protein, allophycocyanin,[12] or an organic dye, Cy5,[13] is used as the energy acceptor. Although there have been many successful applications, as mentioned above, these are limited to systems using the relative changes of donor−acceptor arrangement or distance as a detection principle. They also depend for their specificity upon macromolecular interactions, such as oligonucleotide−oligonucleotide, protein−protein, or receptor−ligand interactions or antigen−antibody reactions.[14−20] In other words, HTRF or LRET is not a suitable detection method for small molecular bioactive substrates. Labeling of macromolecules with donor/acceptor is often complicated, problematic, time-consuming, and expensive.

Here we report a detection method for small bioactive molecules using emission energy transfer and TRF. In this system, europium chelate is used as the energy donor. A novel fluorescent dye, SNR1 [sem*i*naphthor*h*odamine 1 or 3-(diethylamino)-10-tetrahydroquinolizino [1,9-*hi*]-9-(2′-carboxyphenyl)benzo[*c*]xanthylium], was designed and used as the energy acceptor. Since long-lifetime sensitized emission is measured by TRF, background

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(1) Meer, B. W.; Meer, G. C.; Chen, S. Y. S. *Resonance Energy Transfer*; Wiley-VCH: New York, 1994.
(2) Förester, T. *Ann. Phys.* **1948**, *2*, 55−75.
(3) Eis, P. S.; Millar, D. P. *Biochemistry* **1993**, *32*, 13852−13860.
(4) Uchiyama, H.; Hirano, K.; Kashiwasake, J. K.; Taira, K. *J. Biol. Chem.* **1996**, *271*, 380−384.
(5) Miyawaki, A.; Llopis, J.; Heim, R.; McCaffery, J. M.; Adams, J. A.; Ikura, M.; Tsien, R. *Nature* **1997**, *388*, 882−887.
(6) Zlokarnik, G.; Negulescu, P. A.; Knapp, T. E.; Mere, L.; Burres, N.; Feng, L.; Whitney, M.; Roemer, K.; Tsien, R. *Science* **1998**, *279*, 84−88.
(7) Gordon, G. W.; Berry, G.; Liang, X. H.; Levine, B.; Herman, B. *Biophys. J.* **1998**, *74*, 2702−2713.
(8) Mathis, G. *Clin. Chem.* **1993**, *39*, 1953−1959.
(9) Cummings, R. T.; McGovern, H. M.; Zheng, S.; Park, Y. W.; Hermes, J. D. *Anal. Biochem.* **1999**, *269*, 79−93.
(10) Alfred, J. K.; Paul, V. K.; David, J. H.; Young, W. P.; Christine, P.; John, S. M.; Gerard, M. *Drug Discovery Today* **1998**, *3*, 333−342.
(11) Selvin, P. R.; Rana. T. M.; Hearst, J. E. *J. Am. Chem. Soc.* **1994**, *116*, 6029−6030.
(12) Brejc, K.; Fincner, R.; Huber, R.; Steinbacher, S. *J. Mol. Biol.* **1995**, *249*, 424−440.
(13) Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* **1993**, *4*, 105−116.
(14) Cummings, R. T.; McGovern, H. M.; Zheng, S.; Park, Y. W.; Hermes, J. D. *Anal. Biochem.* **1999**, *269*, 79−93.
(15) Farrar, S. J.; Whiting, P. J.; Bonnert, T. P.; McKernan, R. M. *J. Biol. Chem.* **1999**, *274*, 10100−10104.
(16) Heyduk, E.; Heyduk, T. *J. Biol. Chem.* **1999**, 3315−3322.
(17) Heyduk, E.; Heyduk, T.; Claus, P.; Wisniewski, J. R. *J. Biol. Chem.* **1997**, *272*, 19763−19770.
(18) Heyduk, E. Heyduk, T. *Anal. Biochem.* **1997**, *248*, 216−227.
(19) Stenroos, K.; Hurskainen, P.; Eriksson, S.; Hemmila, I.; Blomberg, K.; Lindqvist, C. *Cytokine* **1998**, *10*, 495−499.
(20) Chen, J.; Selvin, P. R. *Bioconjugate Chem.* **1999**, *10*, 311−315.

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fluorescence can be eliminated. The fluorescence property changes evoked by the analyte are monitored as long-lifetime sensitized emission readout by energy transfer from the europium chelate.

EXPERIMENTAL SECTION

Synthetic details are described in Supporting Information.

