ANALYTICAL SCIENCES DECEMBER 1998, VOL. 14

Enhancement of Firefly Bioluminescence Using Liposomes Containing Cationic Components

Tamio KAMIDATE†, Taigo FUJITA, Hirofumi TANI and Hiroto WATANABE

Graduate School of Engineering, Hokkaido University, Sapporo 060–8628, Japan

The effect of cationic components in liposome on the bioluminescence (BL) intensity from the firefly luciferin–luciferase reaction with ATP was investigated by use of vesicles formed by the extrusion technique (VET). Stearyltrimethylammonium chloride (STAC), stearylamine (SA) and tetracaine (TC) were used as cationic components. The VET containing STAC and SA enhanced the maximum BL intensity. In contrast, a lowering of the maximum BL intensity was observed in the presence of the VET containing TC. The sensitivity for ATP in the presence of the VET containing STAC was improved by a factor of 2 and 10 times compared to that in the presence of the VET containing SA and that in water alone, respectively. The differences in the BL enhancement between cationic components could be explained in terms of different membrane surface potentials of the VET containing these cationc components.

Keywords Firefly bioluminescence, adenosine-5′-triphosphate, liposome, stearyltrimethylammonium chloride, stearylamine, membrane surface potential

An assay of adenosine 5′-triphosphate (ATP) is important to estimate the total amount of living cellar material (biomass) as well as the activity or metabolic state of the cells, since all types of living cells use ATP for storage of energy produced *via* metabolic processes. The firefly bioluminescence (BL) method has been widely used for the determination of ATP. In the firefly luciferin–luciferase reaction, luciferase catalyses the oxidative decarboxylation of luciferin in the presence of ATP and Mg^{2+}, with concomitant BL:

$$\text{luciferin}+\text{ATP}+Mg^{2+}+O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin}+\text{AMP}+PP_i+CO_2+\text{light}$$

Firefly BL provides a rapid and very sensitive method for the determination of ATP.[1] Recently, the enhancement of light emission has been noted in view of improving the sensitivity of the firefly BL assay for ATP.

The presence of apporopriate aqueous micellar media can increase the maximum BL intensity. Aqueous nonionic and zwitterionic micellar media increased the BL peak height intensity as well as prolonged the dulation of the BL.[2] In addition, cationic detergents such as benzalkonium chloride increased the firefly luciferin–luciferase reaction rate several-fold, with a sharply defined optimum concentration of detergent.[3] However, the enhancement of light emission in the presence of surfactant micelles was observed only when higher ATP concentrations were used.

Previously, we found that a cationic polysaccharide such as diethylaminoethyl dextran was more effective than other types of charged polysaccharides in enhancing the BL intensity.[4] The detection limit for ATP was improved by a factor of three times compared to that in water alone. In addition, the effect of different charge-types of liposome on the BL intensity was investigated.[5] Cationic liposome containing stearylamine (SA) was superior to anionic and zwitterionic liposomes as an enhancer in the firefly luciferin–luciferase BL reaction. The sensitivity for ATP in the presence of cationc liposome containing SA was improved by a factor of five times compared to that in water alone.

The aim of this work was to prepare newly cationic liposomes using stearyltrimethylammonium chloride and tetracaine as a cationic component for improving the sensitivity of firefly BL assay for ATP and to clarify the differences in the BL enhancement between cationic components by measuring membrane surface potential of the cationic liposomes.

Experimental

Materials

Luciferase from firefly (Photinus pyralis: product number L9009), D-luciferin, adenosine-5′-triphosphate disodium salt (ATP), 4-[butylamino]benzoic acid 2-[dimethyl amino]ethyl ester (tetracaine hydrochloride: TC) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Cholesterol (Chol), egg yolk phosphatidylcholine (PC), stearylamine (SA), magne-

† To whom correspondence should be addressed.

sium acetate, dithiothreitol (DDT), EDTA and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from Wako Pure Chemical Industries. Stearyltrimethylammonium chloride (STAC) and 8-anilino-1-naphthalenesulfonic acid magnesium salt (ANS) were obtained from Nacalai Tesque. The concentration of PC was calculated by use of a molecular weight of 765.[6]

