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Effect of Cationic Surfactants on Enhancement of Firefly Bioluminescence in the Presence of Liposomes

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Firefly bioluminescence (BL) was greatly affected by cationic surfactants coexisting with liposomes containing phosphatidylcholine and cholesterol. In this study, the effects of the type and concentration of cationic surfactants on BL were studied in the presence of the liposomes. Three types of cationic surfactant: benzalkonium chloride (BAC), *n*-dodecyltrimethylammonium bromide (DTAB), and benzethonium chloride (BZC), were used. As a common effect in these surfactants, BL intensity was increased and then drastically decreased with increasing surfactant concentration. This can be explained by the formation of cationic liposomes as BL enhancers at low concentration of the surfactant, and by the transformation into cationic (mixed) micelles as inhibitors at high concentration. The maximal BL intensity and the concentration for the maximal BL were dependent on the type of the surfactants. To explain the differences in these parameters in the enhanced BL, we determined the distribution coefficient, *K*, of the surfactants to the liposomal membrane. The result indicated that the surfactant with higher *K* value gives the maximal BL intensity at lower concentration.

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Introduction

The firefly bioluminescence (BL) is widely applied for sensitive determination of adenosine-5′-triphosphate (ATP).[1] Firefly BL is emitted in the reaction of oxidative decarboxylation of D-luciferin catalyzed by luciferase in the presence of ATP, Mg^{2+}, and molecular oxygen.[2]

$$\text{Luciferin} + \text{ATP} + Mg^{2+} + O_2 - (\text{luciferase}) \longrightarrow \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

Since the BL emission (BL intensity) is in proportion to the ATP concentration, one can determine ATP concentration by measuring BL intensity. Additionally, the BL method can be applied to the estimation of cell number,[3,4] because all living cells contain ATP as an energy source in the same concentration level. In the determination of ATP in cells by the BL method, ATP should be extracted to bulk phase from the inside of the cells by using lysis agents, such as cationic surfactants. *n*-Dodecyltrimethylammonium bromide (DTAB) is one of the effective extractants for this purpose.[5] However, cationic surfactants often show inhibitory effects for luciferase. Thus, before the BL measurement, the cell extract containing ATP and cationic surfactant must be diluted to the concentration level without inhibitory effect of the surfactant, which sacrifices high sensitivity for ATP in the BL method.

Recently, improved BL methods in the presence of cationic surfactants draw the researchers' attention in ATP determination. The inhibitory effect of DTAB for luciferase was eliminated by employing α-cyclodextrin to incorporate DTAB into its pore.[6] On the other hand, a recombinant luciferase with resistance to benzalkonium chloride (BAC) was developed, and applied to the detection of ATP by BL method.[7] However, these approaches are focused on reduction of inhibitory effects of cationic surfactants. No enhanced BL method for highly sensitive determination of ATP even in the presence of cationic surfactants has been reported.

Previously, we have reported enhanced firefly BL in the presence of cationic liposomes containing phosphatidylcholine (PC), cholesterol (Chol), and stearyltrimethylammonium chloride (STAC).[8] The detection limit of ATP in the presence of the cationic liposomes was improved 10-fold compared to that in water alone. Additionally, we have found that zwitterionic liposomes containing PC and Chol incorporate BAC into the liposomal membrane to transform into cationic liposomes.[9] The rate of incorporation into liposomes of BAC was faster than that of inhibition of luciferase by BAC. Thus, when BL reagent containing luciferase and luciferin was added with zwitterionic liposomes to a BAC-containing ATP sample, no inhibitory effect of BAC on luciferase was observed due to rapid incorporation of BAC into the liposomes, but instead, enhanced BL emission by cationic liposomes appeared. We have found the detection limit of ATP in *Escherichia coli* was improved 10-fold even in the presence of BAC by this method.[9]

In the BL-based cell counting method using cationic surfactants as cell lysis agents, liposomes show a dual function for high sensitivity: removing BL-inhibitory cationic surfactants and providing enhanced BL media. For further improvement of the method, it is important to clarify the details of this enhanced BL system. In this study, the effect of molecular structure of cationic surfactant on enhanced BL was investigated by using three types of cationic surfactant: benzalkonium chloride (BAC),

