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Additive effect of heparin on the photoinactivation of *Escherichia coli* using tricationic P-porphyrins

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Keywords: heparin; polycationic P-porphyrin; *Escherichia coli*; photoinactivation

Abstract

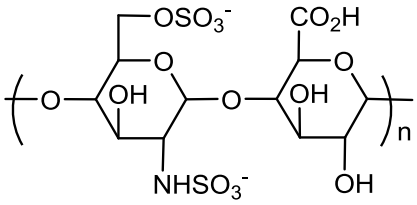
Polycationic porphyrins have received substantial attention in developing singlet oxygen-sensitizers for biological use such as in the photoinactivation of bacteria and photodynamic therapy (PDT) of tumor cells because they have strong binding affinities for DNA and proteins. However, these strong cellular interactions can retard elimination of the drug after PDT. Therefore, the studies on the interactions of porphyrins with other molecules present much interest, in order to modulate the sensitizers` activity or even remove them from the human body after PDT. Here, we studied the additive effect of heparin on the photoinactivation by polycationic porphyrins using *Escherichia coli* as a model cell. Tricationic P-porphyrin sensitizers substituted with an *N*-alkylpyridinium group (alkyl = pentyl (**1a**), hexyl (**1b**), and heptyl (**1c**)) or *N*-hexylammonium (**1d**) as the axial ligand were used. Additionally, dicationic Sb-porphyrin substituted with an *N*-hexylpyridinium group (**1e**) was prepared. We studied the additive effect of heparin on the photoinactivation of *E. coli* by **1a**-**1e**. The bactericidal activities were evaluated using the half-life ($T_{1/2}$ in min) of *E. coli* and the minimum effective concentrations ([*P*]) of the porphyrin sensitizers. In the

absence of heparin, the [*P*] values were determined to be 0.4 - 0.5 μM for **1a**−**1c** and 2.0 μM for **1d**−**1e**. The bactericidal activity of **1a**−**1c** was completely retarded by the addition of heparin (1.0 μM). However, the addition of heparin (1.0 μM) could not completely retard the bactericidal activity of **1d**−**1e** whose [*P*] values were relatively large. It is suggested that tricationic **1a**−**1c** adsorbed onto the anionic heparin through electrostatic interactions. The adsorption of **1** on heparin disturbs the uptake of **1** into *E. coli* cells. Thus, the addition of heparin was found to be a useful method for retarding photoinactivation.

Porphyrins have received substantial attention in developing singlet oxygen sensitizers for the photoinactivation of bacteria[1] and for the photodynamic therapy (PDT) of tumor cells.[2] In particular, it is believed that polycationic porphyrins are promising $^1O_2$-sensitizers, because they are water-soluble and have strong binding affinities for DNA[3-5] and proteins[6]. Polycationic porphyrins can effectively photoinactivate even gram-negative bacteria (e.g., *Escherichia coli*) that have an impermeable barrier to antimicrobial agents.[7] Therefore, the studies on the interactions of porphyrins with other molecules present much interest, in order to modulate the sensitizers' activity or even remove them from the human body after PDT. Herein, we studied the additive effect of heparin on the photoinactivation by polycationic porphyrins using *Escherichia coli* as a model cell.

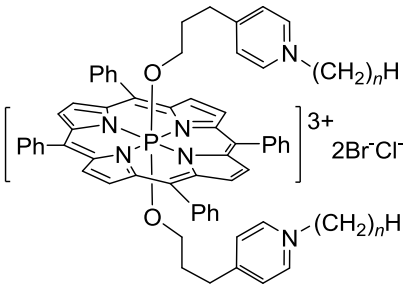
Heparin (**Scheme 1**), a naturally occurring polysaccharide, is often incorporated into cancer therapy in view of its unique biological and physicochemical properties such as anti-inflammation, anti-angiogenesis, and anti-tumor cell proliferation.[8] Huh et al. have extensively prepared heparin-photosensitizer conjugates to control the activity of the sensitizer and develop a new drug delivery system.[9-11] The abundance of sulfate and carboxylate groups endow heparin with high negative charge, mediating its electrostatic

interaction with cationic molecules. As a new functionality of heparin, we studied the scavenging effect of anionic heparin on the photoinactivation of *E. coli* by polycationic porphyrins.



