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Enhanced efficacy of TD53, a novel algicidal agent, against the harmful algae via the liposomal delivery system

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ABSTRACT

The present study aimed to design the liposomal delivery system for TD53, a novel algicial drug in order to improve the delivery properties of TD53 and evaluate its algicidal effects as well as selectivity against harmful and non-harmful algae. Liposomes of TD53 were prepared with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) by a lyophilization, resulting in relatively small size vesicles (234 ± 38 nm) and narrow size distribution (PI = 0.130 ± 0.027). The drug leakage from the liposome was negligible in the F/2 media (<2% during 96 h incubation). Subsequently algicidal activity of liposomal TD53 against harmful and nonharmful algae was evaluated at various concentrations. The IC_{50} values of TD53 in liposome against harmful algae such as *Chattonella marina*, *Heterosigma akashiwo* and *Cocholodinium polykrikoides* were 2.675, 2.029, and 0.480 μM, respectively, and were reduced by approximately 50% compared to those obtained from non-liposomal TD53. In contrast, the algicidal effect of liposomal TD53 was insignificant against non-harmful algae including *Navicula pelliculosa*, *Nannochloropsis oculata* and *Phaeodactylum* EPV. Those results suggested that liposomal delivery systems might be effective to enhance the efficacy of TD53 while maintaining the selectivity to harmful algal species.

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1. Introduction

Harmful algal blooms (HABs) bring a great impact on aquatic-ecosystem sustainability, water resources and human and animal health and economic vitality in worldwide. Many scientists have conducted physiological and ecological studies in the hope of reducing the extent of damages caused by HABs (Kim et al., 2000; Uchida et al., 1999; Yanagi et al., 1995). In an effort to manage and alleviate these vast effects of HABs, several approaches such as chemical algicides, flocculants and other physical manipulations and biological agents are currently under investigation (Doucette et al., 1999; Hennes and Simon, 1995; Hennes et al., 1995; Kim, 2006; Sengco et al., 2001). Among the proposed approaches, the application of chemicals is one of the most common methods of controlling the development of noxious phytoplankton, however, their use has been limited by less selectivity and consequently undesirable toxicity towards non-target species (Jancula et al., 2008; Liu et al., 2004; Zhou et al., 2004). Therefore, considerable effort has been made to identify new compounds that are selectively effective against HABs.

Thiazolidinedione (TD) was introduced in the late 1990s as an adjunctive therapy for diabetes mellitus (type 2) and related diseases. TDs act by binding to PPARs (peroxisome proliferator-activated receptors), a group of receptor molecules inside the cell nucleus (Lehman et al., 1995). Recently, based on the in vitro screening studies, it was found that some of TDs could inhibit the growth of harmful algal species (Kim et al., 2010). Therefore, for the discovery of environmentally safe and selective algicides to manage the HABs, series of TD derivatives were chemically synthesized via the introduction of various substituents to the hydroxyl group of 5-(4-hydroxybenzylidene) thiazolidine-2,4-dione and the effects of these derivatives on the growth of a number of harmful algal species were examined in our previous studies (Kim et al., 2010). As a result, it was found that some of TD derivatives exhibited remarkable algicidal activity against three typical harmful algal species, *Heterosigma akashiwo*, *Chattonella marina* and *Cocholodinium polykrikoides* with low IC_{50} values (0.1–2 μM) while they were safe to non harmful algae (Kim et al., 2010). Particularly, TD53, a cyclopentyl methyl-thiazolidine-2,4-dione (Fig. 1), has been identified as a potent and selective inhibitor against harmful algae (Kim

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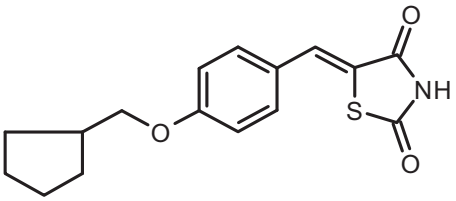

Fig. 1. The structure of TD53.

et al., 2010). However, TD53 is poorly water soluble and it is necessary to improve its solubility to ensure further safety evaluation at high doses and also to maximize its effectiveness as a novel algicidal agent.

