Microenvironmental Effects of Water-Soluble Polymers on the Chemiluminescence of Luminol and Its Analogs

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The effects of water-soluble polymers(WSPs), such as bovine serum albumin(BSA) and polyethylenimine-(PEI), on chemiluminescence(CL) in an aqueous solution have been studied. The CL emission, particularly in the initial stage of the CL reaction, was strongly enhanced by increasing the concentrations of these WSPs. This was attributed to the acceleration of the chemical reactions prior to the formation of the light-emitting species. BSA was also peculiar in that it could enhance CL under neutral pH conditions. It was suggested that these WSPs offered hydrophobic and basic microenvironments well suited to the CL reaction.

The microenvironment of an aqueous solution has a substantial effect on the chemi- and the bioluminescence.[1-4] In particular, the microenvironmental effects of surfactants, such as hexadecyltrimethylammonium bromide(CTAB), polyoxyethylene sorbitan monostearate(Tween 60), polyoxyethylene(10)octylphenyl ether(Triton X-100), polyoxyethylene(23)-dodecanol(Brij-35), and sodium dodecyl sulfate(SDS), to enhance the CL emission of lucigenin(*N,N'*-dimethyl-9,9'-biacridinium dinitrate) and of luminol(5-amino-2,3-dihydro-1,4-phthalazinedione) are interesting from the standpoint of analytical chemistry.[5-8]

CL-enhancement effects can arise from several factors: (1) The rate of the reaction leading to the formation of the light-emitter, (2) the CL excitation efficiency, (3) the fluorescence quantum yield of the emitter, and (4) some combination of these factors.[6]

To date, only a few reports have dealt with the relationship between the CL-emission intensity in the presence of surfactants and the above-mentioned factors.[1,4,6] In the CL of cypridina luciferin, two factors, (1) and (3), have a substantial effects on the enhancement of its CL emission,[1] while the (2) factor is significant in the CL of MAD(9-methylene-10-dodecylacridane).[4] Two factors, (1) and (2), contribute to an increase in the CL-emission intensity of lucigenin.[6]

Such WSPs as BSA and PEI show a strong binding affinity to small aromatic anions.[9,10] These are expected to influence the CL reaction significantly. However, the effects of the WSPs on the CL emission have not yet been studied. In this paper, the effects of BSA and PEI(Mw: 70,000) on the CL emission centering around luminol were studied and were evaluated from the analytical standpoint. The other compounds used in this study were isoluminol(6-amino-2,3-dihydro-1,4-phthalazinedione) and *N*-(4-aminobutyl)-isoluminol derivatives (ABI derivatives: 6-[*N*-(4-aminobutyl)alkylamino]-2,3-dihydro-1,4-phthalazinedione) (Fig. 1).

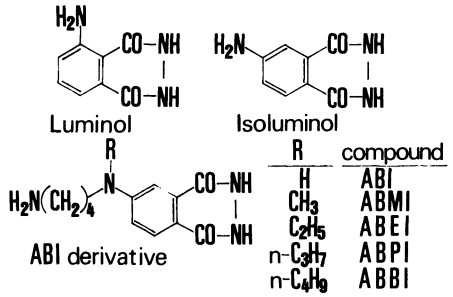
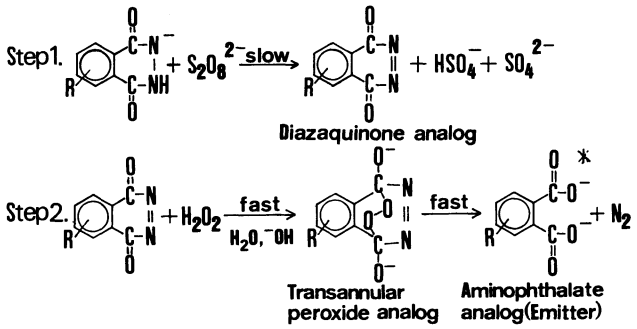


Fig. 1. Chemical structures of luminol and its analogs.