Scheme 1. General structure of heparin

We selected tricationic P-porphyrins (**1a-1c**) bonded with alkylpyridinium groups through axial hydroxo-ligands of $P(tpp)(OH)_2$ complex (tpp = tetraphenylporphyrinato ligand). The structures of bis[3-(1-alkyl-4-pyridinio)-1-propoxo]-5,10,15,20-tetraphenylporphyrinato-phosphorus (V) dibromide, chloride (**1a-1c**) are shown in **Scheme 2**. These P-porphyrins were water-soluble and their water-solubility (C_W) values are shown in Table 1. These axially alkylpyridinium-substituted P-porphyrins have already been successfully applied to the inactivation of *Saccharomyces cerevisiae*[12] and *E. coli*[13] under visible-light irradiation. *S. cerevisiae* was photoinactivated in concentrations that were lower than those in the cases of *E. coli*: the effective minimum concentration of **1b** was found to be 40 nM for *S. cerevisiae* and 500 nM for *E. coli*. Therefore, in the present study, *E. coli* was selected as a model cell in order to examine the additive effect for porphyrin in higher concentration.



	n
1a	5
1b	6
1c	7

Scheme 2 Tricationic P-porphyrins (**1a-1c**) bound axially to the alkylpyridinium group

First, **1** was subjected to the photoinactivation of *E. coli* in the absence of heparin. *E. coli* K-12 (IFO 3335) was cultured aerobically at 30 °C for 8 h in a basal medium (pH 6.5).[13] After the centrifugation of the cultured broth, the harvested cells were washed with physiological saline (NaCl, 7 g L^{-1}) and then the *E. coli* was suspended in physiological saline. The cell concentration was determined by turbidity (absorbance) at 600 nm. A typical procedure for the photoinactivation of *E. coli* using **1b** in the absence of heparin is described as follows:[13] An L-type glass tube containing *E. coli* cells (2×10^4 cell mL^{-1}) and **1b** (0.5 μM) in phosphate buffer solution (0.1 M, pH 7.6, 10 mL) was set on a reciprocal shaker and shaken at 160 rpm at room temperature under dark conditions for 2 h to adsorb **1b** onto the *E. coli* cells. Irradiation was performed using a fluorescent lamp (λ= 400–723 nm, 10.5 W cm^{-2}, maximum intensity: 545 nm) for 2 h at room temperature under aerobic conditions with shaking. Aliquots (0.1 mL) of the reaction mixture were taken from the L-type glass tube at 20-min intervals and plated on a solid agar medium. The amount of living cells (*B*) in colony formation unit (CFU mL^{-1}) was defined as the average number of *E. coli* colonies that appeared after incubation for 24 h at 30 °C in three replicate plates.

Figure 1 shows the time−course plots of the survival ratio ($100B/B_0$) where B_0 is the initial amount of *E. coli*. The survival ratio gradually decreased as irradiation time increased. The bactericidal activity of **1b** was evaluated by the half-life ($T_{1/2}$ in min), which was the time required to reduce *B* to 0.5 B_0. The $T_{1/2}$ was determined from the slope in the time−course plots. Moreover, the minimum concentrations of the porphyrins ([*P*]) were adjusted so that the $T_{1/2}$ had values between 20 and 120 min. Thus, the [*P*] and $T_{1/2}$ values for **1b** were determined to be 0.5 μM and 27 min, respectively. Similarly, the [*P*] and values $T_{1/2}$ were determined for **1a** and for **1c**, as shown in **Table 1**. The survival plots in the absence of heparin are shown in the Supporting Information. Details of the synthesis of **1a**, which was

prepared from $P(tpp)Cl_2^+Cl^-$ according to the preparation method described in a previous report,[14] are also described in the Supporting Information.