Liposomal delivery systems have been adopted as a promising approach to improve the unfavorable physicochemical properties of drugs such as low solubility and poor membrane permeability (Drulis-Kawa and Dorotkiewicz-Jach, 2010; Lian and Ho, 2001; Sharma and Sharma, 1997). Liposomal formulation not only acts as a formulation aid but also provides an opportunity to enhance the efficacy of drugs in such a way that a greater fraction of the dose reaches the target site. Therefore, liposomes have been used as carriers for a wide range of pharmacologically active compounds, such as anticancers, antivirals, antifungals and vaccines. Liposomes are spherical vesicles consisting of relatively biocompatible and biodegradable synthetic and natural phospholipids and drugs with widely varying lipophilicities can be encapsulated in liposomes (Lian and Ho, 2001; Sharma and Sharma, 1997). Therefore, in the present study, in order to improve the delivery properties of TD53, we designed the liposomal delivery system for TD53 and evaluated their algicidal effects as well as selectivity against harmful and non-harmful algae.

2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from the Avanti polar lipid Inc (Alabaster, AL, USA). Tween 80 and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and used without further purification.

2.2. Algal cultures, mediums and culture conditions

Heterosigma akashiwo (CCMP 452) (*H. akashiwo*) and *Cochlodinium polykrikoides* (*C. polykrikoides*) were obtained from the Provasoli–Guillard Center for the Culture of Marine Phytoplankton (CCMP) and National Fisheries Research and Development Institute (NFRDI), respectively. The microalga, *Chattonella marina* (*C. marina*), *Amphidinium* sp. and *Navicula pelliculosa* were kindly provided by Professor M.-S. Han (Hanyang University, Korea). *Nannochloropsis oculata* and *Phaeodactylum* EPV were kindly provided by Professor M.-K. Kim (Young Nam University, Korea).

Harmful algal strains, *H. akashiwo*, *C. marina* and *C. polykrikoides*, and non-harmful algae were grown in a culture flask (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 20 °C under constant light in Guillard's F/2 medium with filtered seawater, as reported previously (Guillard and Keller, 1984).

2.3. Synthesis of TD53

TD53 was synthesized and characterized as described in the previous report (Kim et al., 2010). Briefly, diethyl azodicarboxylate (40% in toluene, 3.7 g, 8.6 mmol) was added slowly to a stirring solution of 2-cyclopentylmethanol (1 g, 7.8 mmol), p-hydroxybenzaldehyde (953 mg, 7.8 mmol) and triphenylphosphine (2.25 g, 8.6 mmol) in THF (20 ml) for 10 min at 0 °C. The mixture was then stirred at room temperature until the starting materials (TLC analysis) began to disappear. The resulting solution was concentrated under reduced pressure and purified using column chromatography through silica gel (elution with hexane/ethyl acetate, 10:1) to produce 1.54 g of the intermediate, 4-(cyclopentylmethoxy)benzaldehyde (86%), a yellow oil. A mixture of 4-(cyclopentylmethoxy)benzaldehyde (1.0 g, 4.3 mmol), 2,4-thiazolidinedione (504 mg, 4.3 mmol), piperidine (0.21 ml, 2.15 mmol) and acetic acid (0.14 ml, 2.15 mmol) in toluene (20 ml) was placed into a round bottom flask fitted with a Dean–Stark water trap and was stirred overnight under reflux. After cooling to room temperature, the precipitate was washed with hexane and dried to produce 5-(4-(cyclopentylmethoxy)benzylidene)thiazolidine-2,4-dione (TD53). This compound was obtained via recrystallization as a yellow solid (1.16 g, 82% yield) with the following characteristics: ^{1}H NMR (300 MHz, DMSO-d_6) δ12.503 (s, 1H), 7.732 (s, 1H), 7.547 (d, J = 9.0 Hz, 2H), 7.094 (d, J = 9.0 Hz, 2H), 3.922 (d, J = 7.2 Hz, 2H), 2.253–2.351 (m, 1H), 1.750–1.770 (m, 2H), 1.525–1.603 (m, 4H), 1.283–1.344 (m, 2H).

