Cobalt(II)-Catalyzed Chemiluminescence in a Dioctadecyldimethylammonium Chloride Bilayer Membranous Medium for the Flow Injection Determination of Phenylpyruvic Acid

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A novel chemiluminescence system is described for the flow injection determination of phenylpyruvic acid (PPA). The presence of both ordered surfactant molecular assemblies and a metal ion catalyst in the system is essential for the phosphorescence of benzaldehyde (emitter) produced by the aerobic oxidation of PPA in alkaline solution. Dioctadecyldimethylammonium chloride bilayer aggregates and cobalt(II) allows PPA to be selectively determined down to 1×10^{-7} M. The linearity is 2 orders of magnitude with a relative standard deviation 3.1% ($n = 10$) for 1×10^{-6} M PPA. Of 28 other species (1×10^{-3} M) tested, only 4-hydroxymanderic acid, 4-hydroxyphenylpyruvic acid, 2,5-dihydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylpyruvic acid provided signals 2-13 times more intense than that for 1×10^{-6} M PPA. PPA present at 10^{-3}-10^{-2} M levels in urine from patients with phenylketonuria can be determined with no special sample pretreatment by using this CL procedure. The mechanistic study of the present luminescent reaction are also undertaken in detail. The bilayer aggregates were found to contribute favorably both to the production of key intermediates and to the efficient phosphorescence emission.

INTRODUCTION

Analytical solution chemiluminescence (CL) has received much recent attention in various fields due to its extremely high sensitivity and other advantages. Established CL systems like luminol, lucigenin, and oxalate esters have been exclusively utilized (*1-4*), although several new CL systems are currently being proposed for selective determination of inorganic and organic analytes (*5-8*). From a practical point of view, these limited CL systems can only partially meet the demands of analytical chemists. It should be noted that the paucity of usable CL systems is a major impediment to the wide spread acceptance of CL methods. The search for new CL systems is of significant importance for widening the scope of CL methods. Our efforts have been devoted to finding such new CL systems by taking note of the fact that there are many oxidative reactions which emit very weak light. It has been previously demonstrated that dramatic improvements in CL characteristics of such poor CL systems can be achieved by use of ordered surfactant assemblies, sensitizers or catalysts (*9, 10*).

It is well-known that the local microenvironment in ordered surfactant assemblies (microheterogeneous phase) can be very different from that in bulk (homogeneous phase). This can greatly alter the chemical and photophysical pathways and rates of solubilized solutes (*11*). The CL quantum yield involves CL parameters such as the efficiency of chemical reaction and the yield of excited state, in addition to a fluorescence quantum yield. Furthermore, in flow CL analysis the signal measured (i.e., emission intensity) depends not only on the CL quantum yield but also on the reaction rate. Thus, the microheterogeneity of ordered surfactant assemblies can result in significant improvements in the analytical performance of CL reaction system. The resultant use of ordered surfactant media such as micelle, reversed micelle, or bilayer membranous aggregates (lamella or vesicle structure) have markedly increased the CL quantum yields and/or the reaction rates (*12, 13*).

Phenylpyruvic acid (PPA) is found in large amounts in the urine of patients with phenylketonurea, a serious inherited disease involving a metabolic disfunction of phenylalanine. The determination of urinary PPA is important for the biochemical diagnosis of phenylketonurea. Although some spectrometric methods are available for the determination of PPA in urine (*14-16*), these are often limited by poor sensitivity (detection limit 10^{-5}-10^{-6} M) and selectivity. High-performance liquid chromatography with fluorescence detection (*17*) is very sensitive (detection limit 10^{-9} M), but the required derivatization and chromatographic steps are time-consuming. There are many reports on CL methods for the determination of biologically and clinically important compounds (*18*). However, no CL methods have been reported for the determination of PPA.

