ORGANIC LETTERS

2010
Vol. 12, No. 8
1704−1707

Sensitive Detection of Acrolein in Serum Using Time-Resolved Luminescence

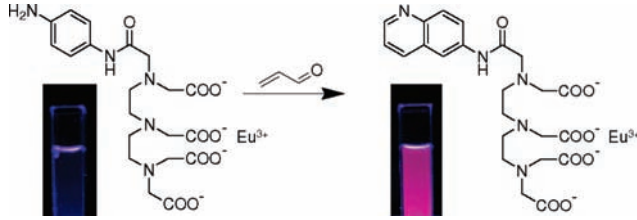
Masataka Togashi,[†,‡] Yasuteru Urano,[§] Hirotatsu Kojima,[||] Takuya Terai,[†,‡] Kenjiro Hanaoka,[†,‡] Kazuei Igarashi,[⊥] Yasunobu Hirata,[‡,#] and Tetsuo Nagano*[,†,‡,||]

Graduate School of Pharmaceutical Sciences, Graduate School of Medicine, and Chemical Biology Research Initiative, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, CREST, Japan Science and Technology Agency, 4-8-1 Honcho, Kawaguchi, Saitama 332-0012, Japan, Department of Clinical Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8675, Japan, and Department of Advanced Clinical Science and Therapeutics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

tlong@mol.f.u-tokyo.ac.jp

Received January 29, 2010

ABSTRACT



A novel lanthanide probe was designed, synthesized, and employed for a sensitive and reliable assay of acrolein based on time-resolved luminescence measurement, which suppresses the background signal of serum.

Acrolein (CH_2CHCHO) is a lipid peroxidation product that can be generated ubiquitously in biological systems.[1] It is a representative carcinogenic aldehyde formed endogenously through oxidative stress, caused by reactive oxygen species and lipid peroxide. Among these aldehydes, acrolein is one of the strongest electrophiles, and it disrupts important cellular functions by covalently modifying biomolecules, including proteins, DNA, and lipids.[2]

Acrolein is becoming of great interest to medical scientists as a potential marker of various diseases, including chronic renal failure,[3] stroke,[4] and cancer.[5] For example, acrolein was reported to be increased in the plasma of stroke patients, so that diagnosis may be possible by quantifying acrolein in plasma or serum. In addition, acrolein is spontaneously formed by polyamines and strongly inhibits cell growth owing to its cytotoxicity.[6] Therefore, a simple and convenient

[†] Graduate School of Pharmaceutical Sciences, The University of Tokyo.
[‡] CREST, Japan Science and Technology Agency.
[§] Graduate School of Medicine, The University of Tokyo.
[||] Chemical Biology Research Initiative, The University of Tokyo.
[⊥] Graduate School of Pharmaceutical Sciences, Chiba University.
[#] Department of Advanced Clinical Science and Therapeutics, Graduate School of Medicine, The University of Tokyo.

(1) (a) Esterbauer, H.; Schaur, R. J.; Zollner, H. *Free Radical Bio. Med.* **1991**, *11*, 81. (b) Girotti, A. W. *J. Lipid Res.* **1998**, *39*, 1529.

(2) (a) Kumagai, T.; Matsukawa, N.; Kaneko, Y.; Kusumi, Y.; Mitsumata, M.; Uchida, K. *J. Biol. Chem.* **2004**, *279*, 48389. (b) Matsumoto, K.; Kumamoto, T.; Ishikawa, T.; Ishii, E.; Tomitori, H.; Igarashi, K. *Heterocycles* **2006**, *67*, 361. (c) Kawai, Y.; Furuhata, A.; Toyokuni, S.; Aratani, S.; Uchida, K. *J. Biol. Chem.* **2003**, *278*, 50346.

(3) Sakata, K.; Kashiwagi, K.; Sharmin, S.; Ueda, S.; Irie, Y.; Murotani, N.; Igarashi, K. *Biochem. Bioph. Res. Co.* **2003**, *305*, 143.

(4) Tomitori, H.; Usui, T.; Saeki, N.; Ueda, S.; Kase, H.; Nishimura, K.; Kashiwagi, K.; Igarashi, K. *Stroke* **2005**, *36*, 2609.

(5) Uchida, K. *Trends Cardiovas. Med.* **1999**, *9*, 109.

