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Notes

Cationic Liposomes Enhanced Firefly Bioluminescent Assay of Bacterial ATP in the Presence of an ATP Extractant

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Cationic liposomes composed of two components, diethylaminoethyl-carbamoyl cholesterol and phosphatidylcholine, were applied to an enhancer for a firefly bioluminescent (BL) assay of bacterial ATP in the presence of an ATP extractant. Trichloroacetic acid (TCA), which inhibits the activity of luciferase, was used as an ATP extractant. Cationic liposomes enhanced the BL intensity as long as luciferase was active. The detection limits for cell numbers of *Escherichia coli* extracts in the presence of cationic liposomes and in water alone were 199 and 897 colony forming units ml^{-1}, respectively. The sensitivity for bacterial ATP in the presence of cationic liposomes was improved by a factor of 2.5 times compared to that in the presence of diethylaminoethyl-dextran.

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Adenosine 5′-triphosphate (ATP) is present in living cells and the amount of ATP per cell remains fairly constant. Therefore, an assay of ATP is responsible for the total amount of bacteria cells. The firefly bioluminescent (BL) assay has been widely used for the determination of ATP in bacterial cells, since ATP can be determined rapidly and sensibly compared with conventional culture techniques.[1] Many ATP extractants are used for the release of ATP from bacterial cells. The extraction of ATP is conducted with dilute acids, such as trichloroacetic acid (TCA); surfactants, such as Triton X-100; and organic solvents, such as ethanol.[1] Among ATP extractants, TCA is widely used because of its effectiveness for the extraction of ATP from many living cells such as microorganisms (*Escherichia coli, Candida albicans, Bacillus cereus*) and somatic cells (whole blood, erythrocytes, granulocytes).[2] However, TCA acts as an inhibitor for the firefly luciferin-luciferase reaction. The extracts containing ATP and TCA must be diluted before the determination of ATP for eliminating the inhibitory effect of TCA on luciferase, resulting in a decrease in the concentration of ATP in the extracts. Therefore, an improvement in the sensitivity of the firefly BL assay is necessary to determine the lower level of the ATP concentration in the presence of TCA.

Previously, we found that diethylaminoethyl-dextran (DEAE-Dx) gave a BL enhancement effect.[3] The detection limit for ATP in the presence of DEAE-Dx was a factor of three-times better than that in water alone. In addition, DEAE-Dx enhanced the BL emission in the presence of TCA and Triton X-100 as long as firefly luciferase was active.[4] On the other hand, cationic liposomes composed of phosphatidylcholine and diethylaminoethyl-carbamoyl cholesterol (DEAE-chol) were also effective as enhancers for the firefly BL assay of ATP in aqueous standard solution.[5] The sensitivity for ATP in the presence of cationic liposomes was improved by a factor of 10-times better than that in water alone. The aim of this work was to improve the sensitivity of the firefly BL assay for bacterial ATP in the presence of TCA by using cationic liposomes.

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Experimental

Materials

Firefly luciferase and luciferin potassium salt were purchased from Promega Co. Adenosine 5′-triphosphate disodium salt (ATP) was purchased from Sigma Chemical Co. Egg yolk phosphatidylcholine (PC, molecular weight 773) was obtained from Nichiyu Liposome Co. DEAE-Dx was purchased from Pharmacia Biotech. Agar for bacterial culture medium and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were obtained from Wako Pure Chemicals. Luria–Bertani (LB) broth base powder was purchased from Difco Laboratory. All of the chemicals used were guaranteed-grade reagents, and were used without further purification. DEAE-chol was synthesized as described.[5]

A 1.0 mM solution of ATP was prepared by dissolving the compound with 25 mM HEPES buffer (pH 7.0) containing 2 mM EDTA. Working solutions of ATP were prepared by serial dilution with the HEPES buffer. A luciferase solution was prepared by dissolving 0.5 mg luciferase in 25 mL of the HEPES buffer containing 0.6 mM luciferin sodium salt, 24 mM magnesium acetate tetrahydrate, 3 mg bovine serum albumin and 2 mM dithiothreitol. Aqueous ATP standard solutions containing TCA (pH 7.0) were prepared with the HEPES buffer. A 10% solution of TCA was prepared with the HEPES buffer for the extraction of ATP in *Escherichia coli* (*E. coli*).