Table 1. Additive effect of heparin on the photoinactivation of *E. coli* by polycationic P-porphyrins (**1a-1e**) [a)]

1		**1a**	**1b**	**1c**	**1d**	**1e**
C_W/mM [b)]		3.8	5.8	6.0	6.1	5.2
[*P*] /μM[c)]		0.5	0.5	0.4	2.0	2.0
$T_{1/2}$ /min [d)]	[Heparin]/μM = 0.0	29	27 [e)]	26 [e)]	43	24 [e)]
	[Heparin]/μM = 1.0	>120	>120	>120	65	73

a) Photoinactivation was performed in buffer solutions (10 mL) containing *E. coli* (1×10^4 cell mL^{-1}), heparin (0 and 1.0 μM), and **1** (0.4, 0.5, and 2.0 μM) under irradiation from a fluorescent lamp.
b) C_W = Water solubility in mM. The C_W values of **1b**, **1c**, and **1e** were obtained from Ref 14.
c) [*P*] = Minimum effective concentration of **1** in μM
d) $T_{1/2}$ = Half-life in min.
e) Data was obtained from Ref 13.

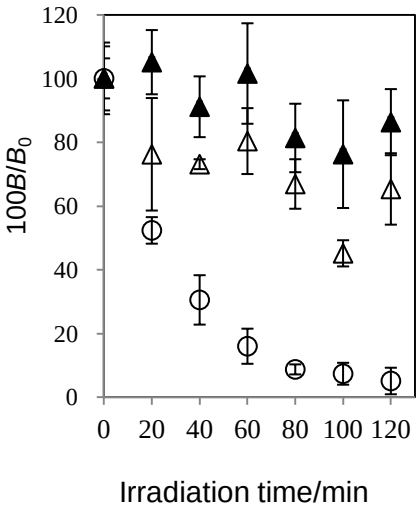


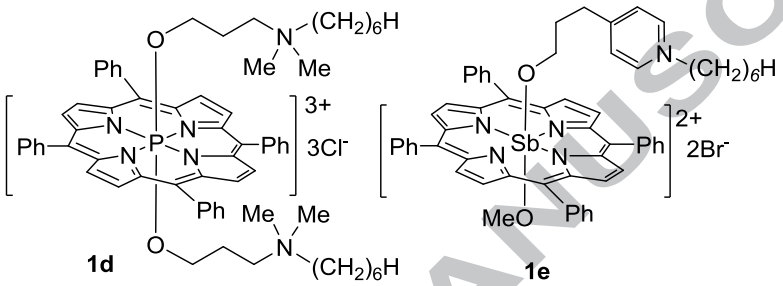
Figure 1. Additive effect of heparin on the photoinactivation of *E.coli* using **1b** (0.5 μM). [Heparin] = 0.0 (○), 0.50 (△), and 1.0 μM (▲).

To examine the additive effect of heparin, the photoinactivation of *E. coli* by **1** was performed in the presence of various heparin concentrations. The concentration of **1** was adjusted to [*P*]. A typical procedure for a run using **1b** (0.5 μM) and heparin (1.0 μM) is