A 1.0 mM solution of ATP was prepared daily by dissolving the compound with 25 mM HEPES buffer (pH 7.8) containing 2 mM EDTA. Working solutions of ATP were prepared by serial dilution with the HEPES buffer. A luciferase solution was prepared by dissolving 1 mg of luciferase in 50 ml of 25 mM HEPES buffer containing 0.6 mM luciferin, 24 mM magnesium acetate, 3 mg BSA, 2 mM DTT and 2 mM EDTA, pH 7.8. The luciferase solution was stored in portions of 1.0 ml at −20˚C. All solutions were prepared with ultrapure deionized water from a Millipore Mill-Q water purification system.

Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared as follows: a mixture (10 μmol PC, 10 μmol Chol, 0 – 15 μmol cationic component) in chloroform was added to a 100 ml round-bottom flask. Chemical structures of cationic components are shown in Fig. 1.

Chloroform was removed by rotary evaporation at 30˚C under reduced pressure and by a stream of nitrogen gas forming a lipid film on the wall of the flask. After at least 2 h *in vacuo*, 1 ml of the buffer solution was added in the flask and all the contents were extensively mixed on a Vortex stirrer for 15 min at 25˚C. The concentrations of PC, Chol and cationic component in the MLVs suspensions were 10, 10 and 0 – 15 mM, respectively.

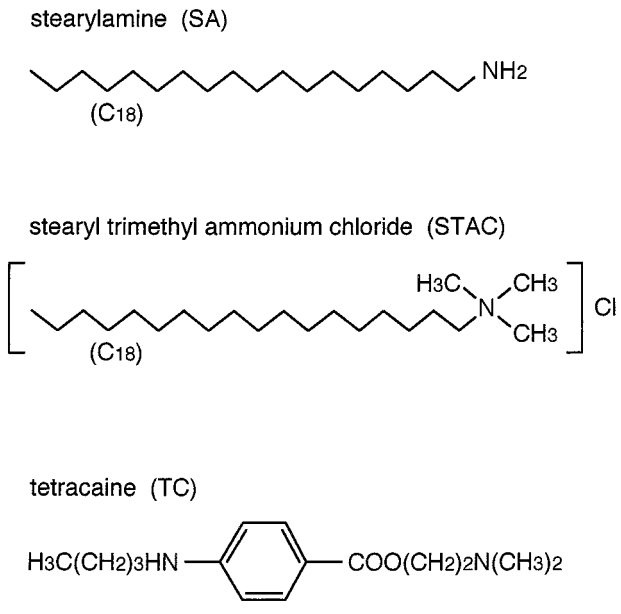

Fig. 1 Chemical structure of cationic components.

The MLVs were extruded through a polycarbonate filter with a pore size of 1000 nm. The polycarbonate filter was obtained from Avestin Inc. The filter was mounted in LiposoFast™-Basic (Avestin Inc.) fitted with two 0.50 ml Hamilton syringes. We subjected samples to 30 passes through a single filter. A numerical subscript indicated the pore size of the polycarbonate filter employed. Thus, a VET_{1000} indicated liposomes extruded through polycarbonate filter with 1000 nm pore size.

The size of the VET_{1000} was estimated by a laser scattering particle size distribution analyzer (LA-910, Horiba). The distribution of particle sizes of the VET_{1000} was in the range from 300 to 670 nm, and the mean diameter was 440 nm.

Bioluminescence detection

The BL experimental procedure consisted in pipetting a 100 μl portion of the luciferase solution and a 50 μl portion of the VET_{1000} suspensions into a plastic cuvette (15 mm i.d.×15 mm: internal volume 800 μl) in a luminometer (TD-3A, Tohoku Denshi Sangyo). Next, a 250 μl portion of ATP solution was injected into the cuvette. The BL emission was detected by a photomultiplier. The resultant photocurrent was converted to a voltage, whose value was displayed on a chart recorder. All BL measurements were made at 25˚C. All glassware used was soaked for overnight in 10% sodium hypochlorite solution and then rinsed in ultrapure water.