Results

The CL reaction in this study was initiated with a hydrogen peroxide(H_2O_2)-potassium peroxodisulfate($K_2S_2O_8$) co-oxidation system in 0.1 mol dm^{-3} aqueous sodium carbonate (Na_2CO_3, pH 11.4). The following scheme, Scheme 1, is proposed as the CL reaction mechanism of luminol(pK_a=6 and 13).[14,15] The CL reaction of the other compounds also seems to follow Scheme 1. The reaction is of the first order in the CL



Scheme 1.

compound and in peroxodisulfate($S_2O_8^{2-}$), but is of zero order in H_2O_2 and at hydroxide(OH^-) concentration under the conditions of this experiment.[14]

The CL quantum yield(ϕ_{CL}) based on the starting luminols is the product of several variables:[15]

$$\phi_{CL} = \phi_r \cdot \phi_{es} \cdot \phi_f, \quad (1)$$

where ϕ_r is the fraction of molecules following the correct chemical reaction path, ϕ_{es} is the fraction of those product molecules that are formed in the excited emitter, i.e., the CL excitation efficiency, and ϕ_f is the fluorescence quantum yield of the emitter. In this study, the ϕ_f values were determined on the basis of the

Table 1. Quantum Yields of Luminol and Its Analogs[a)]

Compound[b)]	$\phi_r \cdot \phi_{es}$[c)]	ϕ_{fl}	ϕ_{CL}
Luminol	0.059	0.16	0.0094[d)]
Isoluminol	0.048	0.034	0.0017[e)]
ABI	0.12	0.040	0.0049
ABMI	0.29	0.053	0.016
ABEI	0.19	0.044	0.0083
ABPI	0.16	0.038	0.0059
ABBI	0.070	0.039	0.0027

a) Oxidation system: H_2O_2–$K_2S_2O_8$. b) [Compound]= 1.6×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} Na_2CO_3. c) The product, $\phi_r \cdot \phi_{es}$, was calculated according to Eq. 1 from the experimentally determined ϕ_{fl} and ϕ_{CL} values. ϕ_r is usually assumed to be approximately unity. Ref. 15. d) Reported value=0.006—0.009. Ref. 14, 20. e) Reported value=0.0012 (it was measured in 0.1 mol dm^{-3} K_2CO_3 with H_2O_2 and hemin.). Ref. 16.

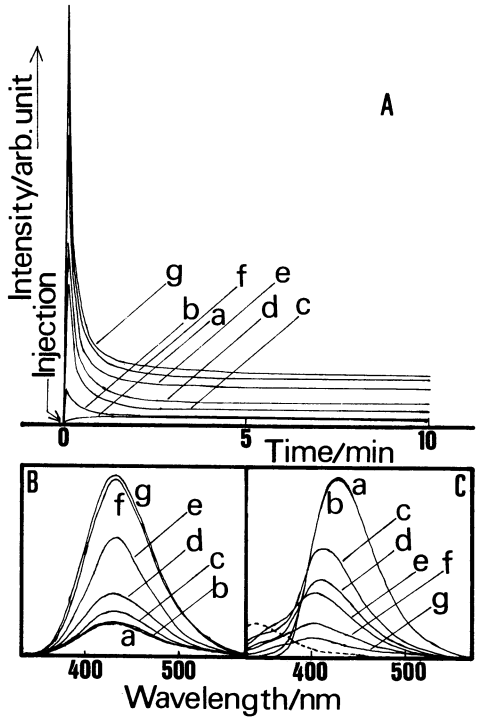


Fig. 2. Time course of luminol chemiluminescence (A), chemiluminescence spectra at 10—12.5 min after the initiation of the reaction (B), and fluorescence spectra of the total reaction mixture at 48 h after the initiation of the reaction (C) with and without BSA. [Luminol]=8.0×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} Na_2CO_3. [H_2O_2]=0.1 mol dm^{-3}. [$K_2S_2O_8$]=2.5×10^{-4} mol dm^{-3}. [BSA]/wt%: a=0, b=0.020, c=0.40, d=0.60, e=1.2, f=2.4, g=4.0. Excitation: 306 nm. Broken line in (C): Fluorescence spectrum of 1.0 wt% of BSA in 0.1 mol dm^{-3} Na_2CO_3 (pH 11.4).

total reaction mixture at the end of the CL reaction(48 h after CL initiation). The values of these variables are summarized in Table 1.