Measurement of the Spectral Properties of SNR1. A 10 μM methanol solution of SNR1 was prepared from a 10 mM DMSO stock solution. Fluorescence spectra were taken on a Hitachi F-4500 fluorometer (scan speed 2400 nm/min, slit width 2.5 nm). The UV−visible spectrum was taken on a Hitachi UV-1600 spectrometer.

Metal Labeling. The binding of metal to DTPA chelate compounds is reported to be insensitive to pH and is completed within 30 min.[21] Equimolar amounts of 10 mM $EuCl_3 \cdot 6H_2O$ solution and 10 mM DTPA-cs124 DMSO stock solution were mixed and diluted with methanol to give a final concentration of 10 μM. After 30 min, the mixture was used as 10 μM EuDTPA-cs124 and the spectra were measured.

Spectral Overlap Integral. Spectral overlap integral J (nm^4 M^{-1}) was calculated using[3]

$$J = \int \epsilon_A(\lambda) F_D(\lambda) \lambda^4 \, d\lambda / \int F_D(\lambda) \, d\lambda \qquad (1)$$

EuDTPA-cs124+SNR1 Diffusion-Enhanced Energy-Transfer System. Ten micromolar EuDTPA-cs124 was prepared, and a 10 mM DMSO stock solution of SNR1 was added to give SNR1concentrations of 0, 1, 2, 3, 5, 10, and 20 μM.

Measurements of Time-Resolved Fluorescence Spectra and Lifetimes. Time-resolved fluorescence spectra and lifetimes were recorded on a Perkin-Elmer LS-50B (Beaconsfield, Buckinghamshire, England). For the time-resolved fluorescence spectra, 10-μs pulsed excitation at 328 nm was used to sensitize the donor, EuDTPA-cs124 (delay time 0.05 ms, gate time 1.00 ms, scan speed 900 nm/min, slit width 5 nm). For the lifetime measurement, 10-μs pulsed excitation at 328 nm was used to sensitize the donor, EuDTPA-cs124. The fluorescence intensities at every 50 μs after the pulsed excitation at 328 nm were measured using a 1-ms gate time. Data were fitted to a single-exponential decay curve.

RESULTS AND DISCUSSION

Development of a Novel Fluorescent Dye with Long-Wavelength Excitation Corresponding to Europium Emission. SNR1 has a long-wavelength excitation maximum (Ex 615 nm, Em 645 nm), and it can be conveniently derivatized at the benzene ring. The spectral characteristics of the europium chelate and SNR1 are shown in Figure 1. The spectral overlap integral J of the donor (europium chelate) emission (F_D) and acceptor (SNR1) absorption (ϵ_A) was calculated as 7.34×10^{15} (nm^4 M^{-1}). This large value of J suggests that SNR1 is an efficient energy acceptor from the europium chelate, resulting in sensitized emission at 655 nm.[11]

Energy-Transfer System for Long-Lived Sensitized Emission. Two general types of fluorescence energy-transfer system have so far been reported, i.e., the proximity-induced type, in which the distance between donor and acceptor is critical, and the diffusion-enhanced type, in which the long-lived excited energy of a lanthanoid chelate is transferred efficiently to the acceptor.[22,23] The sensitized emission of the acceptor is not necessarily observed in the proximity-induced type, since the fluorescence is sometimes quenched by the close interaction of two dye molecules. We examined three types of energy-transfer system from europium chelate to SNR1, as shown in Figure 2.

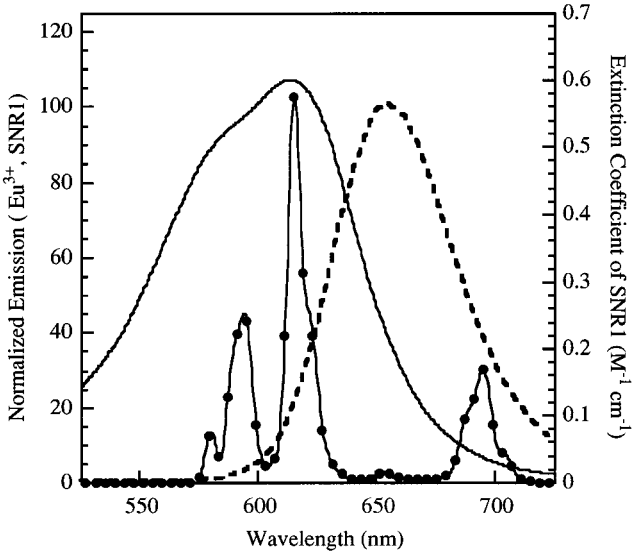

Figure 1. Spectra of EuDTPA-cs124 and of SNR1. Solid and dashed lines show the absorption and emission spectra of SNR1, respectively. The solid line with circles is the emission spectrum of EuDTPA-cs124. All the data shown were obtained at the concentration of 10 μM, in methanol.