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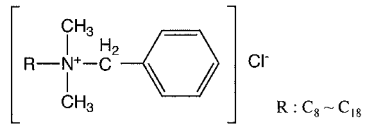
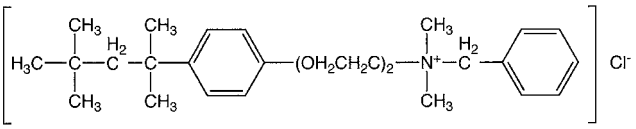
Benzalkonium chloride (BAC)



Benzethonium chloride (BZC)



Dodecyltrimethylammonium bromide (DTAB)

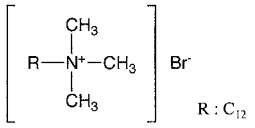


Fig. 1 Chemical structures of cationic surfactants.

n-dodecyltrimethylammonium bromide (DTAB), and benzethonium chloride (BZC), which are frequently used as ATP extractants. The differences in enhancement effect among the surfactants were explained in terms of the distribution ratios of them to the liposomes.

Materials and Methods

Reagents

Firefly luciferase (from *Photinus pyralis*), D-luciferin, and adenosine-5′-triphosphate (ATP) disodium salt were obtained from Sigma. Bovine serum albumin (BSA), dithiothreitol (DTT), and magnesium acetate tetrahydrate were purchased from Wako Pure Chemical (Osaka, Japan). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acid disodium salt (EDTA) were from Dojindo Laboratories (Kumamoto, Japan).

Benzalkonium chloride (BAC) from Kanto Chemical (Tokyo, Japan), *n*-dodecyltrimethylammonium bromide (DTAB) from Tokyo Chemical Industry (Tokyo, Japan), and benzethonium chloride (BZC) from Wako Pure Chemicals were used. The structures of these cationic surfactants are shown in Fig. 1. Benzalkonium chloride is a mixture including a range of alkyl chain lengths from C8 to C18 with an average molecular weight of 368.

Egg yolk phosphatidylcholine (PC) and cholesterol (Chol) were obtained from Wako Pure Chemicals. Potassium phosphate was from Kanto Chemical.

A luciferase-luciferin reagent solution (L-L solution) containing 20 mg/L luciferase and 0.6 mM luciferin was prepared from a 25 mM HEPES buffer solution (pH 7.75) containing 2.0 mM DTT, 2.0 mM EDTA, 24 mM magnesium acetate, and 60 mg/L BSA. The L-L solution was divided into 1.0-mL aliquots and stored at –20°C until use. The concentrations of all the constituents in L-L solution were optimized previously for the determination of ATP.[9] A 1.0 mM stock solution of ATP was prepared every month by dissolving the compound with 25 mM HEPES buffer (pH 7.75) containing 2.0 mM EDTA; this stock solution was stored in a refrigerator. Working solutions of ATP were prepared by serial dilution with the buffer and surfactant solutions.

All solutions were prepared with ultrapure water from a Millipore Direct-Q3 water purification system. All chemicals were reagent grade and were used as received. The PC and surfactant concentrations described and shown in the figures are the final solution concentrations, while the others are the initial concentrations.

Preparation of liposomes

One milliliter of chloroform solution containing 10 μmol PC and 10 μmol cholesterol was placed in a round-bottom flask. Chloroform was removed by rotary evaporation at 30°C under reduced pressure with a nitrogen gas stream, making a lipid film on the wall of the flask. After at least 30 min *in vacuo*, 1 mL of 25 mM HEPES buffer solution (pH 7.75) was added into the flask; this mixture was then vortexed for a few minutes at 25°C to hydrate the film. In this step, multilamellar vesicles (MLVs) were obtained. Next, the MLVs were extruded 20 times through a single polycarbonate filter with a pore size of 100 nm. The filter was obtained from Avestin Inc. and was mounted in LiposoFast-Basic (Avestin) fitted with two 0.50-mL Hamilton syringes. Unilamellar vesicles thus obtained are indicated as VET_{100} (Vesicles by Extrusion Technique through a filter of 100 nm pore size) or simply as liposomes in this paper.

The size distribution of the liposomes was measured immediately after preparation of VET_{100} by a dynamic light scattering technique using a particle size analyzer FPAR-1000 (Otsuka Electronics, Osaka, Japan). In the mixture of liposomes and surfactant, a surfactant solution was added to the liposomal suspension to be an appropriate surfactant concentration followed by the measurement of size distribution.