In this paper, we describe a new CL system capable of directly determining PPA in urine with the aid of ordered surfactant assemblies and a metal ion catalyst. The method is based on the measurement of the CL arising from the oxidation of PPA by dissolved oxygen in an alkaline solution containing dioctadecyldimethylammonium chloride bilayer membranous aggregates in the presence of a cobalt(II) catalyst. The CL mechanism and the function of the surfactant aggregates are also elucidated.

EXPERIMENTAL SECTION

Apparatus. Prior to flow experiments, batch experiments were conducted, as described before (*19*), in order to obtain CL signal profiles. Each 0.1 mL of reagent solutions was transferred to a reaction vessel (ca. 3.5 mL) in various mixing orders at the flow rate of ca. 10 mL min^{-1} by a peristaltic pump.

A schematic diagram of the flow system is shown in Figure 1. Reagent solutions (R_1 and R_2) are delivered by peristaltic pumps (P) through two flow-lines; R_1 is a mixed solution of dioctadecyldimethylammonium chloride (DODAC) and sodium hydroxide and R_2 is a cobalt(II) chloride solution. The solutions of PPA and other compounds are injected by means of a 170-μL rotary valve injector (S) placed close to a luminometer (D, Lumiflow 1000, NITI-ON) with a 70-μL spiral flow cell made of Pyrex glass tubing (1-mm i.d.). Black Teflon tubing (1-mm i.d.) is used for the flow lines. The peak height of the signal recorded was measured as a CL signal.

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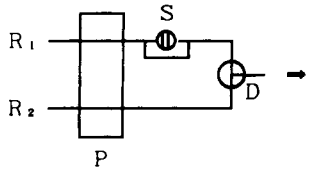


Figure 1. Schematic diagram of the flow system (for key, see text). Recommended conditions: R_1 1×10^{-3} M DODAC/1×10^{-2} M NaOH, R_2 1×10^{-5} M Co(II), flow rate 2.2 mL min^{-1}.

Table I. Effect of the Mixing Order of Reagents on the CL Signal for PPA[a]

mixing order							CL
1	+	2	+	3	+	4	signal[b]
PPA		NaOH		DDDAB		Co(II)	100
DDDAB		Co(II)		PPA		NaOH	14
NaOH		DDDAB		Co(II)		PPA	9
PPA		NaOH		Co(II)		DDDAB	ND[c]

[a] Conditions: 1×10^{-4} M PPA, 1×10^{-3} M DDDAB, 1×10^{-2} M NaOH, 1×10^{-3} M Co(II). [b] Normalized with respect to the signal (=100) in the best mixing order. [c] Not detected.

UV and visible spectra of PPA and its reaction products were measured by a spectrophotometer (Shimadzu UV-240), and fluorescence spectra of reaction products, by a fluorescence spectrometer (Shimadzu RF-540). The spectra of CL were taken by use of a flow cell with the fluorescence spectrometer by closing an excitation slit.

Reagents. Chemicals of reagent grade were used as received, except for a surfactant DODAC, which was recrystallized several times from acetone when used in the flow experiments. The water used was prepared by deionization of distilled water from a stainless steel apparatus. PPA solutions were prepared freshly from its sodium salt (PP^-). Surfactant solutions were prepared daily by ultrasonic treatment. Other reagent solutions were prepared daily from 10^{-2} M stock solutions.

RESULTS AND DISCUSSION

Mixing Order of Reagents. The mixing order of reagent solution plays a key role in CL reaction; no or weakened light emission may often be observed with different mixing orders, and also how fast the CL reaction proceeds is a dominant factor that decides sensitivity in flow CL analysis because transient light emission is monitored.

Thus, time courses of CL from the system were measured by the batch method. The experiments were performed under the presence of didodecyldimethylammonium bromide (DDDAB) and Co(II) ion because both ordered surfactant assemblies and a metal ion catalyst are essential for light emission. The results are shown in Table I, indicating that the CL signal was strongly dependent on the mixing order of the reagent solutions. The mixing order, PPA + NaOH + DDDAB + Co(II), gave the highest signal. Interestingly, the replacement of DDDAB and Co(II) in the above mixing order resulted in the complete disappearence of light emission. This suggests that for the light emission a key intermediate must be produced in the ordered medium before the addition of Co(II). For further batch experiments, the above best mixing order was employed.