First, proximity-induced energy transfer was examined. The donor and the acceptor were conjugated via two types of linker, to transfer the excited energy efficiently. In system A, a flexible linker (**6**) was used, allowing hydrophobic interaction of the donor and the acceptor. In system B, we used a rigid steroid linker to prevent direct interaction of the donor and the acceptor. In both systems, the conjugates were nonfluorescent in time-resolved fluorescence measurement with 50-μs delay with 328-nm excitation.

It is considered that the emission of both the donor and the acceptor was quenched because of the very large energy-transfer rate constant, and this resulted in too short lifetime of the excited state of system B.

Next, we investigated diffusion-enhanced energy transfer. The emission energy-transfer system without covalent linkage of the donor and acceptor, system C, was used to detect the sensitized emission of the acceptor in TRF. The time-resolved fluorescence spectra are shown in Figure 3. The donor emission (615 nm) intensity was decreased and the acceptor emission (655 nm) intensity was increased dose-dependently by the addition of SNR1. The decrease of the 615-nm band and the corresponding increase of the 655-nm band are considered to be the sensitized emission of SNR1 via the diffusion-enhanced energy transfer.

(21) Li, M.; Selvin, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 8132−8138.

(22) Thomas, D. D.; Carlsen, W. F.; Stryer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5746−5750.

(23) Stryer, L.; Thomas, D. D.; Meares, C. F. *Annu. Rev. Biophys. Bioeng.* **1982**, *11*, 203−222.

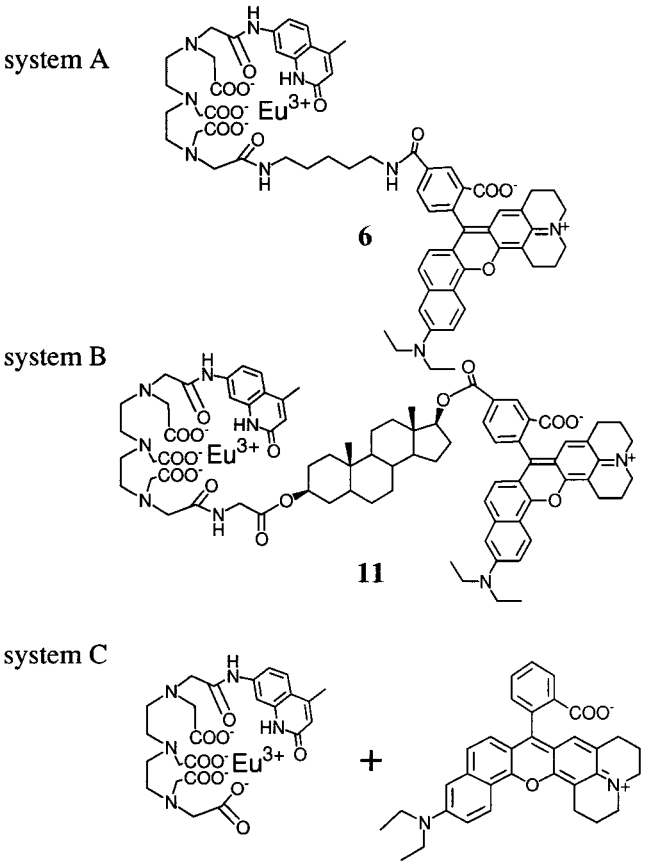

Figure 2. Energy-transfer systems for long-lived sensitized emission.