Figure 2 shows the effects of BSA on the CL of luminol. In the presence of BSA, the CL emission reached its maximum intensity at about 10 s after the initiation of the reaction and thereafter rapidly to a constant level. The CL emission intensities in both the initial stage and in the plateau region increased with the increase in the BSA concentration. The CL-spectrum maximum from 10 to 12.5 min after the initiation of the reaction was affected little by the addition of BSA, whereas the fluorescence-spectrum maximum of the total reaction mixture at the end of the reaction, 48 h after the initiation of the reaction, shifted considerably to shorter wave lengths and its intensity was lower than that obtained for the total reaction mixture in the absence of BSA. The inherent fluorescence of BSA in the total reaction mixture also

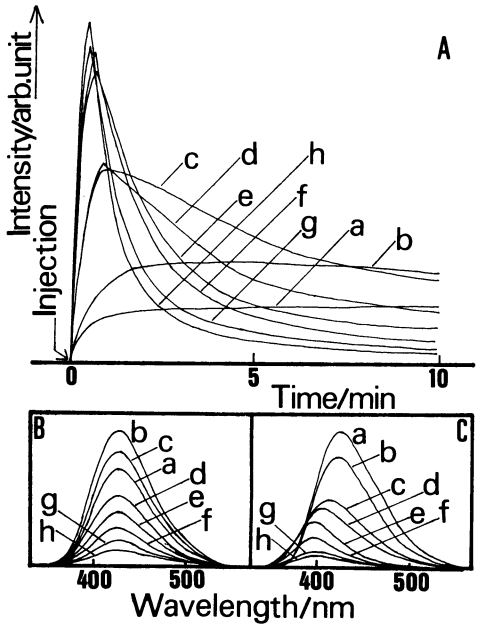


Fig. 3. Time course of luminol chemiluminescence (A), chemiluminescence spectra at 10—12.5 min after the initiation of the reaction (B) and fluorescence spectra of the total reaction mixture at 48 h after the initiation of the reaction (C) with and without BSA. [Luminol]=8.0×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} Na_2CO_3. [H_2O_2]=0.10 mol dm^{-3}. [$K_2S_2O_8$]=2.5×10^{-4} mol dm^{-3}. [PEI]/wt%: a=0, b=0.010, c=0.050, d=0.10, e=0.25, f=0.50, g=1.0, h=2.0. Excitation: 306 nm.

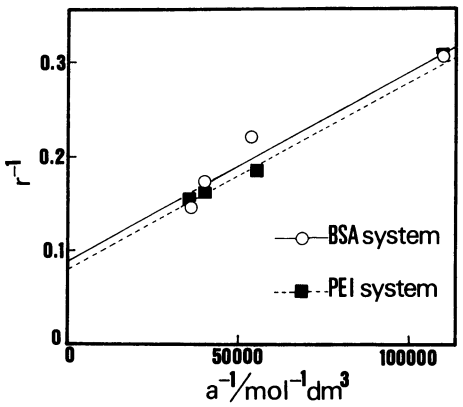


Fig. 4. Reciprocal of average mole ratio of bound luminol monoanions to WSP ($1/r$) vs. reciprocal of equilibrium concentration of luminol monoanions ($1/a$). Solvent: 0.1 mol dm^{-3} Na_2CO_3. [WSP]=0.10 wt%. [Luminol]=1.0×10^{-5}—3.0×10^{-5} mol dm^{-3}.

become weaker than in the 0.1 mol dm^{-3} Na_2CO_3 solution.

PEI also influenced the CL emission, particularly in the initial stage, as is shown in Fig. 3. In this case, much more time was required for the attainment of the CL-emission peak than in the BSA system. By analogy to the BSA, the fluorescence spectrum shifted to a shorter wavelength, and its intensity decreased, with the increase in the PEI concentration. In this case, PEI was non-fluorescent, unlike BSA.

The binding constant(K, mol^{-1} dm^3) between the luminol monoanions and the WSPs and the maximum possible number of bound luminol monoanions per WSP molecule(n) were determined using the equilibrium dialysis method following Eq. 2, as suggested by Klotz et al.:[9]

$$1/r = 1/n + 1/n \cdot K \cdot a, \quad (2)$$

where r is the moles of luminol monoanions bound to the total moles of WSP and where a is the molar concentration of free luminol monoanions at equilibrium. The measurements were made in 0.1 mol dm^{-3} aqueous Na_2CO_3. Both K and n can be calculated from a linear plot of $1/r$ vs. $1/a$. Figure 4 shows the results of the analysis of the relationship between the luminol monoanions and the WSPs. In this system containing 0.1 wt% of BSA, the K and n values were 4.41×10^4 mol^{-1} dm^3 and 11 respectively, while they were 3.72×10^4 mol^{-1} dm^3 and 12 respectively in the presence of 0.1 wt% of PEI(Mw: 70000).