Reaction Medium and Catalyst. First, various surfactants and organic solvents were subjected to test for the selection of reaction medium. The results are summarized in Table II, showing that a cationic surfactant DODAC provided the highest CL signal, about 150 times higher than those in organic solvents. This type of surfactant with two long alkyl chains in the molecule spontaneously forms bilayer membranous aggregates (vesicle or lamella), which are more ordered, rigid, and stable than micelles (*20*). Another dialkyl type surfactant DDDAB used above is also an effective medium, giving the signal of one-third that in the DODAC medium. The lower signal in the DDDAB medium lies conceivably in the difference in the phase-transition temperature (<10 °C for DDDAB and ca. 45 °C for DODAC) (*9*).

On the other hand, no or very weak light emission was observed in the anionic (SDS), nonionic (Brij 35), and some cationic (DTAB, TTAB, TDBAC, HPC) micellar media and in the organic solvent (ca. 30%) media. The absorption spectra of PPA (PP^-) in these media (except DTAB, TTAB, and HPC) without Co(II) did not show any appreciable formation of enolate ion (PP^{2-}) resulting from the tautomerization of PPA that was needed for the subsequent CL reaction (see CL reaction scheme shown later). In contrast, the DTAB, TTAB, and HPC media brought about PP^{2-} in fair amounts. Accordingly, it can be said that the DTAB, TTAB, and HPC micelles are favorable media for the PP^{2-} formation but not for the subsequent process. In the case of HPC, the dynamic quenching due to the pyridinium ion (*21*) is responsible in part for the weak light emission. Among the cationic surfactants tested, only the TDBAC medium did not facilitate the PP^{2-} formation. The reason lies presumably in the suppressed association of PP^- with the micelle owing to the geometrical constraint caused by the benzyl group of TDBAC projected into the bulk water. Of course, the formation of PP^{2-} was sufficiently recognized, as well as in the DODAC and DDDAB media, in the HTAC, HEDAB, and OTAC media, which gave much higher signals than other cationic micellar media. It is conceivable that the PP^{2-} formation is favored by the electrostatic interactions between the positively charged

Table II. Effect of Reaction Media on the CL Signal for PPA[a]

medium	concn[b]	CL signal[c]
water		ND[d]
methanol		0.6
acetonitrile		1.0
acetone		1.5
dodecyltrimethylammonium bromide (DTAB)	50 (16)	2.1
didodecyldimethylammonium bromide (DDDAB)	0.6 (0.18)	56
tetradecyltrimethylammonium bromide (TTAB)	11 (3.5)	0.7
tetradecyldimethylbenzylammonium chloride (TDBAC)	1.1 (0.37)	1.0
hexadecyltrimethylammonium chloride (HTAC)	3 (0.9)	34
hexadecylpyridinium chloride (HPC)	3 (0.9)	0.7
hexadecylethyldimethylammonium bromide (HEDAB)	3	56
octadecyltrimethylammonium chloride (OTAC)	1 (0.34)	29
dioctadecyldimethylammonium chloride (DODAC)	1	150
sodium dodecyl sulfate (SDS)	24 (8)	ND
polyoxyethylene(23)dodecanol (Brij 35)	0.2 (0.06)	ND

[a] Mixing order: 1×10^{-3} M PPA + 1×10^{-2} M NaOH + medium + 1×10^{-4} M Co(II). [b] 10^{-3} M (values in parentheses show critical micelle concentration). [c] Normalized with respect to the signal (=1.0) when the reaction medium is acetonitrile. [d] Not detected.