Cationic liposomes composed of PC and DEAE-chol were prepared according to the procedure described before.[5] A polycarbonate filter with a pore size of 100 nm was used to prepare unilamellar vesicles. Each concentration of PC and DEAE-chol in liposome suspensions were 45 mM and the total lipid concentrations of both PC and DEAE-chol was 90 mM.

Preparation of E. coli samples

E. coli strain JM109 was used. The culture was grown in a 5 ml of LB broth (1.0% tryptone, 0.5% yeast Extract, and 1% NaCl, pH 7.0 – 8.0) overnight at 37˚C. The culture was diluted 1:100 with fresh LB broth, and grown to the midlog phase

(OD_{600} = ~0.4).

E. coli suspensions were collected by centrifugation at 10000*g* for 5 min. The pellet was resuspended in a 1.0 ml portion of the HEPES buffer or a 1.0 ml portion of a 0.15 M NaCl solution. These procedures were repeated three times. The optical density of *E. coli* suspensions prepared with the HEPES buffer was the same as those prepared with a NaCl solution. Thus-prepared *E. coli* suspensions were used for working cell suspensions by serially diluting with the HEPES buffer or the NaCl solution. The HEPES buffer and the NaCl solution were used to prepare working cell suspensions for the firefly BL assay of bacterial ATP and for the determination of cell numbers by a colony-counting method, respectively.

In a colony-counting method, working cell suspensions of *E. coli* were plated onto an LB broth containing 1.5% agar and left standing at 37˚C for 24 h. The bacterial numbers in sample dilutions were determined as colony-forming units (CFU) ml^{-1} from triplicate plate counts.

BL measurements

The BL experimental procedure consisted in pipetting a 100 µl portion of the luciferase solution and a 50 µl portion of liposome suspensions into a plastic cuvette (15 mm i.d. × 15 mm: internal volume 500 µL) in a luminometer (BLD-100HU, Tohoku Densi Sangyo Co.). Next, a 250 µL portion of an ATP solution was injected into the cuvette and the BL reaction was initiated. The BL emission was detected by a photomultiplier. The resultant photocurrent was converted to counts per second (cps), whose value was displayed on a personal computer.

In the extraction of ATP in *E. coli*, a 500 µl portion of 10%(w/v) TCA solution was added to an equal volume of *E. coli* suspensions. The mixture was allowed to stand for 3 min to extract ATP from *E. coli* cells. Next, the extracts (1 ml) were diluted with a 29 ml portion of the HEPES buffer containing 0.306 mmol NaOH that was added to neutralize TCA. The pH value of the extract was adjusted to 7.0. A 250 µl portion of the diluted extracts was injected into the cuvette and the light emission was measured at 30˚C.

Results and Discussion

Cationic liposomes enhanced BL in the presence of TCA

The BL response curve was measured in water alone according to a procedure in which a 0.1 nM solution of ATP was injected into the cuvette and the buffer solution was used in place of liposome suspensions. The light emission appeared immediately after the start of the reaction, and reached the maximum intensity in 30 s, after which the light emission decayed rapidly. No BL emission was observed in the blank solution, which contained no ATP. We then defined the maximum light emission as a BL intensity. The effect of TCA on the BL intensity was examined by using a 0.1 nM solution of ATP containing 0.1% TCA. The BL intensity in the presence of TCA decreased by comparing with that observed in water alone.

Next, we measured the BL response curve using a 0.1 nM solution of ATP containing 0.1% TCA in the presence of cationic liposome suspensions. Cationic liposomes were prepared with PC:DEAE-Chol molar ratio of 1:1, since its molar ratio was most effective for the BL enhancement compared with other molar ratio of PC and DEAE-Chol.[5] We then used cationic liposome suspensions containing 10 mM PC and 10 mM DEAE-chol. The BL intensity increased remarkably in the presence of cationic liposomes. The BL intensity in the presence of cationic liposomes was 2.5 and 3 times greater than those observed in water alone and in the ATP solution containing TCA, respectively.