Figure 5 indicates the relationship between the initial CL-emission intensity(CL intensity, I; at 10 s after the initiation of the reaction) and the concentration of WSP. The ratio of the CL intensity in the presence of WSP to that in the absence of WSP was plotted as a function of the WSP concentration.

Figure 6 indicates the effects of various surfactants on the CL emission. As is shown in Fig. 6, the surfactants did not affect the initial CL emission nearly as much as the WSPs. In addition, unlike the system containing the WSPs, the shifts of the fluorescence-spectrum maximum were not observed.

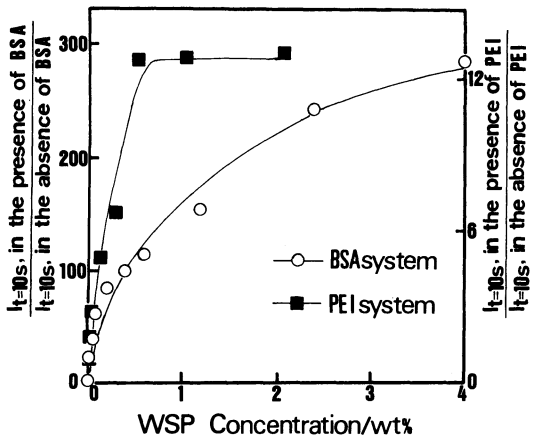


Fig. 5. Effects of BSA and PEI on the initial chemiluminescence intensity($I_{t=10s}$). [Luminol]= 8.0×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} Na_2CO_3. [H_2O_2]=0.10 mol dm^{-3}. [$K_2S_2O_8$]=2.5×10^{-4} mol dm^{-3}. [BSA]=0.10 wt%. [PEI]=0.10 wt%.

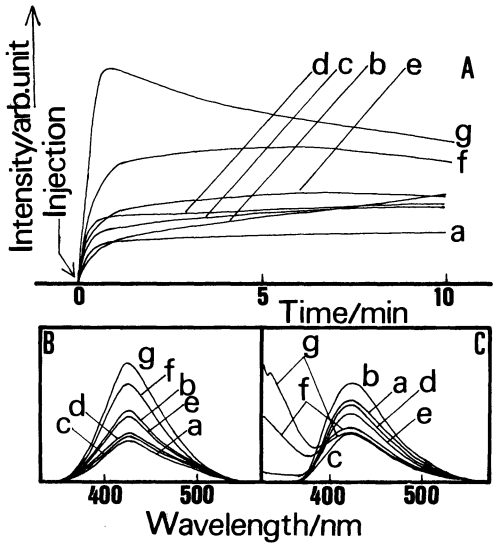


Fig. 6. Time course of luminol chemiluminescence (A), chemiluminescence spectra at 10—12.5 min after the initiation of the reaction (B) and fluorescence spectra of the total reaction mixture at 48 h after the initiation of the reaction (C) with and without surfactants. [Luminol]=8.0×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} Na_2CO_3. [H_2O_2]=0.10 mol dm^{-3}. [$K_2S_2O_8$]=2.5×10^{-4} mol dm^{-3}. [Surfactant]/mol dm^{-3}: a; surfactant free, b; [CTAB]=1.8×10^{-3}, c; [SDS]=1.6×10^{-2}, d; [Brij 35]=2.0×10^{-4}, e; [Triton X-100]=8.0×10^{-4}, f; [Triton X-100]=8.0×10^{-3}, g; [Triton X-100]=2.0×10^{-2}. a—e: cmc×2, f: cmc×20, g: cmc×50. These cmc values are in water (Refs. 6, 8).

Table 2. Effect of BSA on ϕ_{fl} and on CL Intensity(I). $\phi_{fl,BSA\text{-free}}$, $\phi_{fl,BSA}$, and Initial I Ratio

Compound[a]	$\phi_{fl,BSA\text{-free}}$	$\phi_{fl,BSA}$[b]	$I_{BSA}/I_{BSA\text{-free}}$[c]
Luminol	0.16	0.17	66.7
Isoluminol	0.034	0.030	40.3
ABI	0.040	0.050	5.3
ABMI	0.053	0.045	10.3
ABEI	0.044	0.043	9.7
ABPI	0.038	0.043	4.5
ABBI	0.039	0.040	5.5

a) [Compound]=1.6×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} Na_2CO_3. b) [BSA]=0.10 wt%. c) I in the presence of BSA to I in the absence of BSA at 10 s after the initiation of the reaction.