Table III. Effect of Metal Ion Catalysts on the CL Signal for PPA[a]

catalyst	CL signal[b]	catalyst	CL signal[b]
none	ND[c]	Zn(II)	3.9
Co(II)	40	Cr(III)	1.4
Fe(II)	31	Fe(III)	1.0
Ni(II)	6.5	Ag(I)	1.0
Cu(II)	6.2	Cr(VI)	0.5
Mn(II)	5.8	Mo(VI)	0.5

[a] Mixing order: 1×10^{-5} M PPA + 1×10^{-2} M NaOH + 1×10^{-3} M DODAC + 1×10^{-3} M catalyst. [b] Normalized with respect to the signal (=1.0) when the metal ion catalyst is Fe(III). [c] Not detected.

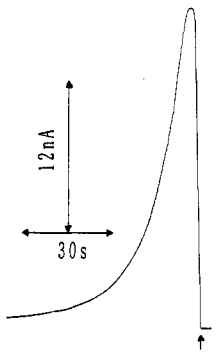


Figure 2. CL profile of PPA (batch method): mixing order, 1×10^{-4} M PPA + 1×10^{-3} M DODAC/1×10^{-2} M NaOH + 1×10^{-4} M Co(II). The arrow indicates the transfer of Co(II) solution to the reaction vessel.

surface of surfactant assemblies and the negatively charged PP^-.

Next, metal ion catalysts other than Co(II) were examined in the DODAC medium. The results are shown in Table III. The ferrous ion (Mohr's salt) also exhibited a prominent catalytic effect, providing a signal comparable to that for Co(II). It is known that Co(II) and Fe(II) often function as catalysts of aerobic oxidations through the activation of substrates or oxygen (22). The catalytic effect of Co(II) will be briefly discussed later. Anyhow, the employment of Fe(II) catalyst should be resigned from the stability of view, i.e., its gradual deactivation owing to the oxidation to Fe(III).

For the subsequent experiments, DODAC and Co(II) were used throughout as the reaction medium and catalyst, respectively. The CL profile of PPA obtained by the batch method is depicted in Figure 2, showing that the present CL reaction proceeds relatively fast.

Optimization of the Flow System. Based on the above batch experiments, a flow system was assembled as shown in Figure 1. Separate streams of the NaOH and the DODAC solutions provided higher signal but higher noise, resulting in lower signal-to-noise ratio, compared with the stream of their premixed solution. The signal for PPA (1×10^{-6} M) gradually increased with an increase in the DODAC concentration ($\sim 10^{-3}$ M). The dependency of NaOH concentration on the signal exhibited a maximum at about 1×10^{-2} M. With an increase in the Co(II) concentration, the signal slightly increased; the best signal-to-noise ratio was obtained at 1×10^{-5} M. On the other hand, the signal is little affected by the flow rate (~ 7 mL min^{-1}) of each reagent solution. Thus optimum operating conditions were determined as shown in Figure 1.

Characteristics of the System. Under the optimum conditions, the analytical characteristics of the system were investigated. The system provided fairly low background current (3×10^{-12} A) and hence low noise current (3×10^{-12} A). A logarithmic calibration graph for PPA exhibited a straight line with a slope of unity between 1×10^{-5} and 1×10^{-7} M (the determination limit at $S/N = 3$). When the concentration is higher than 1×10^{-5}, the graph deviates from a straight line because of an increase in the peak width of the signal with an increase in the concentration. The narrow linearity seems to be caused by the lower Co(II) concentration (10^{-5} M) compared with the PPA concentration. However, it was not improved by the increase in the Co(II) concentration ($\sim 10^{-3}$ M). The increased Co(II) may catalyze the dark reaction without light emission (23) or deactivate the excited-state product. The relative standard deviation ($n = 10$) was 3.1% for 1×10^{-6} M PPA. The sample throughput was ca. 60 h^{-1}.