(6) Sharmin, S.; Sakata, K.; Kashiwagi, K.; Ueda, S.; Iwasaki, S.; Shirahata, A.; Igarashi, K. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 228.

10.1021/ol1002219
Published on Web 03/16/2010

detection method for low concentrations of acrolein would find applications in medical, biological and other fields.

A currently used conventional method for acrolein detection is fluorescence analysis based on the Skraup reaction with *m*-aminophenol.[7] In this method, the plasma of patients is reacted with *m*-aminophenol, the product, 7-hydroxyquinoline, is separated by HPLC, and its fluorescence is quantified. HPLC separation cannot be avoided, because (i) the background fluorescence of plasma excited at 350−400 nm, which is the excitation wavelength of 7-hydroxyquinoline, is quite high and (ii) the quantum yield of 7-hydroxyquinoline is quite low. However, HPLC separation is not suitable for high-throughput measurement of multiple samples using microplates. Acrolein−protein adducts can also be detected using monoclonal antibody,[5,8] but the method is costly and needs complicated procedures. Additionally, there is a lag of several hours between production of acrolein and that of acrolein−protein adducts. Here we report a novel luminescence-based method of acrolein detection that is suitable for high-throughput microplate assays without HPLC separation.

The method of time-resolved fluorescence (TRF) measurement was selected as a basis for the development of the assay. It makes use of the characteristic long-lived luminescence of certain compounds, especially lanthanide complexes.[9] In TRF measurement, the luminescence signal is collected for a certain gate time after an appropriate delay time, following a pulsed excitation. Lanthanide complexes have long luminescence lifetimes of the order of milliseconds, in contrast to usual organic compounds, which have luminescence lifetimes of the order of nanoseconds. By taking advantage of this unique character, the influence of short-lived background fluorescence and scattered light can be reduced to a negligible level, and the long-lived lanthanide luminescence can be distinguished from background signals.[10] Lanthanide complexes also have large stokes shifts, which can further decrease the background. Because of these advantages, TRF measurements are employed in various fields where both small assay scale and high sensitivity are required, especially immunoassays[11] and high-throughput screening.[12]

To achieve sensitive detection, we must control the luminescence properties of our probe, which should have no luminescence itself, but should become highly luminescent upon reaction with acrolein. Our design strategy is summarized in Scheme 1. A lanthanide complex for TRF measurement usually consists of two parts, an antenna moiety and a chelator moiety. We thought that the luminescence properties could be precisely controlled if the absorbance spectrum of the antenna moiety changed as a result of reaction with acrolein. We chose an aniline structure as the antenna and the Skraup reaction as the controlling reaction (Scheme 1). The Skraup reaction of aniline is well-known as being highly specific for acrolein, and the λ_{max} of the aniline moiety is greatly increased by quinoline formation. In addition, the quinoline ring is a good energy transfer donor to lanthanide complex.[13] As the chelator, we selected diethylenetriaminepentaacetic acid (DTPA) because (i) DTPA has the ability to form a strong complex with europium ion and to emit bright luminescence[14] and (ii) it has high solubility in aqueous solution. The probe composed of aniline and DTPA moieties, *p*-NH_2PhDTPA, was designed according to Scheme 1. Before the Skraup reaction, the probe is expected to emit no luminescence because it is not able to absorb at the excitation wavelength for quinoline. In contrast, the product of the reaction with acrolein can absorb the light and emit luminescence via energy transfer to the europium

Scheme 1. (A) Schematic Representation of the Mechanism of Our Probe. Skraup Reaction Forms an Extended-Conjugate System. The Product Is Strongly Luminescent when Excited at the Appropriate Wavelength. (B) Reaction of the Designed Probe with Acrolein

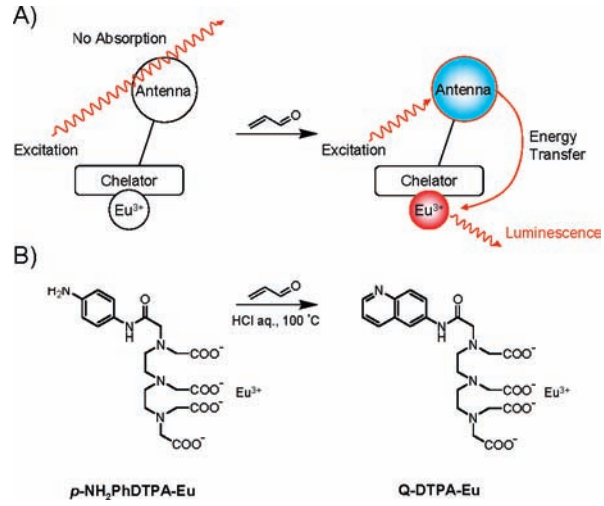


Table 1. Photochemical Properties of the Probes

	Abs_{max} (nm)	E_m(max) (nm)	Φ_{fl}[a] (%)
p- NH_2PhDTPA-Eu	243	n.d.[b]	n.d.[b]
Q-DTPA-Eu	320	618	4.6