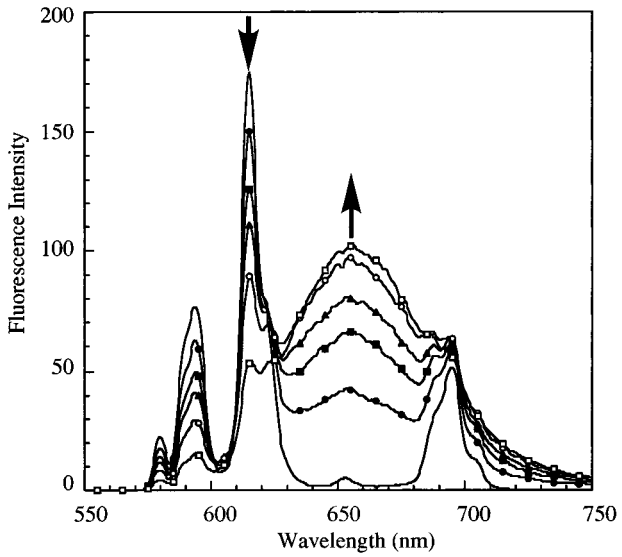

Figure 3. Time-resolved fluorescence spectra obtained with the diffusion-enhanced energy-transfer system. EuDTPA-cs124 (10 μM, solid line) was mixed with 1.0 (closed circle), 2.0 (closed square), 3.0 (closed triangle), 5.0 (open circle) or 10 μM (open square) SNR1 in methanol. All the data were obtained using a delay time 0.05 ms, gate time 1.00 ms, scan speed 900 nm/min, and slit width 5 nm.

Fluorescence Lifetime Measurement. Emission at 615 nm is due to the europium chelate and that at 655 nm corresponds to SNR1. The fluorescence intensity was measured at every 50 μs after pulsed excitation at 328 nm, using a 1-ms gate time. This

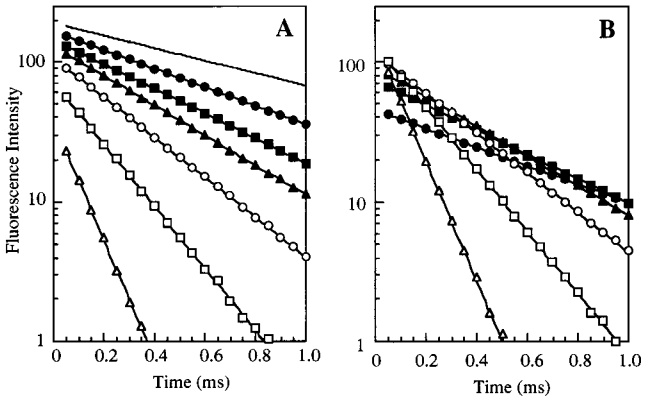

Figure 4. Fluorescence decay curves of diffusion-enhanced energy transfer recorded at (A) 615 and (B) 655 nm. The fluorescence intensities were measured periodically at 0.05-ms intervals after pulsed excitation (10 μs, 328 nm) using a 1.00-ms gate. The data were fitted to a single-exponential decay curve, $I = I_0 \exp(-t/\tau)$. The symbols used are the same as in Figure 3.

Table 1. Changes of the Parameters of the Decay Curves in the Diffusion-Enhanced FRET System[a]

		615 nm		655 nm	
run	sample	I_0	τ_{615} (ms)	I_0	τ_{655} (ms)
1	10 μM EuDTPA-cs124	191	0.96		
2	run 1 + 1 μM SNR1	166	0.65	45	0.65
3	run 1 + 2 μM SNR1	145	0.49	73	0.49
4	run 1 + 3 μM SNR1	129	0.41	91	0.41
5	run 1 + 5 μM SNR1	107	0.31	114	0.31
6	run 1 + 10 μM SNR1	72	0.20	131	0.20
7	run 1 + 20 μM SNR1	38	0.10	140	0.10

[a] The fluorescence intensities were measured periodically in every 0.05 ms after pulsed excitation (10 μs, 328 nm), using a 1.00-ms gate. The data were fitted to a single-exponential decay curve, $I = I_0 \exp(-t/\tau)$.

relatively long gate time was employed, since it enables collecting reliable data around lower emission intensity. The decay curve obeys in a single-exponential manner when the energy is transferred without quenching by random collision. The possibility of random collision is negligible, since when a small molecule acceptor without spectral overlap of europium emission, such as fluorescein or rhodamine, was mixed with the donor, there was no effect on lifetime of donor emission. It also supports the single-exponential decay that the data were fitted well to single-exponential decay curves (Figure 4), the time constant τ was calculated based on the curves. Values of I_0 and τ at both wavelengths are summarized in Table 1. The data were collected after 50 μs and all the emission from organic compounds are diminished during this time, so the preexponential factor I_0 was almost meaningless for the energy transfer. The lifetime of the emission at 615 nm, derived from EuDPTA-cs124, decreased dose-dependently upon addition of SNR1. The lifetime of the emission at 655 nm coincided with the lifetime at 615 nm. The decrease of the donor luminescence lifetime is due to the energy transfer. The coincidence of the luminescence lifetimes at 615 and 655 nm is evidence of the energy transfer in this system. The Stern−Volmer constant K_{SV} was determined as 4.2×10^5 M^{-1} (data not shown), and k_T, a rate constant of energy transfer, was calculated as 4.3×10^8 M^{-1} s^{-1}, based on the equation $k_T = K_{SV}/\tau$.