Effect of Other Species. For evaluation of the selectivity, 1×10^{-3} M solutions of PPA-related and biologically important compounds were injected and the signals were compared with that for 1×10^{-6} M PPA. The present system is fairly selective. Of 28 compounds tested, 4-hydroxymanderic acid, 4-hydroxyphenylpyruvic acid, 2,5-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylpyruvic acid, 4-hydroxyphenylacetic acid, and indole-3-acetic acid gave signals 1300, 1200, 490, 210, 45, and 45% of that for PPA, respectively. PPA and these compounds possess an active methylene group whose proton tends to be deprotonated. The methylene group of PPA is more active than those of the above compounds because of the absence of any electron-donating group(s) and the presence of the electron-withdrawing carbonyl group in the neighborhood of the active methylene group. As stated later, the deprotonation (i.e., the enolization of the carbonyl group) is essential for the present CL reaction. This is the reason for the realization of selectivity. The following compounds were found not to cause light emission themselves: glyoxylic acid, pyruvic acid, oxalacetic acid, ketomalonic acid, 2-ketoglutaric acid, 2-ketovaleric acid, 4-hydroxycinnamic acid, indole-3-pyruvic acid, 2,3-dihydroxymandelic acid, 4-hydroxy-3-methoxyphenylacetic acid, mandelic acid, hippuric acid, 3-benzoylpropionic acid, benzoylacetone, phenylalanine, tyrosine, histidine, epinefrine, serotonin creatinine, caffeine, urea, and glucose.

In order to check the effect of concomitant species on the signal for PPA, a 1×10^{-6} M PPA solution containing each of species which coexist normally in human urine was injected. The results are shown in Table IV. There are many species showing interferences, 2,5-dihydroxyphenylacetic acid, amino acids, ascorbic acid, and some inorganic salts completely suppressing the signal for PPA. Such severe interferences seem to be due mainly to the depression of the catalytic effects of DODAC assemblies and/or Co(II) based on the deterioration of the microenvironment in the vicinity of the bilayer membranous aggregates by the interferents at high concentration levels. In fact, it was found from the absorption spectra that the formation of PP^{2-} was remarkably inhibited by the interferents. For instance, it is considered that the inhibition of the PP^{2-} formation by the amino acids and ascorbic acid (anionic species) is attributed to the competition with PP^- for the binding sites on the surface of DODAC assemblies. The deactivation of the Co(II) catalyst due to the complexation is not responsible for the interferences from amino acids whose concentrations are much higher than that for Co(II). This idea is not in conflict with the fact that the increased Co(II) concentration ($\sim 10^{-2}$ M) does not reduce the interference at all. On the other hand, no benzaldehyde was produced with the 2,5-dihydroxyphenylacetic acid interference. The less interferences from Ca(II), Mg(II), and NH_4^+ (NH_3) cannot readily be interpreted, but the coordination to PP^- might relate to no or less inhibition of the PP^{2-} formation.

Applicability to Urine Sample. The direct injection of normal urine freshly taken to the system provided a signal

Table IV. Effect of Concomitant Species on the CL Signal for PPA[a]