[a] Quantum yields were determined using $[Ru(bpy)_3]Cl_2$ ($\Phi = 0.028$ in water) as a standard. Measurements were performed in 50 mM sodium phosphate buffer (pH 3.5). [b] Not determined.

(7) (a) Alarcon, R. A. *Anal. Chem.* **1968**, *40*, 1704. (b) Bohnenstengel, F.; Eichelbaum, M.; Golbs, E.; Kroemer, H. K. *J. Chromatogr. B* **1997**, *692*, 163.

(8) Sakata, K.; Kashiwagi, K.; Sharmin, S.; Ueda, S.; Igarashi, K. *Biochem. Soc. Trans.* **2003**, *31*, 371.

(9) Mizukami, S.; Kikuchi, K.; Higuchi, T.; Urano, Y.; Mashima, T.; Tsuruo, T.; Nagano, T. *FEBS Lett.* **1999**, *453*, 356.

(10) (a) Parker, D.; Dickins, R. S.; Puschmann, H.; Crossland, C.; Howard, J. A. K. *Chem. Rev.* **2002**, *102*, 1977. (b) Døssing, A. *Eur. J. Inorg. Chem.* **2005**, 1425.

(11) Johansson, M. K.; Cook, R. M.; Xu, J.; Raymond, K. N. *J. Am. Chem. Soc.* **2004**, *126*, 16451.

(12) Yuan, J.; Wang, G.; Majima, K.; Matsumoto, K. *Anal. Chem.* **2001**, *73*, 1869.

(13) Hanaoka, K.; Kikuchi, K.; Kojima, H.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 12470.

(14) (a) Moeller, T.; Thompson, L. C. *J. Inorg. Nucl. Chem.* **1962**, *24*, 499–510. (b) Terai, T.; Kikuchi, K.; Iwasawa, S. Y.; Kawabe, T.; Hirata, Y.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 6938.

ion. Simultaneously, we also synthesized quinoline−DTPA adduct (Q-DTPA), which is the expected product of the Skraup reaction of acrolein and *p*-NH_2PhDTPA.

The photochemical properties of the probe and product are summarized in Table 1. As expected, λ_{max} of Q-DTPA-Eu was at 77 nm longer wavelength than that of *p*-NH_2PhDTPA-Eu. In addition, Q-DTPA-Eu emitted strong luminescence, indicating that energy transfer occurred from the quinoline ring to the lanthanide complex. Luminescence of *p*-NH_2PhDTPA-Eu was too low to allow determination of the quantum yield. These results showed that our rational design strategy worked very well. Absorption and emission spectra of *p*-NH_2PhDTPA-Eu (green line) and Q-DTPA-Eu (red line) are shown in Figure 1.

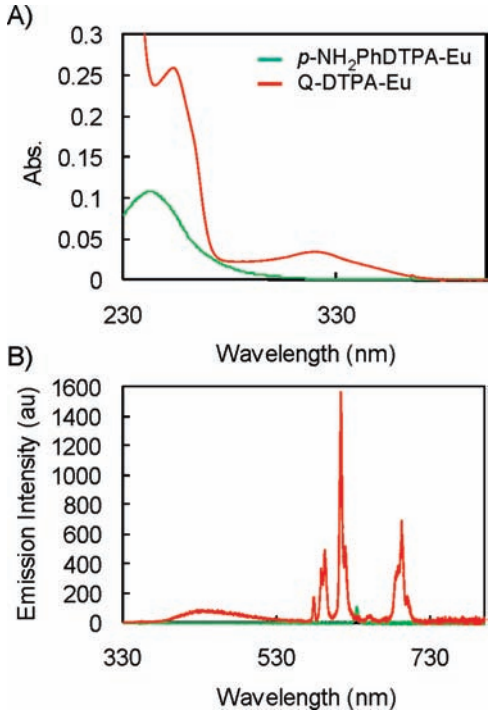


Figure 1. Absorption (A) and emission (B) spectra of *p*-NH_2PhDTPA-Eu (green) and Q-DTPA-Eu (red) 16.4 μM with europium ions in aqueous solution (100 mM sodium phosphate buffer, pH 3.5).