Bioluminescence measurement

The BL measurement in the presence of liposomes and surfactant was carried out as follows: a 100-μL L-L solution and a 50-μL VET_{100} suspension were added into a plastic cuvette. The cuvette was placed in a luminometer (BLD-100HU, Tohoku Electronic Industry, Japan), and then 250 μL of 1.0×10^{-10} M ATP solution containing surfactant was injected into the cuvette from the outside of the luminometer. The time course of light emission (BL response) was recorded as photon counts per second (cps) by a computer. The maximal light emission in the BL response was defined as BL intensity, while the integrated photon counts during whole BL reaction were defined as total light emission. All BL measurements were made at 25°C. All glassware used was soaked overnight in 10% sodium hypochlorite solution and then rinsed in ultrapure water.

Turbidity measurement

The turbidity of the liposomal suspension and of the mixture of liposomes and surfactant was measured as optical density at 660 nm. Each measurement was performed at least three times by using a double beam spectrophotometer U-2000 (Hitachi, Japan) and a 1-cm quartz cuvette.

Results and Discussion

Effect of BAC on BL with and without liposomes

To reveal the effect of BAC on luciferase activity, we measured the BL response at various BAC concentrations. A 100-μL L-L solution and a 50-μL BAC solution were mixed and left for 2 min in a cuvette. Then, the BL reaction was initiated by the addition of 250 μL of 1.0×10^{-10} M ATP solution. The BL response curves thus obtained are shown in Fig. 2A. In the presence of 2.7×10^{-6} M of BAC, the same response curve was

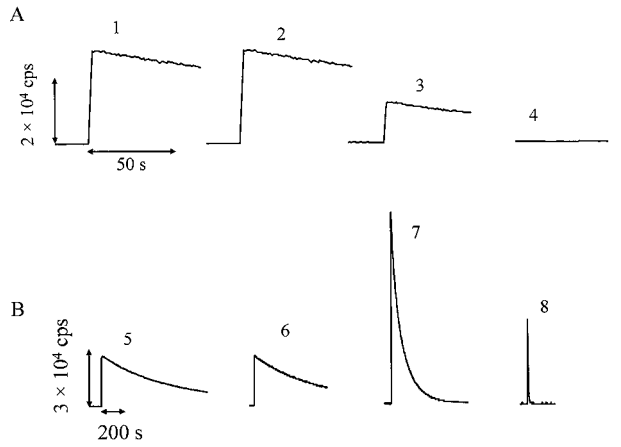


Fig. 2 Typical BL response curves in the presence of BAC (A) and of both BAC and VET_{100} (B). In A, BAC concentrations are 0 (curve 1), 2.7×10^{-6} (2), 2.7×10^{-5} (3), and 2.7×10^{-4} M (4). In B, BAC are 0 (curve 5), 2.7×10^{-6} (6), 2.7×10^{-4} (7), and 2.7×10^{-3} M (8), all containing VET_{100} at 1.25×10^{-3} M PC and 1.25×10^{-3} M Chol.

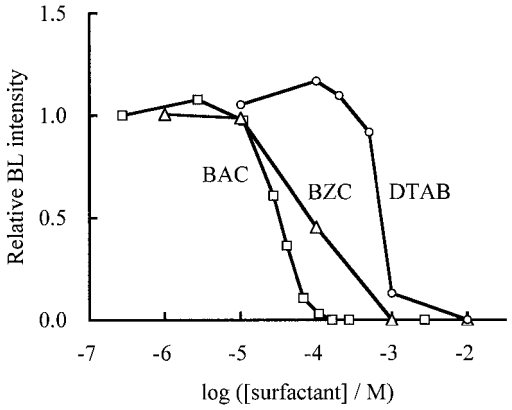


Fig. 3 Effect of cationic surfactant concentration on relative BL intensity.

obtained as that without BAC (curves 1 and 2). At 2.7×10^{-5} M BAC, both BL intensity and total light emission were decreased due to inhibition of luciferase by BAC (curve 3). The BL was completely diminished at 2.7×10^{-4} M BAC (curve 4). As with BAC, the effects of BZC and DTAB on the BL response were also studied. The relationship between relative BL intensity and cationic surfactant concentration is shown in Fig. 3. The relative BL intensity is the ratio of BL intensity in the presence of surfactants to that without them. In Fig. 3, BZC and DTAB showed the same effect on BL as BAC, that is, the BL intensity was decreased with increasing of surfactant concentration. In the case of BZC, the BL decreased from 1.0×10^{-5} M and disappeared at 1.0×10^{-3} M. On the other hand, in DTAB it decreased from 1.0×10^{-4} M and disappeared at 1.0×10^{-2} M.