The effects of BSA on various CL reactions were also examined. Table 2 shows the ϕ_{fl} values with and without BSA and the initial I ratio(I in the presence of BSA to I in the absence of BSA) at 10 s after the initiation of the reaction. As has been referred to earlier, the ϕ_{fl} values were determined based on the total reaction mixture at the end of the CL reaction(48 h after CL initiation). The concentration of BSA was 0.10 wt%. In such a fairly dilute system, BSA had only a small influence on the fluorescence of the total reaction mixture.

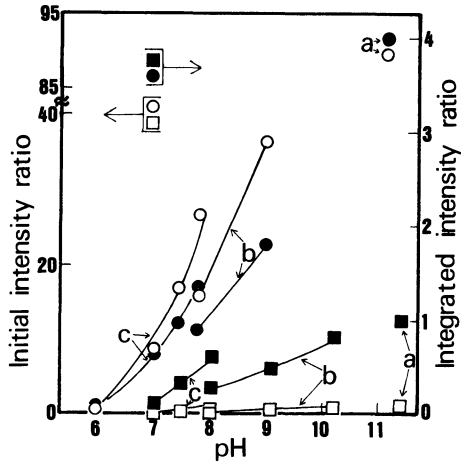


Fig. 7. Comparison of the chemiluminescence-emission intensity of luminol under various pH conditions with and without BSA. [Luminol]=8.0×10^{-5} mol dm^{-3}. [H_2O_2]=0.10 mol dm^{-3}. [$K_2S_2O_8$]=2.5×10^{-4} mol dm^{-3}. [BSA]=0.50 wt%. a: Prepared with 0.1 mol dm^{-3} Na_2CO_3, b: prepared with 0.05 mol dm^{-3} borate/NaOH buffer solution, c: prepared with 0.1 mol dm^{-3} phosphate buffer solution. ○, ●: With BSA. □, ■: Without BSA. The intensity ratios were calculated on the basis of the initial intensity (t=10 s) or the integrated intensity (during 10 min after the initiation of the reaction) measured in 0.1 mol dm^{-3} Na_2CO_3 without BSA.

Figure 7 shows the CL-emission intensities measured under various pH conditions in the presence of BSA.

Discussion

Time Course of CL Emission. The effects of BSA and PEI on the time course will be described briefly, since it is important to evaluate the features of the time course of CL emission if we are to use CL for analytical chemistry.

In the presence of BSA (Fig. 2A), the time required to reach the CL-emission peak is nearly constant and is independent of the BSA concentration, irrespective of the peak intensity. In the decay part in Fig. 2A, there are at least two decay components, one of them decays rapidly, while the other one lasts more than 10 min.

On the other hand, in the presence of PEI (Fig. 3A), the time required to reach the CL-emission peak depends on the PEI concentration and becomes shorter, approaching an almost constant value, as the PEI concentration increases. The decay feature with no prolonged emission is also different from that in the presence of BSA.

It is difficult to elucidate the time course in detail, since the interaction between these WSPs and the dissolved chemical species may well be very intricate. However, the effects of these WSPs, especially BSA, on enhancing the initial CL emission seem to have a great practical value from the analytical standpoint.

Enhancement of CL Emission. The most remarkable feature of the absorption spectra of luminol and its analogs in the 0.1 mol dm^{-3} Na_2CO_3 solution containing BSA or PEI was that the absorption peak near 230 nm becomes small and shifts to the longer wavelength side by 5—10 nm. This suggests that the monoanions of these CL compounds are bound to the hydrophobic region of BSA or PEI, like other aromatic anions.[9-13] In this connection, it was worth noting that most of the acidic groups in BSA are dissociated in the 0.1 mol dm^{-3} Na_2CO_3 solution. A similar thing is true with the ammonium protons on PEI(pK_a≈6). Therefore, BSA and PEI under the present reaction conditions are likely to afford basic binding sites(for binding acidic CL compounds) in addition to their inherent hydrophobic sites within the polymer matrix. The binding affinities of the luminol monoanions to both BSA and PEI seem similar, for there is no marked difference in the K values or n values determined in the two WSP systems.