The emission spectrum of Q-DTPA is characteristic of a europium complex.

We investigated suitable reaction conditions of acrolein and the probe. First, *p*-NH_2PhDTPA and HCl were added to acrolein in sodium phosphate buffer, and the mixture was heated for 30 min at 100 °C and then cooled in tap water. Europium was added to the diluted mixture, and the luminescence intensity was measured. Optimum conditions were evaluated by measuring the luminescence of reaction solutions. The results indicated that the optimum concentraion of the probe was 10 mM, and a suitable reaction time was 30 min.

Figure 2 shows the results of assays of various concentrations of acrolein under optimal conditions. A dose-dependent

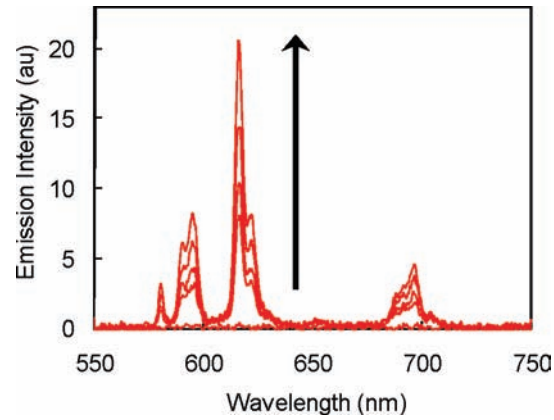


Figure 2. Reaction of *p*-NH_2PhDTPA (final concentration of 10 mM) with acrolein (0, 0.5, 1.0, 1.5, 2.0 mM). Emission spectra of the reaction mixture diluted with 200 mM sodium phosphate buffer, pH 3.5. Time-resolved luminescence intensity (ex = 320 nm) was recorded 30 min after the initiation of the reaction.

increase of luminescence intensity was observed. We also carried out HPLC analysis to confirm that the reaction of *p*-NH_2PhDTPA and acrolein produced Q-DTPA. The main product of the reaction was indeed Q-DTPA (Supporting Information). These results suggest that the reaction proceeded as expected, and the luminescence was greatly increased following the reaction of acrolein and *p*-NH_2PhDTPA. We thus considered that *p*-NH_2PhDTPA is potentially useful for the sensitive detection of acrolein and next addressed acrolein assay with microplates. Microplates (96-well, 384-well, etc.) are an indispensable tool for biological assays. They can handle a large number of samples simultaneously with a small sample volume (microliter order) and are suitable for automated assay systems. They are, however, more likely to suffer from autofluorescence of the device and scattered light. Therefore, TRF measurements

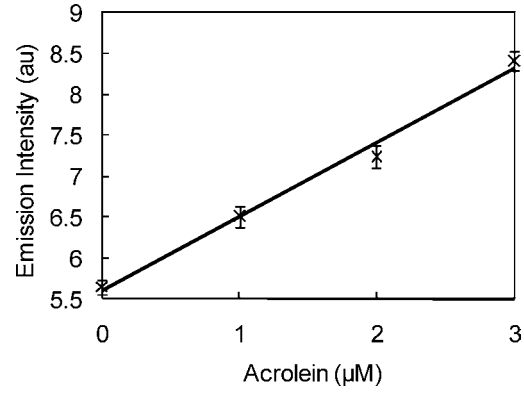


Figure 3. Microplate assays of acrolein (0, 1, 2, 3 μM) in aqueous solution with *p*-NH_2PhDTPA (final concentration of 10 mM). Time-resolved luminescence intensity (ex/em = 340/615 nm, measured after dilution with 200 mM sodium phosphate buffer, pH 3.5) was recorded 30 min after the initiation of the reaction. Data are shown as mean ± SD (n = 3).

(15) (a) Yuan, J.; Wang, G.; Majima, K.; Matsumoto, K. *Anal. Chem.* **2001**, *73*, 1869. (b) Gudgin Dickson, E. F.; Pollak, A.; Diamandis, E. P. *J. Photochem.Photobiol. B* **1995**, *27*, 3.