Effect of the TCA concentration on the BL intensity

TCA solutions in the range of 1 to 10% are widely used to extract ATP from various living cells. We thus examined the effect of the TCA concentrations on the BL intensity in water alone and in the presence of cationic liposomes. Figure 1 (curve 1) shows the relative BL intensity-TCA concentration profiles. The relative BL intensity was defined as the ratio of the BL intensity obtained in a solution containing TCA to that obtained in water alone. The inhibitory effect of TCA on the BL reaction appeared above 0.01% TCA.

Next, the effect of the TCA concentrations on the BL intensity was observed in the presence of cationic liposomes. The dependence of TCA concentrations on the relative BL intensity is shown in Fig. 1 (curve 2). The relative BL intensity was defined as the ratio of the BL intensity obtained in a mixture containing TCA and cationic liposomes to that obtained in water alone. The enhancement of the BL emission by cationic liposomes efficiently appeared below 0.01% TCA. However, the enhancement effect decreased remarkably, accompanied by the occurrence of an inhibitory effect of TCA (>0.01% TCA) on the BL reaction. No BL enhancement was observed above 1.0% TCA. These results suggest that the BL enhancement by cationic liposomes could appear as long as luciferase is active in the presence of TCA.

The effect of the total lipids concentration on the BL intensity was examined in the range of 10 to 100 mM under the following condition: a PC:DEAE-chol molar ratio of 1:1. A 0.1 nM solution of ATP containing 0.2% TCA was used, since luciferase is active in the presence of 0.2% TCA. The BL intensity increased with an increase in the total lipids concentration, and was broadly maximal at 90 mM. Thus, the optimum total concentration of PC and DEAE-chol was chosen to be 90 mM.

Dilution of an ATP sample

The ATP extractants from bacterial cells containing a high concentration of TCA must be diluted so as to eliminate the inhibitory effect of TCA on the BL reaction. To determine the optimal dilution, we examined the BL measurement with serial dilutions of a 0.1 nM ATP solution containing 5% TCA. As can be seen from Fig. 1, when 5% TCA was used, luciferase was completely inhibited. BL measurements were also carried out for diluted solutions in the presence of cationic liposome suspensions containing 45 mM PC and 45 mM DEAE-chol. The dependence of the dilution factor on the relative BL intensity is shown in Fig. 2. The relative BL intensity was defined as the ratio of the BL intensity observed in the absence (Fig. 2, curve 1) and presence (Fig. 2, curve 2) of cationic liposomes to that obtained in water alone. The relative BL intensity was maximal at 15- and 30-fold dilution in both the absence and presence of cationic liposomes, respectively. These results can be explained in terms of the luciferase activity and the concentration of ATP. The concentration of TCA decreases by the dilution of the ATP extracts, thus resulting in an increase in the BL intensity. However, the dilution leads to a decrease in the concentration of ATP. As a result, the BL intensity decreases accompanying dilution. Therefore, the BL intensity can exhibit a maximum at which those two factors compensate each other. The optimal condition was thus determined to be 15- and 30-fold dilution in both the absence and presence of cationic liposomes, respectively. The final concentration of TCA was 0.16% in the presence of cationic

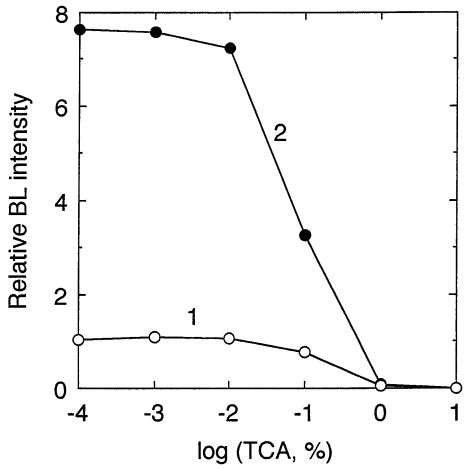

Fig. 1 Effect of the TCA concentrations on the relative BL intensity. 1, ATP in water alone; 2, ATP solutions containing liposomes. [PC] = 10 mM, [DEAE-chol] = 10 mM, [ATP] = 0.1 nM.