Measurement of membrane surface potential of the VET_{1000}

Changes in the membrane surface potential of the VET_{1000} were monitored by measuring the fluorescence intensity of ANS.[7] The measurements were carried out in a fluorescence spectrophotometer (F-2000, Hitachi) with an excitation wavelength of 385 nm and an emission wavelength of 480 nm. To 400 μl of the VET_{1000} suspensions, a 400 μl portion of 0.1 mM ANS solution was added; then the fluorescence intensity was measured.

Fluorescence intensity, F, is defined as

$$F=F_{l-a}-(F_l+F_a) \quad (1)$$

where F_{l-a}, F_l and F_a are fluorescence intensity in the mixture of VET_{1000} and ANS suspensions, the VET_{1000} suspensions alone and ANS alone, respectively. The relative membrane surface potential (Ψ_{rel}) was calculated by the following equation:

$$\Psi_{rel}=F_s/F_b \quad (2)$$

where F_s and F_b stand for fluorescence intensity calculated by Eq. (1) using the VET_{1000} containing PC, Chol and cationic component, and the VET_{1000} containing PC and Chol.

Results and Discussion

BL intensity–time profiles in the presence of the cationic VET_{1000}

In order to investigate the effect of cationic components in the VET_{1000} on the BL intensity, the BL response curves were measured in water alone and in the presence of the cationic VET_{1000} suspensions. In measurements in water alone, the buffer solution was used in place of the VET_{1000} suspensions.

Typical BL response curves are shown in Fig. 2. A 100 pM solution of ATP was added to the mixture at the arrow. In each reaction media, the light emission appeared rapidly after the start of the reaction and reached maximum intensity in 30 s after which the intensity of the light emission decayed rapidly. A maximum light emission is referred to as a BL intensity. No BL emission was observed in the blank solution, which contained no ATP. The presence of the VET_{1000} containing STAC and SA enhanced remarkably the BL intensity when compared with that in water alone. In constract, the BL intensity in the presence of the VET_{1000} containing TC was lower than that in water alone. The relative BL intensity is defined as the ratio of the BL intensity in the presence of the VET_{1000} containing cationic components to that in water alone. As can be seen in Fig. 2, the relative BL intensities of the VET_{1000} containing STAC, SA and TC were 5.0, 3.8 and 0.5, respectively. STAC was most effective with respect to the ability to amplify BL.

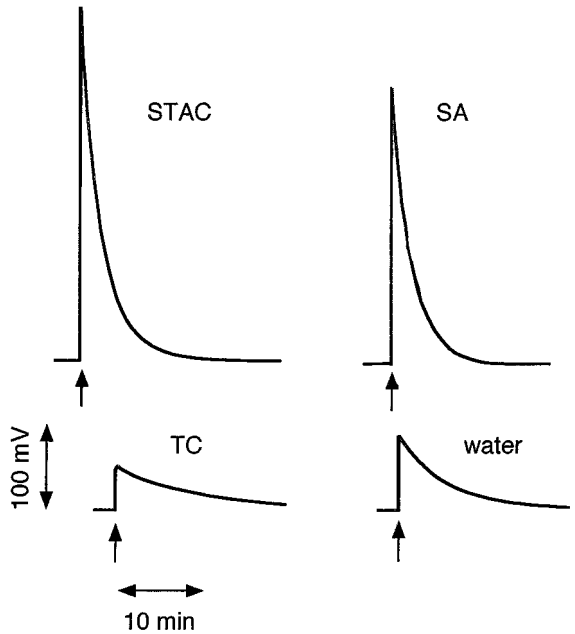

Fig. 2 Typical BL response curves in the presence of cationic VET_{1000}. Concentrations of the components in the cationic VET_{1000} suspensions: [PC]=10 mM, [Chol]=10 mM, [cationic component]=10 mM; pH 7.8.