species[c]	concn, M	relative CL signal[b] [PPA] = 1×10^{-6} M	relative CL signal[b] [PPA] = 1×10^{-5} M
histidine	1×10^{-6}	120	
albumin	3×10^{-5} [d]	110	
creatinine	1.5[d]	110	
urea	2.5×10^{-1}	97	
3-indoleacetic acid	1×10^{-6}	93	
4-hydroxyphenylpyruvic acid	1×10^{-6}	89	
4-hydroxy-3-methoxyphenyl-pyruvic acid	1×10^{-6}	83	
hippuric acid	1×10^{-4}	82	
4-hydroxyphenylacetic acid	1×10^{-5}	78	
indican	1.4×10^{-5}	66	
serotonin creatinine sulfate	1×10^{-6}	52	
alanine	1.2×10^{-2}	0	18
	1.2×10^{-3}		78
leucine	8.4×10^{-3}	0	18
	8.4×10^{-4}		43
valine	9.4×10^{-3}	0	14
	9.4×10^{-4}		60
phenylalanine	6.7×10^{-3}	0	16
	6.7×10^{-4}		33
tyrosine	1×10^{-3}	0	9
	1×10^{-4}		25
ascorbic acid	1.4×10^{-4}	0	3
	1.4×10^{-5}		12
2,5-dihydroxyphenylacetic acid	1×10^{-6}	0	92
$MgCl_2$	4.1×10^{-3}	170	
NH_4Cl	2×10^{-2}	110	
$CaCl_2$	2.5×10^{-3}	68	
NaCl	4.3×10^{-1}	0	3
	4.3×10^{-2}		45
Na_2SO_4	2.5×10^{-2}	0	9
	2.5×10^{-3}		81
KCl	5.1×10^{-2}	0	33
	5.1×10^{-3}		108
KH_2PO_4	2×10^{-2}	0	9
	2×10^{-3}		36

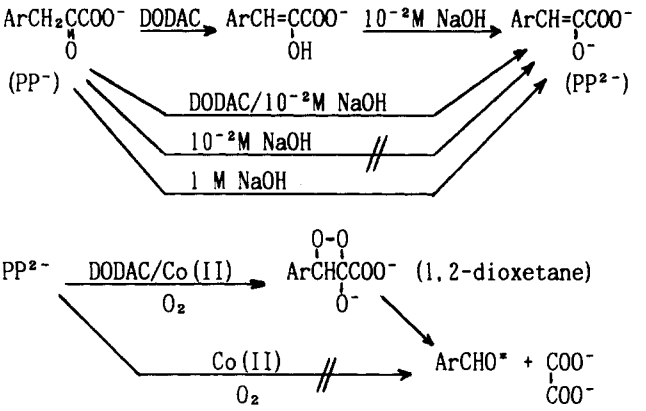
[a] Coditions are as in Figure 1. [b] Normalized with respect to the signal (=100) for 1×10^{-6} (or 1×10^{-5}) M PPA in the absence of concomitant species. [c] Added to 1×10^{-6} (or 1×10^{-5}) M PPA solution. [d] Grams per liter.

about 5 times that for 1×10^{-6} M PPA standard. This native signal does not come from PPA itself in the urine because the concomitant species should completely suppress the signal for PPA present at 10^{-6} M levels in normal urine of adults. The native signal disappeared with the 1000-times diluted urine sample; in this diluted urine sample the signal for 1×10^{-5} M PPA standard reduced to ca. 30%. This implies that some modifications of the flow system may enable PPA (10^{-3}–10^{-2} M) in urine from patients with phenylketonuria to be determined with no special sample pretreatment.

The flow system was modified in such a way that a carrier stream (5×10^{-2} M NaCl, 2.2 mL min^{-1}) into which the urine sample was injected was newly assembled so as to join the DODAC/NaOH stream. The injection of the 100-times diluted urine sample no longer yielded the native signal. Calibration data for PPA standards in the 100-times diluted urine was linear over the range (0–5) $\times 10^{-4}$ M, the limit of determinations ($S/N = 3$) was 1×10^{-5} M, and the relative standard deviation ($n = 10$) for 4×10^{-5} M PPA was 3.4%. The spiked PPA (2×10^{-5} M) was successfully recovered (100, 100, 102%) from the 100-times diluted urine sample. This indicates the direct determination of PPA in phenylketonuria urine only with dilution.

Mechanistic Study of the CL Reaction. PPA gives benzaldehyde by the aerobic oxidation in aqueous NaOH solution, although severe reaction conditions (e.g., 2 M NaOH, 95 °C, 30 min) are needed for the quantitative oxidation (*15*). It is reported that benzaldehyde is produced with a weak light emission by the aerobic oxidation of PPA in acetate buffer containing peroxidase (*24*) or in dimethyl sulfoxide containing potassium *tert*-butoxide (*25*). On the basis of these information, we attempted to investigate the mechanistic details of the present CL reaction and discussed the roles of the ordered DODAC assemblies and the Co(II) catalyst.