Next, the effect of the surfactant on BL response in the presence of liposomes was examined. First, a 100-μL L-L solution and 50 μL VET_{100} suspension were added into a cuvette; then, 250 μL of 1.0×10^{-10} M ATP solution containing surfactant was injected into the cuvette for BL initiation. Figure 2B shows the BL response curves obtained at various concentrations of BAC in the presence of VET_{100}. At 2.7×10^{-6} M BAC, the BL response remained unchanged from that without

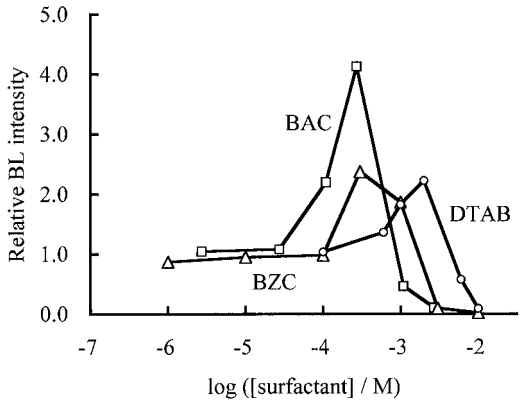


Fig. 4 Effect of cationic surfactant concentration on relative BL intensity in the presence of VET_{100}. Both PC and Chol concentrations are 1.25×10^{-3} M.

BAC (VET_{100} alone) (curves 5 and 6), as seen in Fig. 2A, curves 1 and 2 without VET_{100}. On the other hand, increased BL intensity and total emission were observed at 2.7×10^{-4} M BAC (curve 7) compared to those in VET_{100} alone. However, at 2.7×10^{-3} M BAC, the total emission was drastically decreased (curve 8). Figure 4 shows the effect of the concentrations of BAC, BZC, and DTAB on relative BL intensity in the presence of VET_{100}. As in the case with BAC, BZC and DTAB with VET_{100} also showed enhancement and reduction of BL intensity. In BZC and DTAB, enhanced BL was observed even at concentrations at which BAC inhibited luciferase activity.

The results indicate that cationic surfactants having inhibitory effect on luciferase can function as a BL enhancer, working together with liposomes. Additionally, the degree of enhancement of BL and the effective concentration of surfactant are dependent on the type of surfactant.

Turbidity and dynamic light scattering in the mixture of liposomes and surfactant

Previously, we have found that cationic liposomes are essential in the enhancement of firefly BL. That is, since BL reactants such as luciferase, luciferin, and ATP are all anionic in the reaction pH, electrostatic interactions should take place between the reactants and cationic liposomes. We have considered that localization of the reactants on the liposomal surface leads to acceleration of BL reaction, resulting in enhancement of BL intensity.[10] Additionally, we have found an increase of the membrane surface potential of liposomes in the presence of BAC, monitored by using a fluorescence probe, 8-anilino-1-naphthalenesulfonate.[9] This implies the distribution of BAC into the bilayer of liposomes and the formation of cationic liposomes. To clarify the formation of cationic liposomes directly, we measured the turbidity and the dynamic light scattering of the mixture of VET_{100} and the surfactant at various concentrations.

The turbidity of the mixture as a function of surfactant concentrations is shown in Fig. 5. In Fig. 5, the relative BL intensity is also indicated for comparison. In each surfactant, the turbidity of the mixture was increased and then decreased with an increase in the concentration. This result can be explained as follows: an increase in the turbidity should be due to the VET_{100} growth on incorporating cationic surfactants into the lipid bilayer to form cationic liposomes. Meanwhile, beyond a critical point of surfactant/lipid molar ratio, the liposomes would collapse and transform into the mixed micelles of lipid