Effect of cationic component concentration and pH on the BL intensity

The dependence of the BL intensity upon the concentration of cationic components was examined in the range of 2 – 15 mM in the VET_{1000} suspensions. The concentrations of PC (10 mM) and Chol (10 mM) were constant in the VET_{1000} suspensions. Figure 3 shows the relative BL intensity–cationic component concentration profiles. When STAC was used, the relative BL intensity was maximal at 8 mM. In the case of SA, the relative BL intensity increased to a maximal value at 10 mM SA. In contrast, the relative BL intensity decreased gradually with an increase in the concentration of TC. The optimum concentrations of STAC and SA were chosen to be 8 and 10 mM, respectively.

Next, the effect of pH on the BL intensity was examined in the pH range from 5.0 to 11.0. Figure 4 shows the influence of pH on the BL intensity in the presence of the cationic VET_{1000} suspensions and water alone. In Fig. 4, the relative BL intensity was defined as the ratio of the BL intensity in the presence of the cationic VET_{1000} suspensions to that at pH 7.8 in water alone. The relative BL intensity was constant in the pH range from 7.8 to 10.5 in water alone. On the other hand, the relative BL intensity exhibited a maximum at pH 7.8 in the presence of the VET_{1000} containing STAC and SA. The optimum pH was thus chosen to be 7.8 for STAC and SA.

Calibration curves for ATP in the presence of the cationic VET_{1000}

The optimum concentrations of luciferin, Mg^{2+}, EDTA, BSA, and DTT in the luciferase solution were determined previously.[5] The calibration curves for ATP were prepared under the optimum conditions thus established. Logarithmic calibration curves in the presence of the VET_{1000} containing STAC and SA were lin-

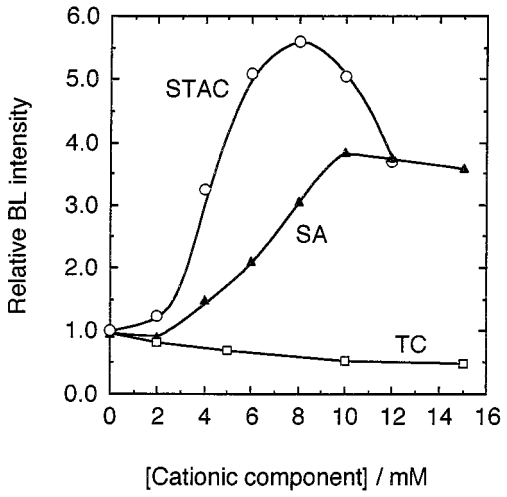

Fig. 3 Effect of cationic component concentrations in the VET_{1000} suspensions on relative BL intensity. Concentrations of PC and Chol in the cationic VET_{1000} suspensions: [PC]=10 mM, [Chol]=10 mM; pH 7.8.

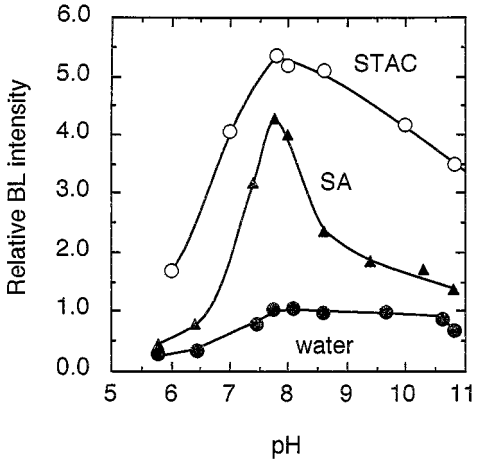

Fig. 4 Effect of pH in the cationic VET_{1000} suspensions on relative BL intensity. Concentrations of the components in the cationic VET_{1000} suspensions. STAC: [PC]=10 mM, [Chol] =10 mM, [STAC]=8 mM; SA: [PC]=10 mM, [Chol]=10 mM, [SA]=10 mM.