Scheme I



In order to serve as an aid in the interpretation of the luminescence reaction mechanism, the present CL system was subjected to spectroscopic studies, i.e., measurements of the CL spectrum and the fluorescence and absorption spectra of phenylpyruvate (PP^-) before and after CL reaction. In water, PP^- has no prominent absorption maximum in the wavelength range 250–400 nm because it exists mostly as the keto form (*26*). This is also the case in the present weakly alkaline medium without DODAC. In contrast, in the DODAC medium there appeared three distinct absorption maxima (275, 286, and 298 nm) whose intensities gradually increased with time. These absorptions are assigned to the conjugation between the aromatic ring and the olefin group, which is produced by the enolization resulting from the binding of PP^- to the hydrophobic surface of cationic bilayer membranes owing to its charge. In the alkaline DODAC medium, these absorptions almost disappered and a strong absorption maximum appeared at 335 nm, which results from the dissociation of the enol proton.

In general, strong base can abstract an acidic α-proton from a carbonyl compound to form an enolate ion. In the absence of DODAC, such base-catalyzed formation of the enolate ion (PP^{2-}) did not occur in the present 10^{-2} M NaOH solution because of weak basicity, although it did in a 1 M NaOH solution. The equilibrium for acid dissociation (the value of pK_a) is often altered by the association with ordered surfactant assemblies whose microenvironment is much different from that in bulk water (*27*). Therefore, it is conceivable that the decrease in the pK_a in the DODAC medium facilitates both the abstraction of the acidic α-proton and the dissociation of the enol proton by base, resulting in the acceleration of the PP^{2-} formation. The addition of Co(II) to the alkaline DODAC medium made the absorption for PP^{2-} (λ_{max} = 335 nm) disappear and then that for benzaldehyde (λ_{max} = 248 nm) appear. Judging from the fact that the addition of Co(II) to the 1 M NaOH solution of PP^- (i.e., the PP^{2-} solution) in the absence of DODAC yields benzaldehyde very slowly, it can be said that the ordered DODAC assemblies also facilitate the aerobic oxidation of the PP^{2-} catalyzed by Co(II). Cobalt(II) will exert the catalytic effect through the salt and/or complex formation with PP^{2-} in the DODAC assemblies, although the details cannot be offered at present.

Thus, the CL reaction mechanism involving the 1,2-dioxetane formation can be written as Scheme I. The light em-

Table V. Effects of DODAC Bilayer Membranous Aggregates and Co(II) Catalyst on the Present CL Reaction[a]

CL system[b]	CL parameter		
	k, s^{-1}	Φ_{CL}[c]	I_{max}[c]
PPA + H_2O + t-BuO^-/DMSO[d]	1	1	1
PPA + Co(II) + t-BuO^-/DMSO	0.02	70	1.4
PPA + Co(II) + OH^-/DODAC	0.03	840	25
PPA + Co(II) + OH^-/OTAC	0.03	210	6.3

[a] Based on batch experiments. CL decay curve: $I_t = \Phi_{CL}k[PPA]_o \exp(-kt)$, $I_{max} = \Phi_{CL}k[PPA]_o$. [b] DMSO was used as the homogeneous medium because of no light emission in aqueous medium. Each of the reagent solutions (10^{-4} M PPA, 10^{-4} M Co(II), 10^{-2} M alkali, 10^{-3} M surfactant) was mixed in that order, as PPA emitted light without Co(II) in the t-BuO^-/DMSO medium. [c] Normalized with respect to the value (=1) for the CL system, PPA + H_2O + t-BuO^-/DMSO. [d] Potassium *tert*-butoxide/dimethyl sulfoxide.