described as follows: An aqueous heparin solution (500 μM) was prepared by dissolving 50 mg of sodium heparin (average MW 2.0×10^4, Wako Chemicals, Japan) in water (5 mL). The aqueous heparin solution (500 μM, 80 μL) was mixed with an aqueous solution of **1b** (50 μM, 400 μL) in measuring flask; then, the volume was adjusted to 2.0 mL using phosphate buffer (0.1 M, pH 7.6). The solution was allowed to stand for 12 h. The resulting **1b**/heparin solution (0.5 mL) and an *E. coli* suspension (2×10^5 cells mL^{-1}, 1.0 mL) were mixed in phosphate buffer (8.5 mL) in an L-type glass tube. The L-type glass tube containing *E. coli* cells (2×10^4 cells mL^{-1}), **1b** (0.5 μM), and heparin (1.0 μM) in buffer (10 mL) was shaken at 160 rpm under dark conditions for 2 h and then irradiated with a fluorescent lamp for 2 h. Time−course plots of survival ratios for photoinactivation by **1b** in the presence of heparin (1.0 μM) were overlaid onto **Fig. 1**. The $T_{1/2}$ values were drastically changed from 27 min to >120 min due to the addition of 1.0 μM of heparin. Thus, the addition of 1.0 μM of heparin completely retarded the bactericidal activity of **1b**. When 0.50 μM of heparin was added, the $T_{1/2}$ value was determined to be 101 min. The addition of 0.50 μM of heparin increased the $T_{1/2}$ value but did not completely retard it. Therefore, the additive effects of heparin towards **1a** and **1c** were examined using 1.0 μM of heparin. Survival plots in the presence of heparin are shown in the Supporting Information. It was found that the $T_{1/2}$ values in the presence of heparin (1.0 μM) were > 120 min and that they were larger than the cases without heparin. Thus, the bactericidal activity of tricationic **1a**−**1c** was markedly retarded by the addition of 1.0 μM of heparin.

Moreover, the additive effect of heparin was applied to other types of polycationic P-porphyrins (**Scheme 3**): tricationic bis[3-(hexyldimethylammonio)-1-propoxo]-5,10,15,20-tetraphenylporphyrinatophosphorus (V) trichloride (**1d**) and dicationic α-(methoxo)-β- [3-(1-hexyl-4-pyridinio)-1-propoxo]-5,10,15,20-tetraphenylporphyrinatoantimony (V) dibromide (**1e**). The details of the preparation and the spectral data of **1d** and **1e** are shown in the

Supporting Information. The survival plots of **1d** and **1e** are shown in **Fig. 2A** and **2B**, respectively. In the absence of heparin, the [*P*] values of **1d** and **1e** were determined to be 2.0 μM. The [*P*] and $T_{1/2}$ values are summarized in **Table 1**. In the cases of **1d** and **1e** in the presence of heparin (1.0 μM), the $T_{1/2}$ values were 65 and 73 min, respectively. The addition of heparin could not prolong the $T_{1/2}$ of **1d** and **1e** to >120 min, despite the addition of 1.0 μM of heparin completely retarding the bactericidal activity with **1a**-**1c**.



Scheme 3.Tricationic P-porphyrin bound axially to ammonium (**1d**) and dicationic Sb-porphyrin (**1e**).

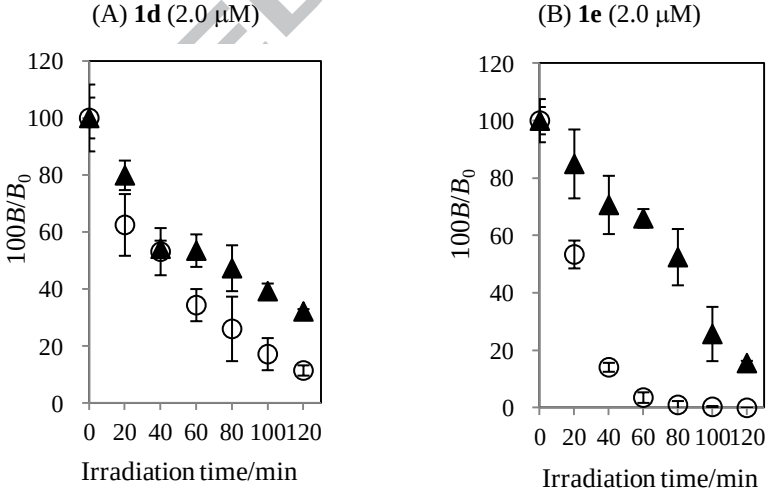


Figure 2. Additive effect of heparin on the photoinactivation of *E. coli* using **1d** (A) and **1e** (B). [Heparin] = 0.0 (○) and 1.0 μM (▲), [**1**] = 2.0 μM.