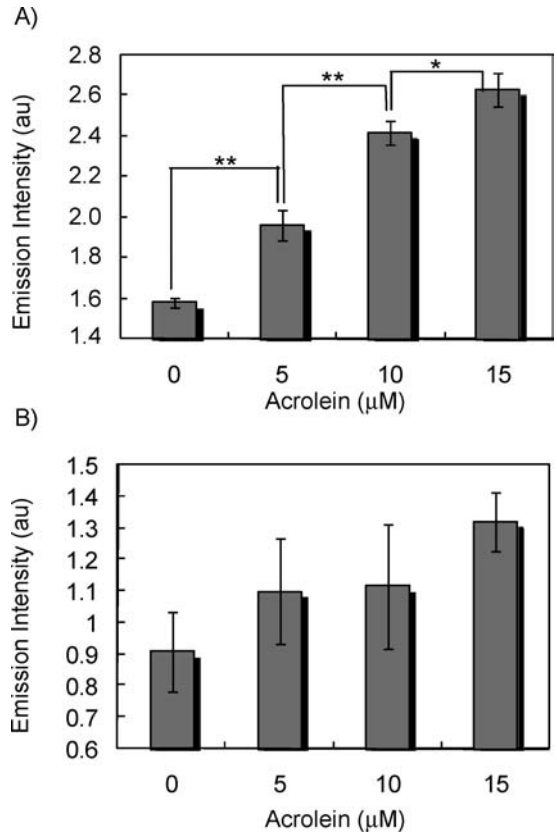


Figure 4. (A) Microplate assays of acrolein (0, 5, 10, 15 μM) in serum with *p*-NH_2PhDTPA (final concentration of 10 mM). Luminescence intensity (ex/em = 340/615 nm, measured after dilution with 200 mM sodium phosphate buffer, pH 3.5) was recorded 30 min after the initiation of the reaction. (B) fluorescence assay of acrolein in serum with the conventional method. Data are shown as mean ± SD ($n = 3$). ** indicates $P < 0.01$; * indicates $P < 0.05$.

with low background signals are expected to be highly advantageous compared with the use of organic fluorescent compounds.[15] Figure 3 clearly shows that *p*-NH_2PhDTPA worked as a reliable probe for acrolein. As expected, a dose-dependent increase of luminescence intensity was observed. The assay also showed good linearity over the concentration range examined. The results showed that our probe worked well in aqueous buffer. Therefore, we applied the method to assays with human serum.

Serum itself has absorbance in UV−vis region and emits fluorescence in the range of 300−500 nm. Sensitive and reliable detection using the conventional method is prevented by the background fluorescence of serum. However, in the case of our probe, the background signal can be suppressed by the use of TRF measurements. Thus, our probe should be especially suitable for assays in serum. The following experiments were performed with human serum spiked with acrolein. As can be seen in Figure 4A, as little as 5 μM acrolein could be detected with this probe, and the luminescence increased linearly with respect to acrolein concentration. A concentration of 5 μM acrolein could not be detected with *m*-aminophenol, which is the probe for the conventional method, without HPLC separation (Figure 4B). In the conventional method, reliable assays are impossible in the range of low concentration of acrolein, because of the strong background fluorescence of serum. With our method, the detection limit of 0.96 μM was achieved. This is 1 order of magnitude better than that of the conventional method (15 μM) without HPLC separation, and the detection limit appears to be appropriate for measuring acrolein concentrations found in disease states (normal, 0.5 μM; patient, 1.4 μM).[8] In conclusion, we have developed a novel probe for acrolein by using TRF measurement, which can eliminate background fluorescence due to serum. Our probe permits sensitive and reliable detection of acrolein in serum, and its performance is superior to that of the conventional method.

Acknowledgment. This research was supported in part by a Grant-in Aid for JSPS Fellows (to M.T.), by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant Nos. 19205021 and 20117003 to Y.U., 20689001 and 21659024 to K.H., and 21750135 to T.T.), and by the Industrial Technology Research Grant Program in 2009 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan (to T.T.). K.H. was also supported by the Mochida Memorial Foundation for Medical and Pharmaceutical Research and Sankyo Foundation of Life Science.

Supporting Information Available: Synthesis and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

OL1002219