Scheme II

$$PP^- \xrightarrow{\phi_R} [\text{dioxetane}] \xrightarrow{\phi_{EX}} ArCHO^* \xrightarrow{\phi_P} h\nu(I\text{max})$$

itted would seem to be the phosphorescence from the excited triplet benzaldehyde produced by the decomposition of the dioxetane. This is deduced from the fact that the CL spectrum has an emission maximum at about 490 nm, a much longer wavelength than that for the benzaldehyde fluorescence (ca. 370 nm in nonpolar solvent) whose quantum efficiency is very low ($<10^{-6}$ in nonpolar solvent and 0 in polar solvent) (*28*). In order to confirm the above interpretation, the phosphorescence spectrum was measured by using a standard benzaldehyde solution under exactly the same experimental conditions, except for being degassed with nitrogen gas. However, no phosphorescence spectrum was observed. This does not mean that the CL emission is not benzaldehyde phosphorescence. It should be noted that chemiexcitation is different from photoexcitation in the excitation process and that the present chemiexcited benzladehyde is not equivalent to the photoexcited benzaldehyde in the standard solution with respect to the binding to the bilayer membrane. The chemiexcited triplet benzaldehyde produced from the anionic intermediate that is strongly bound to the rigid bilayer membrane will be protected from the deactivation by O_2, an effective triplet quencher, and H_2O molecules, resulting in the occurrence of phosphorescence.

Finally, the roles of the DODAC assemblies and the Co(II) catalyst were quantitatively evaluated from the CL decay curves obtained by batch experiments, provided that the present CL reaction is pseudo-first-order with respect to the concentration of PPA (*29*). The results are shown in Table V, in which the CL parameters such as rate constant k, CL quantum yield Φ_{CL} ($=\phi_R\phi_{EX}\phi_P$, see Scheme II) and maximum CL intensity I_{max} are estimated in various media. It can be seen from the table that both the DODAC assemblies and the Co(II) catalyst contribute to great increase in Φ_{CL}, resulting in the increase in I_{max} in spite of significant decrease in k. The catalytic effect of Co(II), which is exerted on the increase in ϕ_R (the yield of dioxetane), is exhibited more efficiently in the microheterogeneous media (DODAC and OTAC) rather than in the homogeneous medium (DMSO). On the other hand, the surfactant media appear to induce the increase in all of ϕ_R, ϕ_{EX} (the yield of ArCHO*), and ϕ_P (the phosphorescence yield of ArCHO), the extent of which is greater in DODAC than in OTAC. Judging from the fact that both surfactant media are comparable in the formations for PP^{2-} and benzaldehyde, the difference in Φ_{CL} will be ascribable to that in ϕ_P. The DODAC bilayer membrane is kinetically more stable and hence offers a more protective environment for excited triplet benzaldehyde than the OTAC micelle.

In conclusion, it has proved from the mechanistic study of the PPA CL that ordered surfactant assemblies play dramatic roles both in the production of key intermediates and in the protection of the emitter, resulting in the great increase in CL quantum yield. Such a chemical amplification of ultraweak light offers a way to the analytical use of low-level CL from many oxidative reactions with no use of special equipments and techniques. Thus it may safely be said that the greatest advantage of using ordered molecular assemblies lies in the capability of developing new CL systems rather than improving CL characteristics of established CL systems. On the other hand, the present work has also shown that the enhanced CL is subject to severe interferences from concomitant substances which cause the structural deterioration of ordered surfactant assemblies. The interferences could be reduced by the reinforcement of surfactant molecular assemblies, e.g., immobilization or polymerization of surfactant molecules. Such devices will also offer reaction media that are applicable to CL sensors.

Registry No. PPA, 156-06-9; DODAC, 107-64-2; DDDAB, 3282-73-3; HTAC, 112-02-7; HEDAB, 124-03-8; Co, 7440-48-4; benzaldehyde, 100-52-7.

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