The interaction of porphyrin with heparin was examined via monitoring the porphyrin absorption spectral changes after the addition of heparin. The peak width (HW) at half height of the Soret band was measured at 415 nm after the addition of aqueous heparin solution (0 −

20 nM) to an aqueous solution of tricationic **1b** (0.5 μM) and dicationic **1e** (0.5 μM). **Figure 3** shows the plots of HW against the concentration of heparin. The addition of heparin caused 6 nm of HW broadening for **1b** (from 19 nm to 25 nm). The HW broadening was due to the interaction of heparin with **1b**.[15] Therefore, it is suggested that tricationic **1b** was adsorbed onto anionic heparin and this intervened between the aggregation of heparins to each other through electrostatic interactions. The adsorption onto heparin induced aggregation of **1b**, resulting in a broadening of the HW. In the case of **1e**, broadening of the HW from 13 to 19 nm occurred. However, the spectral change of **1e** was more gentle compared with tricationic **1b**, suggesting that the electrostatic force of dicationic **1e** was weak for heparin aggregation. It is suggested that the adsorption of **1** onto heparin disrupts the uptake of **1** into *E. coli* cells.

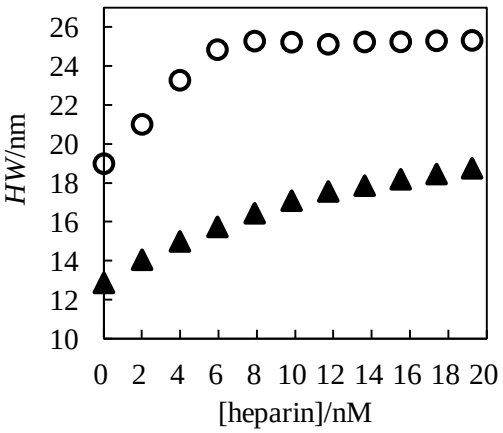


Figure 3. Dependence of peak width at half height (HW) of the Soret band on heparin concentration (0-20 nM) in the absorption spectra of **1b** (0.5 μM, ○) and **1e** (0.5 μM, ▲).

The incorporation of porphyrins inside *E. coli* was confirmed from fluorescence microscopy images as follows: A phosphate buffer solution containing **1b** (1 μM), *E. coli* (ca. 2×10^5 cell mL^{-1}), and heparin (0 and 1 μM) was left to stand for 2 h at 25 °C. The mixture (1 mL) was centrifuged for 15 min at 12,000 rpm and the supernatant solution (0.9 mL) was removed to obtain a sample solution. The sample solution (2 μL) was placed on an agar

pad[13,16] set on a cover glass (24 mm × 50 mm) and covered with another cover glass (18 mm × 18 mm). Fluorescence microscopy images were obtained with an Olympus FV-300 confocal laser scanning microscope (CLSM) equipped with a 60× water immersion objective lens (numerical aperture = 1.2) under laser excitation at 543 nm. As shown in **Fig. 4A**, a fluorescence image depicting the emission of **1b** was observed inside *E. coli* in the absence of heparin. It was found that **1b** was incorporated inside the cell in the absence of heparin. On the contrary, in the presence of heparin (1 μM), fluorescence from within cells was not observed, as shown in **Fig. 4D**. Therefore, in the presence of heparin, **1b** was not incorporated within the cells. These observations demonstrate that the aggregation of **1b** with heparin disturbs the uptake of **1b** into *E. coli*. Thus, photoinactivation was retarded via the addition of heparin.