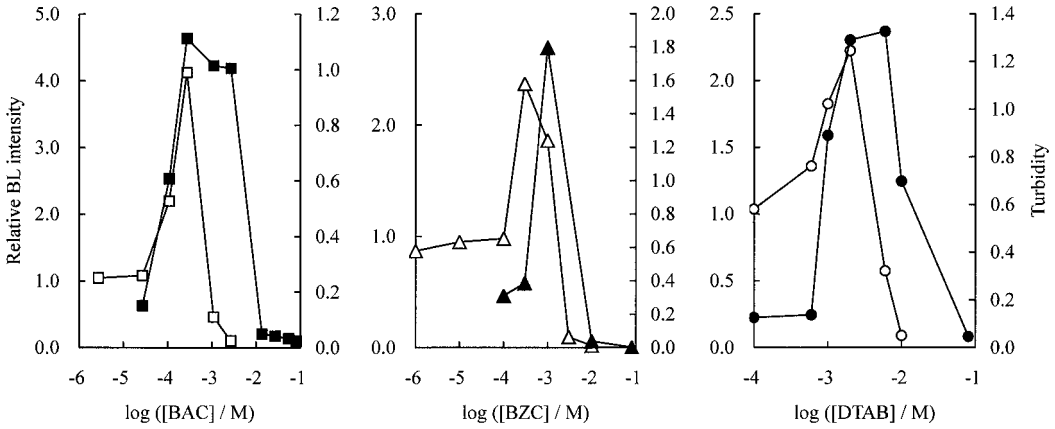


Fig. 5 Effect of cationic surfactant concentration on relative BL intensity (open) and turbidity (filled). Both PC and Chol concentrations are 1.25×10^{-3} M.

and surfactant, which will result in a decrease in the turbidity.[11] Further increase in surfactant concentration would lead to the appearance of surfactant micelles.

The relationship between the relative BL intensity and the turbidity suggests that, with the increase in each surfactant concentration, cationic liposomes that appeared in the mixture of VET_{100} and the surfactant have enhanced the BL emission. The decrease in the BL intensity is ascribed to an increase in the free surfactant (or the micelles) as seen in the case of using surfactant alone.

In BAC and DTAB, the maximal turbidity was obtained in a wide concentration range, while the maximal BL intensity was obtained in a narrow range. It is considered from this difference between turbidity and BL intensity that the inhibition of luciferase by free molecular BAC and DTAB has occurred before the collapse of cationic liposomes by the surfactants. In contrast to BAC and DTAB, free BZC can inhibit the luciferase before saturation of the liposomes.

Liposomal size and its distribution were measured by a dynamic light scattering technique. The average size of VET_{100} without surfactant was about 160 nm, with a distribution range from 100 to 200 nm. In the presence of the surfactant at the concentration giving the maximal turbidity, the size increased up to about 200 nm average with a wide distribution from 100 to 1000 nm in all surfactants tested. This also indicates growth of the liposomes with incorporating cationic surfactants. However, in the case of BAC at 2.7×10^{-3} M, the size distribution separated into two parts with averages of about 160 and 2300 nm. The larger part of them may not be in a spherical liposomal structure, but in a lamellar structure. At this concentration of BAC, the turbidity was high but BL was diminished in Fig. 5. Such structure could affect the BL reaction, but this remains to be seen. Further increase in the surfactant concentration led to the decrease in the size with a single narrow distribution. These results are in good agreement with those of the turbidity measurement.

Effect of distribution coefficient of surfactants on BL intensity

As shown in Fig. 4, the maximal BL intensity and the surfactant concentration for the maximal BL are dependent on the type of surfactant. We have considered the reason starting from the distribution coefficient of the surfactant into the lipid bilayer.

The mass balance of the surfactant can be expressed as

$$[\text{surfactant}]_T = [\text{surfactant}]_w + [\text{surfactant}]_b, \quad (1)$$

where subscripts b, w, and T indicate lipid bilayer phase, bulk aqueous phase, and whole system, respectively. In the lipid-surfactant mixture system, Lichtenberg[12] defined partition coefficient, K, which describes the distribution of the surfactant between the lipid bilayers and the aqueous phase, as

$$K = [\text{surfactant}]_b/([\text{surfactant}]_w[\text{PC}]_T). \quad (2)$$

Additionally, the effective ratio was defined by[12]

$$Re = [\text{surfactant}]_b/[\text{PC}]_T. \quad (3)$$

Therefore, by using Eqs. (1) – (3), total surfactant concentration is given by

$$[\text{surfactant}]_T = Re/K + Re[\text{PC}]_T. \quad (4)$$

When the lipid bilayer is saturated with the surfactant, Eq. (4) can be rewritten by using superscript sat as

$$[\text{surfactant}]_T^{sat} = Re^{sat}/K + Re^{sat}[\text{PC}]_T. \quad (5)$$

Equation (5) demonstrates that the dependence of the saturating surfactant concentration on the lipid concentration intersects with the lipid axis at $-1/K$.