Explanation of Diffusion-Enhanced Energy Transfer and Quenching Mechanism in the Proximity-Induced System. The lifetime of EuDTPA-cs124 in methanol is 0.96 ms, and the corresponding radiative rate constant for depopulation of the excited-state k_f is calculated as 1.0×10^3 s^{-1}. When the acceptor concentration was 20 μM, the first rate constant was calculated as 8.3×10^3 s^{-1} based on k_T . This value is larger than k_f, thus resulting in emission energy transfer.

When an organic fluorescent compound is used as an energy donor, intermolecular energy transfer cannot take place simply upon mixing of the donor and acceptor, as in system C. The reason is that the lifetime of organic fluorescent compounds is very short, and the corresponding radiative rate constant for the depopulation of the excited-state k_f (s^{-1}) is estimated to be of the order of 1.0×10^8−1.0×10^9. Since k_T (M^{-1} s^{-1}) does not exceed the diffusion control rate, 1.0×10^{10} M^{-1} s^{-1}, the upper limit of k_T[A] (s^{-1}) for organic donor and acceptor molecules is calculated to be 2.0×10^5 s^{-1}, for an acceptor concentration of 2.0×10^{-5} M. This value is much smaller than the k_f (s^{-1}) of organic donors, so the decrease of donor emission is much faster than the energy-transfer process. If k_T[A] (s^{-1}) exceeds k_f (s^{-1}), a 1.0×10^{-2} M acceptor concentration would be necessary, and this is inappropriate for practical usage, since the fluorescence can be quenched at this high concentration. However, if a donor with long-lived fluorescence is used, emission energy transfer is observed without direct coupling of the donor and acceptor.

Development of Diffusion-Enhanced Energy-Transfer−TRF System with Synthetic Small Molecules. The changes of fluorescence characteristics of the acceptor molecule can be used as a sensor of biological molecules in our diffusion-enhanced energy-transfer system. If a nonfluorescent acceptor molecule is converted to a fluorescent product by enzymes or other biological molecules, the system can be used to monitor the reaction. It is known that aminofluorescein is nonfluorescent, while acetamidefluorescein is fluorescent.[24] The acetylation of aminofluorescein therefore leads to a drastic increase of the fluorescence quantum yield. To investigate whether the diffusion-enhanced energy-transfer system can indeed detect a chemical reaction that would change the quantum yield of the acceptor, amino-SNR1 (**3d**) and amide-SNR1 (**3e**) were synthesized and the rate constants of diffusion-enhanced energy transfer were measured. While the emission of the europium chelate was quenched dose-dependently by the addition of amino-SNR1, emission from the acceptor molecule was not observed (data not shown). On the other hand, emission energy transfer was observed upon addition of amide-SNR1, a finding that was confirmed by a consideration of the lifetime changes (Table 2). The fluorescence lifetime at 655 nm coincided with the lifetime at 615 nm.

(24) Munkholm, C.; Parkinson, D. R.; Walt, D. R. *J. Am. Chem. Soc.* **1990**, *112*, 2608−2612.

Table 2. Changes of the Lifetime of Fluorescence in the Diffusion-Enhanced FRET System with SNR1 Derivatives[a]

run	sample	amino-SNR τ_{615} (ms)	amide-SNR1 τ_{615} (ms)	amide-SNR1 τ_{655} (ms)
1	10 μM EuDTPAcs-124	0.96	0.96	
2	run 1 + 1 μM SNR1 derivative	0.60	0.56	0.56
3	run 1 + 2 μM SNR1 derivative	0.49	0.41	0.41
4	run 1 + 3 μM SNR1 derivative	0.41	0.33	0.33
5	run 1 + 5 μM SNR1 derivative	0.32	0.24	0.24
6	run 1 + 10 μM SNR1 derivative	0.20	0.15	0.15
7	run 1 + 20 μM SNR1 derivative	0.09	0.07	0.07

[a] The fluorescence lifetimes were measured by the same method as in Table 1.

CONCLUSION

In a mixture of europium chelate and a small molecular organic acceptor without covalent bonding, efficient energy transfer occurred via the diffusion-enhanced energy transfer when the europium chelate was excited. The long-lived europium excitation energy is transferred to the acceptor. If the fluorescence properties of the acceptor molecule are changed by chemical reaction, the changes can be detected by the TRF method.

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SUPPORTING INFORMATION AVAILABLE

Synthetic details. This material is available free of charge via the Internet at http://pubs.acs.org.

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