As is shown in Figs. 2A and 3A, the microenvironments offered by BSA or PEI mentioned above caused remarkable CL-enhancing effects in the initial stage of the CL reaction. Such effects were remarkable, especially in the presence of BSA, as is shown in Fig. 5. The difference in the effects of WSPs on the initial CL emission seems to depend significantly on their basic properties rather than on their hydrophobic properties. This seems to be supported by the fact that no strong initial CL emission is observed in the presence of surfactants without basic groups in their micelles (Fig. 6A).

It should also be emphasized that the CL-enhancing effects were even observed in a very dilute WSP. Such was not the case with surfactants. They usually did not show any CL-enhancing effects below their critical micelle concentrations(cmcs).[5,6]

As is listed in Table 2, the initial I ratios in various CL reactions increased in the presence of BSA. The introduction of the electron-donating substituent to the amino group of isoluminol results in the higher ϕ_{es}.[15] This was also observed in the CL of ABI derivatives, as is summarized in Table 1. This is because the electron-donating property of the aminobutylalkyl substituents of the ABI derivatives is advantageous for the intramolecular chemically initiated electron-exchange luminescence(CIEEL) mechanism.[17,18] In the BSA molecule, various basic groups carrying lone-pair electrons seem to be able to interact with the protons of the amino and hydrazide groups of luminol, as is generally observed in the interaction between enzymes and substrates.[19] In other words, BSA in a basic solution is considered to be able to give an advantageous reaction field to the intramolecular CIEEL mechanism, particularly in the CL of luminol or isoluminol. However, in this study it is difficult to relate the microenvironmental effects to ϕ_{es} directly.

On the other hand, the effects of the WSPs on ϕ_{fl} are rather detrimental to the enhancement of the CL emission, as is shown in Figs. 2C and 3C. This was also

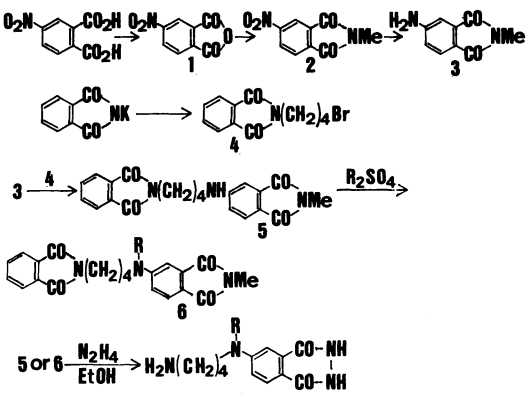
observed in the presence of a surfactant (Fig. 6C). The fluorescence intensity of 3-aminophthalate dianions (8.0×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} Na_2CO_3 solutions containing various concentrations of BSA or PEI also decreased compared with that in the absence of BSA or PEI. However, the shifts in the fluorescence spectra shown in Figs. 2C and 3C were in conflict with the fact that there was no difference in the fluorescence-spectra maxima of 3-aminophthalate dianions in the system both with and without BSA or PEI (the shift was also not observed at 48 h after the preparation of the test solution). The shifts shown in Figs. 2C and 3C are thus at present difficult to explain.

Effects of WSP from the Analytical Standpoint. For the application of CL to clinical and biochemical analysis, it is important to obtain a strong CL emission, even under neutral pH conditions.[20] As is well known, the CL-emission intensity of luminol is much weaker under neutral conditions than under strongly alkaline conditions.[21] Thus, BSA was studied under neutral or weakly alkaline conditions in order to enhance the CL emission. As is shown in Fig. 7, the microenvironments of BSA were highly effective in producing a sufficient CL emission, even under neutral pH conditions. This indicates that BSA can be very useful enhancer of CL emission for analytical application. As is shown in Table 2, the effects of BSA on the CL emission of ABI derivatives are smaller than those of BSA in the CL emission of luminol, but BSA seems to be of practical value as a CL-enhancer with ABI derivatives also.

Experimental

Instruments. The UV and visible absorption spectra were measured by using a JASCO-610C spectrophotometer. The fluorescence and CL spectra were recorded with a Shimadzu RF-540 spectrofluorometer. In recording the CL spectra, the window of the excitation-light source was shut. In the measurements of the time course of the CL emission, the CL emission was received by a EMI 9893A/100 photomultiplier, and then the output photocurrent was amplified by a home-made preamplifier and recorded by a Matsushita VP-6431B recorder. The operational amplifier used in the I-V converter part of the preamplifier was an AD515KH apparatus. The guard ring was placed around the input terminals, and they were put on stand-off terminals made of Teflon for perfect insulation. In the measurements of the monochromatic-light-emission intensity, a Jovan-Yvon H-20 monochromator was set between the photomultiplier and a black box equipped with the CL reaction cell(inner-volume: 5 cm^3). The temperature in the black box was controlled using a thermistor. All the measurements were done at 25 °C. Mass-spectral analysis was performed by means of a Hitachi M-80B mass spectrometer.