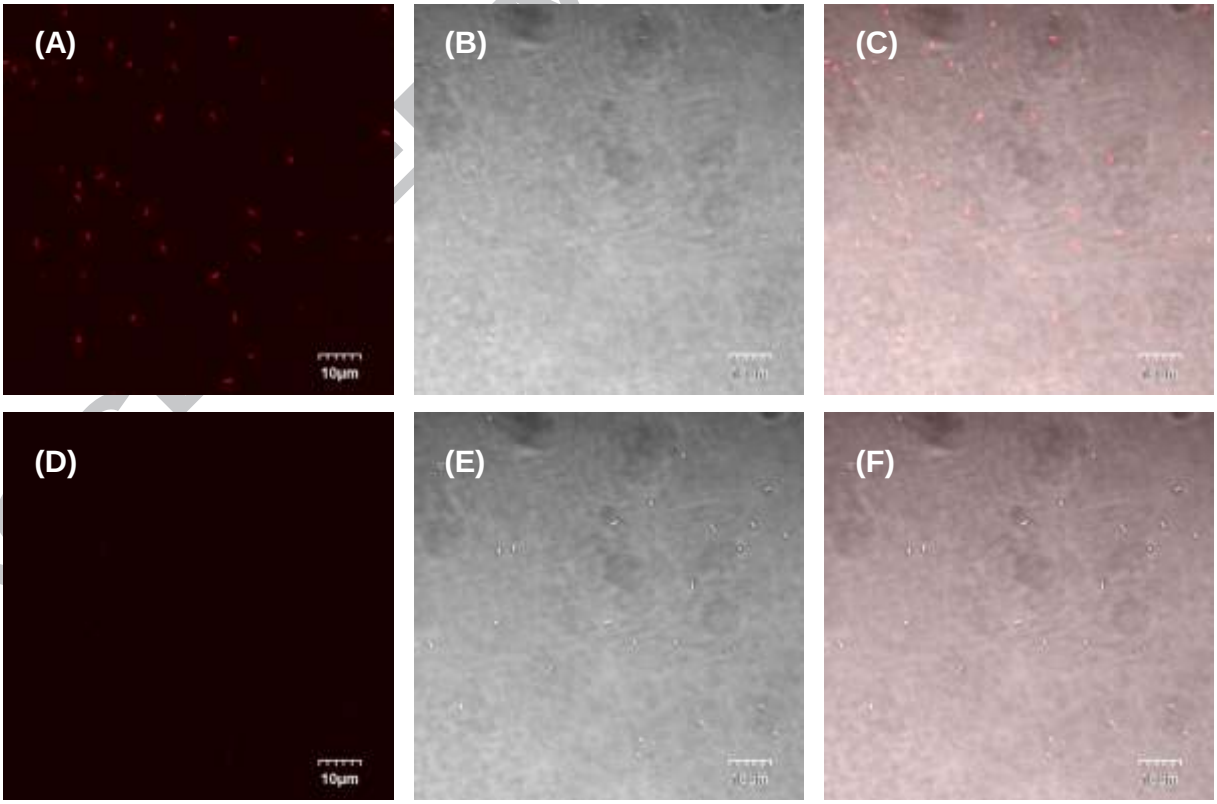


Figure 4. Fluorescence images of *E. coli* with the addition of **1b**. These images were obtained with a CLSM under laser-excitation at 543 nm. Fluorescence from within the cells was observed in the absence of heparin (A) but not observed in the presence of heparin (D). Transmission images of *E. coli* containing **1b** (E) and **1b** and heparin (B). The image in C was

obtained by overlapping images A and B. The image in F was obtained by overlapping images D and E.

In conclusion, we succeeded to control the bactericidal reactivity of the polycationic porphyrins through the addition of heparin. The additive effect of heparin to photoinactivation using various porphyrins is currently underway in our laboratories. In a future, the studies on the use of heparin for the controlled release and activity modulation will continue. In addition, we will study the possible use of heparin for elimination of the sensitizer from a human body after PDT. These studies will be reported in due course.

Acknowledgments

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Highlights

* Heparin could strongly interact with polycationic P-porphyrins through Coulombic interactions.

* Heparin (1.0 μM) could retard the bactericidal activity of tricationic P-porphyrins.

* This is an interesting finding on the scavenging effect of heparin on the photoinactivation of *E. coli*.