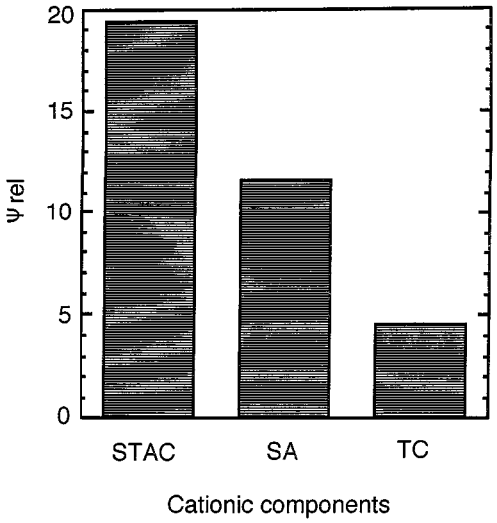

Fig. 5 Relative membrane surface potentials of cationic VET_{1000}. Concentrations of components in cationic VET_{1000} suspensions. STAC: [PC]=10 mM, [Chol]=10 mM, [STAC]=8 mM; SA: [PC]=10 mM, [Chol]=10 mM, [SA]=10 mM.

ear over the range from the detection limit of 0.5 and 1.0 pM up to 3.0 nM with a slope of 0.96, respectively. The detection limit for ATP was defined as the concentration of ATP that produced the BL intensity equal to triple the blank intensity in the mixture containing no ATP. The detection limit for ATP was 5.0 pM in water alone.[5] The detection limit of ATP in the presence of the VET_{1000} containing STAC is improved by a factor of 10, compared with that in water alone, and is improved by a factors of 2 in the presence of the VET_{1000} containing SA. The relative standard deviation of the BL intensity for five successive experiments in the presence of STAC and SA was 4.3 and 5.3% at 0.1 nM of ATP, respectively. On the other hand, the relative standard deviation in water alone was 4.2% under the similar condition. Therfore, the presence of the cationic VETs had no effect on the reproducibility of the BL intensity.

Effect of membrane surface potential of the VET_{1000} on the BL enhancement

In order to elucidate the differences in the BL enhancement between the cationic components, the fluorescence intensity of ANS in the VET_{1000} suspensions was measured according to the procedure. Changes in the fluorescence intensity of ANS reflect the changes in the membrane surface potential of the VET_{1000}.

Figure 5 shows the relative membrane surface potentials (Ψ_{rel}) calculated by Eqs. (1) and (2). As can be seen in Fig. 5, the fluorescence intensity of ANS in the cationic VET_{1000} was greater than that in VET_{1000} composed of PC and Chol. The Ψ_{rel} increased in the following order: STAC>SA>TC. The order of increasing Ψ_{rel} corresponded to that of the enhacement of the BL intensity in the presence of the cationic VET_{1000} suspensions. The surface of the VET_{1000} becomes more positive along with the increase of the Ψ_{rel}. Therefore, these results suggest that cationic components which make the membrane surface more positive could be more effective for enhancers in the firefly BL.

As shown in Fig. 5, the surface of the VET_{1000} containing TC was more positive than that of the VET_{1000} composed of both PC and Chol. The BL intensity in the presence of the VET_{1000} composed of PC and Chol was about the same as that in water alone.[5] However, the presence of the VET_{1000} containing TC reduced the BL intensity to one-half when compared with that in water alone, as shown in Fig. 2. The reason for the decrease of the BL intensity in the presence of the VET_{1000} containing TC is still not clear.

Next, we examined the effect of pH on the Ψ_{rel} of the VET_{1000} containing STAC and SA. Figure 6 shows the dependence of the Ψ_{rel} upon pH in the range of pH from 5.0 to 11.0. The Ψ_{rel} decreased with an increase in pH. These results may be interpreted as follows. The increase in pH could be responsible for the decrease of positive species of STAC and SA existing in the VET_{1000}. Most ANS exists as anionic species under the experimental conditions. Consequently, uptake amount of ANS decreases in propotion to the increase in pH, thus resulting in the decrease of the Ψ_{rel} accompanying the increase in pH.

By comparing Figs. 4 and 6, the relative BL intensity was found to be dependent on the Ψ_{rel} above pH 7.5. On the other hand, the relative BL intensity in the presence of the cationic VET_{1000} decreased markedly below pH 7.5, though the Ψ_{rel} in the presence of the VET_{1000} containing STAC is almost constant and the Ψ_{rel} in the presence of the VET_{1000} containing SA increases gradually below pH 7.5. This is probably attributable to the decrease in the activity of luciferase below pH 7.5, as shown in Fig. 4.