Reagents. The BSA(F-V)(Mw: 70,000, Nakarai) was used as received. The PEI(Mw: 70,000, Wako) was dialysed against cellophane membrane for 3 days in distilled water, and then the solvent was evaporated to give a highly viscous liquid. The CTAB, SDS, Triton X-100, and Brij-35 were all purchased from Wako and used as received. The luminol (Wako), isoluminol(Tokyo Kasei), and 3-aminophthalate (Kanto Kagaku) were used after recrystallization from 0.6 mol dm^{-3} HCl. The ABI, ABMI, ABEI, ABPI, and ABBI were synthesized by a modification of the method of Schroeder et al. for ABEI.[22] The melting-point data are uncorrected values. These compounds were synthesized according to Scheme 2.



Scheme 2.

4-Amino-*N*-methylphthalimide (3). A modification of the method of Nicolet et al. was used for the syntheses of 4-nitrophthalic anhydride (**1**).[23] Compound **2** was synthesized as has been described in the literature.[22] Concentrated HCl (303 cm^3) and 48 cm^3 of water was added, drop by drop and successively, to a mixture of 50 g (0.24 mol) of **2** and 215 g (0.95 mol) of $SnCl_2\cdot2H_2O$, and then the reaction mixture was vigorously stirred for 1 h at 85 °C, resulting in a pale yellow solution. This solution was allowed to stand at room temperature over night. The product was precipitated as a white powder. Filtration and thorough washing with much water gave **3** as a yellow powder. This powder was dried at 120 °C for several hours; mp 246—248 °C.

***N*-(4-Bromobutyl)phthalimide (4).** This compound was synthesized according to the procedure in the literature.[24]

***N*-Methyl-4-*N*[4-(*N*-phthalimide)butyl]aminophthalimide (5).** This compound was likewise synthesized as has been described in the literature.[22]

The *N*-alkylation of **5** was performed by a reaction with dialkyl sulfate by reference to the literature.[22] To synthesize ABMI, a mixture of 7.5 g (0.02 mol) of **5** and 25 cm^3 (0.15 mol) of dimethylsulfate was stirred for 60 min at 140 °C under anhydrous conditions. After it had cooled to room temperature, the mixture was poured into 500 cm^3 of ice water to deposit a dark green-colored viscous fluid. This product was recrystallized from 50% H_2O-acetic acid several times to give yellow green crystals. Next, a mixture of 5 g (0.012 mol) of **6**, 14 cm^3 of anhydrous hydrazine, and 50 cm^3 of absolute ethanol was stirred for 4 h at 85 °C. Then the reaction mixture was cooled to room temperature. In the next step, after the excess hydrazine and ethanol had been removed by vaccum distillation, ABMI including the by-product was deposited as a faintly gray-colored solid. After filtration, it was mixed with 100 cm^3 of 3 mol dm^{-3} HCl to dissolve the ABMI. An insoluble by-product was removed by filtration. The filtrate was adjusted at pH 7—9 with 5 mol dm^{-3} potassium hydroxide, and soon ABMI precipitated as white crystals. They were recrystallized from 50% H_2O-*N*,*N*-dimethylformamide. Other ABI derivatives were

synthesized similarly.

Mass-Spectral Data. ABI; MS (70 eV) m/z (rel intensity) 248 (M^+; 45), 231 (15), 204 (48), 190 (100), and 177 (37); Found: m/z 248.1272. Calcd for $C_{12}H_{16}O_2N_4$: M, 248.2738. ABMI; MS (70 eV) m/z (rel intensity) 262 (M^+; 28), 218 (7), 204 (100), and 191 (11); Found: m/z 262.1415. Calcd for $C_{13}H_{18}O_2N_4$: M, 262.3007. ABEI; MS (70 eV) m/z (rel intensity) 276 (M^+; 26), 232 (5), 218 (100), 204 (8), and 190 (28); Found: m/z 276.1587. Calcd for $C_{14}H_{20}O_2N_4$: M, 276.3276. ABPI; MS (70 eV) m/z (rel intensity) 290 (M^+; 4), 248 (58), 204 (43), 190 (100), and 177 (21); Found: m/z 290.1739. Calcd for $C_{15}H_{22}O_2N_4$: M, 290.3545. ABBI; MS (70 eV) m/z (rel intensity) 304 (M^+; 34), 246 (68), 204 (34), 190 (50), and 72 (100); Found: m/z 304.1898. Calcd for $C_{16}H_{24}O_2N_4$: M, 304.3814.