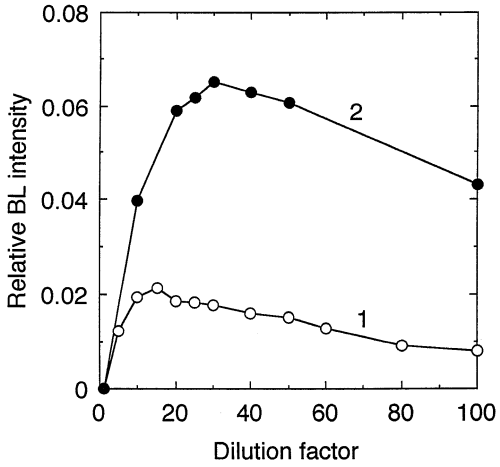

Fig. 2 Effect of the dilution factor on the relative BL intensity. 1, ATP solutions containing TCA; 2, ATP solutions containing both TCA and liposomes. 1. [PC] = 45 mM, [DEAE-chol] = 45 mM. [ATP] = 0.1 nM, TCA = 5%.

liposomes.

Calibration curves for aqueous ATP standard solutions containing TCA

The calibration curves for ATP were prepared by using ATP solutions containing 5% TCA under the thus-established optimum conditions. The logarithmic calibration curve obtained in the absence of the cationic liposomes was linear over the range from the detection limit of 4 pM up to 10 nM with a slope of 0.92 and a correlation coefficient (R_2) of 0.999. The detection limit for ATP was defined as the concentration of ATP yielding an analytical signal equal to triple the standard deviation of the blank BL intensity in the mixture containing no ATP. On the other hand, the logarithmic calibration curve obtained in the presence of cationic liposomes was linear over the range from the detection limit of 0.5 pM up to 2.0 nM with a slope of 0.86 and R_2 of 0.996. The relative standard deviations of the BL intensity for five successive experiments without and with cationic liposomes were 1.9 and 0.86% at 0.1 nM ATP, respectively. In aqueous ATP standard solutions containing TCA, the detection limit of ATP in the presence of cationic liposomes was improved by a factor of 8, compared with that in the absence of cationic liposomes.

Measurement of ATP in E. coli extracts

Working cell suspensions of *E. coli* were prepared according to the procedure. The cell numbers of *E. coli* in the working cell suspensions were determined by measuring the colony-forming units (CFU) ml^{-1} with a conventional colony-counting method. The BL measurements were carried out after the extraction of ATP from working cell suspensions with a 10% TCA solution and 15- and 30-fold dilution of *E. coli* extracts in the absence and the presence of cationic liposomes, respectively. The BL intensities obtained from those ATP extracts were plotted as a function of the cell numbers. The logarithmic curve in the presence of cationic liposomes was linear over the range from 199 to 7.9×10^4 CFU ml^{-1} with a slope of 0.737 and R_2 of 0.995. On the other hand, the logarithmic curve in the absence of cationic liposomes was linear over the range from 897 to 2.5×10^5 CFU ml^{-1} with a slope of 0.827 and R_2 of 0.995. The relative standard deviations of the BL intensity for three successive experiments without and with cationic liposomes were 4.6 and 3.9% at 2×10^4 CFU ml^{-1}. The detection limit for the cell numbers in *E. coli* extracts in the presence of cationic liposomes was improved by a factor of 4.5 compared with that in the absence of cationic liposomes.

In order to clarify the usefulness of cationic liposomes as an enhancer for the determination of bacterial ATP in the presence of TCA, we carried out a firefly BL assay of ATP in *E. coli* extracts in the presence of DEAE-Dx. The BL measurements were made according to a procedure in which HEPES buffer containing 1.0% DEAE-Dx was used in place of liposomes suspensions.[4] The logarithmic curve in the presence of DEAE-Dx was linear over the range from 500 to 1×10^5 CFU ml^{-1} with a slope of 0.852 and R_2 of 0.993. The relative standard deviation of the BL intensity for three successive experiments with DEAE-Dx was 4.4% at 1×10^4 CFU ml^{-1}. Therefore, the sensitivity for bacterial ATP in the presence of cationic liposomes was a factor of 2.5 times better than that in the presence of DEAE-Dx.

In conclusion, cationic liposomes were more effective than DEAE-Dx as an enhancer for the firefly BL assay of bacterial ATP in the presence of TCA. This proposed method would be highly useful for hygiene monitoring in the food-processing industry in which the detection of much lower bacterial contamination is required.

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