Experimentally, $[\text{surfactant}]_T^{sat}$ was determined as the concentration that gives the maximal turbidity of the mixture at each lipid concentration. As seen in Fig. 6, a good linear relationship was observed between $[\text{BAC}]_T^{sat}$ and $[\text{PC}]_T$. In BZC and DTAB, the same results were obtained. From these dependences, K was determined for each surfactant. The K values were 1.5, 0.33, and 0.19 mM^{-1} for BAC, BZC, and DTAB, respectively. The distribution coefficient of BAC was greater that those of BZC and DTAB. The difference in the distribution coefficient among the surfactants should be due to the differences in their hydrophobicity and molecular structure. For example, BAC is a molecular mixture with an average alkyl chain length of C16, while DTAB has a C12 chain. In addition, there is a phenyl group in BAC, but not in DTAB. These indicate BAC could be more hydrophobic than DTAB. On the other hand, BZC has a branched alkyl chain, which would be a disadvantage for entrance into the packed lipid bilayers compared to a linear alkyl chain.

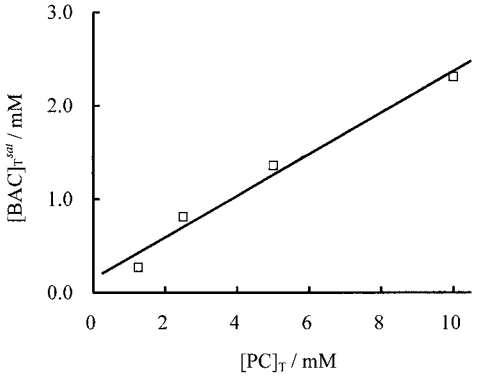


Fig. 6 Relationship between PC and saturating BAC concentrations.

As mentioned above, with increasing in surfactant concentration, the VET_{100} grows while incorporating the surfactant into the lipid bilayer until saturation. The cationic liposomes thus formed enhance the BL emission. After saturation, the liposomes collapse and inhibitory free surfactants and/or micelles increase, resulting in an decrease in the BL intensity. In terms of the distribution coefficient, one can presume that the greater the coefficient is, the lower is the surfactant concentration at which the saturation of the bilayer occurs. Thus, in BAC with the high *K* value, the concentration for the maximal BL intensity was lower than that in BZC and DTAB. The difference in the maximal BL intensity among the surfactants can be also explained by the difference in the distribution coefficient. As seen in Fig. 4, the greater the *K* value, the greater the maximal BL intensity. Before saturation, surfactants in the lipid bilayer are essential for the BL enhancement as cationic components, while those in the bulk aqueous phase act as inhibitors. That is, the large distribution coefficient means the large proportion of the surfactant that contributes to the BL enhancement. The maximal intensity in BAC is therefore greater than those in BZC and DTAB.

The differences of the inhibitory and enhancement effects of each surfactant could be the reason for the variation of the maximal BL. For example, BAC should have a stronger inhibitory effect on the luciferase compared to other surfactants because it reduced BL intensity at lower concentration range as seen in Fig. 3. However, it acts as the most effective enhancer in the presence of VET_{100}. From these results, we conclude that the distribution coefficient into the bilayer is an essential factor for determining the maximal BL intensity and the concentration at which it occurs.

Conclusion

By using three types of cationic surfactant: BAC, BZC, and DTAB, with different molecular structures, we studied the effect of the surfactant concentration on the enhancement of BL intensity in the presence of VET_{100}. The cationic liposomes consist of VET_{100} and the cationic surfactant were found to be effective for the BL enhancement. The maximal BL intensity and the surfactant concentration for it appeared to be closely related with the distribution coefficient of the surfactant into the lipid bilayer.

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