The other reagents used in this study were of a reagent grade.

Determination of ϕ_{fl} and ϕ_{CL}. The fluorescence and CL spectra were corrected according to the method in the literature.[25] The correction of the intensity of the excitation light was made on the basis of the excitation spectrum of Rohdamine B (3 g dm^{-3} in ethylene glycol). The ϕ_{fl} was determined relative to the quantum yield of quinine sulfate.[25] The unknown ϕ_{fl} was given by the following equation:

$$\phi_{fl,sample} = \frac{A_{standard} \cdot N^2_{sample} \cdot I_{sample}}{A_{sample} \cdot N^2_{standard} \cdot I_{standard}} \cdot \phi_{fl,standard}, \quad (3)$$

where A, I, and N are, respectively, the absorbance, the fluorescence intensity (I=the area under the fluorescence spectrum), and the refractive index of the solution. $\phi_{fl,standard}$ is 0.55 at 25 °C. ϕ_{CL} was determined relative to the ϕ_{CL} of luminol in dimethyl sulfoxide. The unknown ϕ_{CL} was given by:[26]

$$\phi_{CL,sample} = \frac{q_{\lambda max,sample} \cdot A_{standard} \cdot f_{\lambda max,standard}}{q_{\lambda max,standard} \cdot A_{sample} \cdot f_{\lambda max,sample}} \cdot \phi_{CL,standard}, \quad (4)$$

where $q_{\lambda max}$ is the time integral of the measured phototube current at the peak wavelength of the CL spectrum, and where A is the number of the molecules reacted in the CL reaction. The fractional CL emission at λ_{max}, $f_{\lambda max}$, is calculated by integrating the area under the portion of the curve at the CL-spectrum maximum bound by the exact wavelength-band pass and by dividing it by the total area under the CL spectrum. $\phi_{CL,standard}$ is 0.0125 at 25 °C.[27]

Initiation of the CL Reaction. The CL reaction was initiated with a H_2O_2-$K_2S_2O_8$ co-oxidation system. The H_2O_2 and the $K_2S_2O_8$ were mixed just before use and then immediately injected into the CL reaction cell using a microsyringe while being stirred by the use of a magnetic stirrer for 30 s. The time required to make the solution homogeneous was within about 3 s in all CL reaction systems. In this study, a mixture of 40 μdm^3 of 35% H_2O_2 and 20 μdm^3 of 0.05 mol dm^{-3} aqueous $K_2S_2O_8$ was injected against 4 cm^3 of the solution of the CL compound in 0.1 mol dm^{-3} aqueous Na_2CO_3 (pH 11.4) (or in an appropriate pH-buffer solution). The concentrations of H_2O_2 and $K_2S_2O_8$ in the CL reaction mixtures were 0.10 and 2.5×10^{-4} mol dm^{-3} respectively.

Determination of pK_a. The pK_a values of the protonated PEI were determined by the titration of 0.46 wt% aqueous PEI (pH 10.2) with 0.05 mol dm^{-3} hydrochloric acid.

Determination of K and n. Both the K and n values were determined by reference to the literature.[9,28] The pretreatment of dialysis bags (Union Carbide, Cellophane Tubing-Seamless 30/32 inch for dialysis) was done according to the usual method.[29] The dialysis bags were filled with 20 cm^3 of the test solution, which contained 0.1 mol dm^{-3} Na_2CO_3, 0.1 wt% WSP, and various known concentrations of luminol (1.0×10^{-5}—3.0×10^{-5} mol dm^{-3}). The solution in the outside compartment (in which the dialysis bags were immersed) was the same as the test solution in volume, in Na_2CO_3 concentration, and in luminol concentration, but it contained no WSP. Three days were allowed for equilibration at 25 °C. The solution in the outside compartment was then analyzed by absorption spectrophotometry. In all systems, corrections were applied to take into account the absorption due to the WSP and due to the leachable material from the dialysis bag. The interaction between luminol and the dialysis bag was found to be negligible in the concentration range of luminol studied.

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