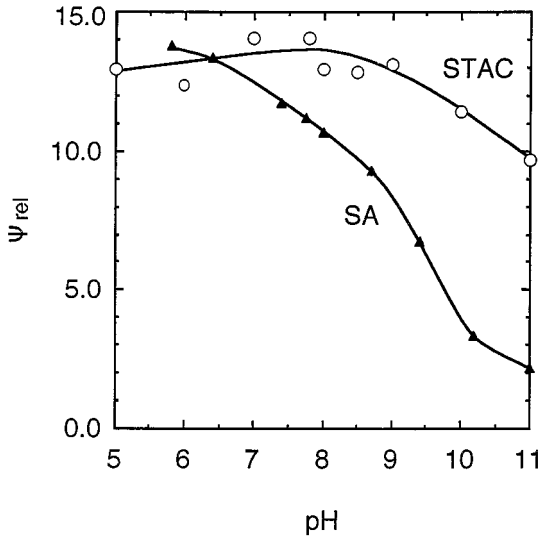

Fig. 6 Effect of pH on relative membrane surface potential of VET_{1000} containing STAC and SA. Concentrations of components in cationic VET_{1000} suspensions. STAC: [PC]=10 mM, [Chol]=10 mM, [STAC]=8 mM; SA: [PC]=10 mM, [Chol] =10 mM, [SA]=10 mM.

Firefly luciferase (*Photinus pyralis*) is a macromolecule having an approximate molecular weight of 100000. Therefore, very little luciferase may distribute into the VET_{1000}. This suggests that the BL enzymatic reaction could proceed on the surface of the cationic VET_{1000} rather than in the interior of the VET_{1000}. We then explained the BL enhancement in the presence of the cationic VET_{1000} in terms of electrostatic interaction between the surface of the cationic VET_{1000} and BL reactants. That is, the deprotonated forms of luciferin and ATP are expected to be the main species in the reaction mixture.[8,9] Consequently, the effective local concentration of luciferin and ATP at the positive surface of the cationic VET_{1000} is greater than their stoichiometric concentrations in bulk water alone. Thus, the rate of the BL reaction could be greater in the solution containing the cationic VET_{10000} than in water alone, resulting in the increase of the BL intensity in the presence of the cationic VET_{1000}.

In conclusion, a highly sensitive BL method was developed for the determination of ATP by the use of the cationic VET_{1000} containing STAC. STAC is superior to other cationic components such as SA and TC in the BL enhancement. The enhancement of the BL intensity is dependent on the Ψ_{rel}. Therefore, the estimation of the Ψ_{rel} of liposomes will be useful for the development of the effective enhancer in the firefly BL reaction.

References

1. J. J. Webster and F. R. Leach, *J. Appl. Biochem.*, **2**, 469 (1980).
2. L. J. Kricka and M. DeLuca, *Arch. Biochem. Biophys.*, **217**, 649 (1982).
3. W. J. Simpson and J. R. Hammond, *J. Biolumin. Chemilumin.*, **6**, 97 (1991).
4. T. Kamidate, T. Kinkou and H. Watanabe, *Chem. Lett.*, **1996**, 237.
5. T. Kamidate, T. Kinkou and H. Watanabe, *Anal. Biochem.*, **244**, 62 (1997).
6. H. Scott and A. A. F. Sayead, *J. Colloid Surface Sci.*, **9**, 274 (1986).
7. T. Aiuchi, N. Kamo, K. Kurihara and Y. Kobatake, *Biochemistry*, **16**, 1626 (1977).
8. O. A. Gandelman, L. Yu. Brovko, N. N. Ugarova, A. Yu. Chikishev and A. P. Shkurimov, *J. Photochem. Photobiol. B: Biol.*, **19**, 187 (1993).
9. N. N. Ugarova, *J. Biolumin. Chemilumin.*, **4**, 406 (1989).

(Received June 3, 1998)
(Accepted August 10, 1998)