2.4. Preparation of liposomes

1.1 mg of TD53 was dissolved in tertiary butyl alcohol in the presence of appropriate amount of DMPC. After rapid freezing at −70 °C, mixtures were subjected to freeze-drying by freeze dryer (EYELA FDU-1200, Japan). Finely dispersed lipid cakes obtained after overnight drying were dispersed in 1 ml of saline. To facilitate the homogeneous dispersion, the lipid suspensions were placed in a bath-type sonicator for 20 min at 37 °C. To remove the unentrapped/precipitated TD53 from TD53-incorporated liposomes, the liposome dispersions were immediately filtered through a 0.8 μm membrane filter. TD53-entrapped liposomes were stored at 4 °C until use.

2.5. Characterization of liposomes

Aliquots of liposome dispersions containing TD53 were freeze-dried, redissolved in 1 ml of the mobile phase and then filtered through a 0.45 μm membrane filter. The amount of TD53 in the resultant clear filtrate was determined by HPLC analysis as described in the following section.

The mean particle size and polydispersity index (PI) of liposome dispersions were determined by dynamic light scattering method using fiber-optics particle analyzer (FPAR-1000, Otsuka Electronics, Japan). Prior to measurement, dispersions were diluted with filtered saline. The system was used in the auto-measuring mode. The PI is a measure of the uniformity of the particle size distribution in a system studied (Lim and Kim, 2002).

The stability of liposomes was evaluated by determining the release of liposome-entrapped TD53 in the F/2 media at room temperature. To determine the release of TD53 from liposomes, 200 μl liposomes and 600 μl F/2 media were mixed and dialyzed against F/2 media supplemented with 1% Tween80 to avoid the precipitation of TD53 after being released from liposomes. Aliquots were taken from dialysates containing released TD53 at designated time intervals and analyzed by HPLC after appropriate concentration and filtration.

2.6. Algicidal activity tests

The algicidal activity of TD53 and liposomal formulation of TD53 against *H. akashiwo*, *M. aeruginosa* and *C. polykrikoides* were exam-

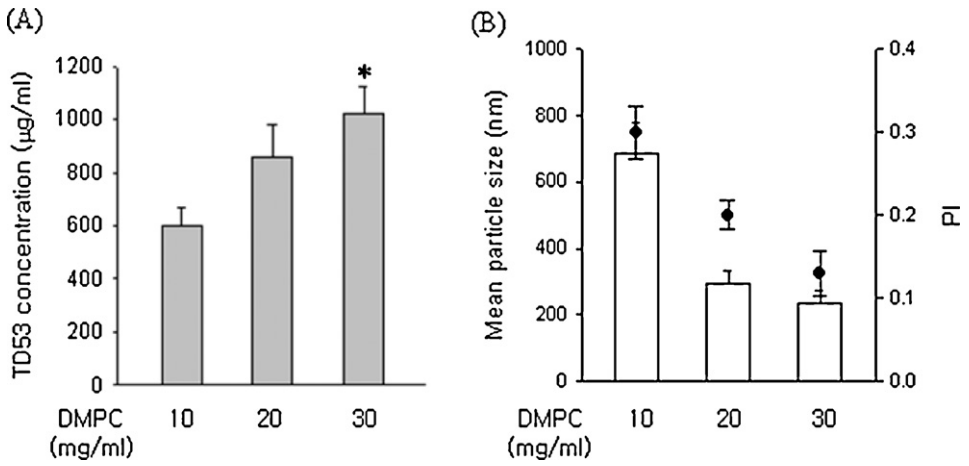

Fig. 2. Effect of DMPC content on the TD53 concentration loaded in liposomes (A) and mean particle size (□) and polydispersity index (●) of resultant liposomes (B). Liposomes were prepared from TD53 and DMPC mixture (1.1 mg TD53 with 10, 20 or 30 mg DMPC) as described in the text. Statistically significant differences compared with liposomes prepared with 10 mg DMPC are indicated by asterisks: $^{*}P < 0.05$. Data are presented as means ± S.D. ($n = 3$).

ined at various concentrations. TD53 was initially dissolved in dimethyl sulfoxide (DMSO) to prepare 50 mM of TD53 stock solution and then underwent the serial dilution with F/2 medium for the desired concentrations. The equivalent amount of DMSO used in the final drug solutions for the algicidal activity tests was also added to the control. Each experiment was carried out in 24 well tissue culture test plates (SPL, Pocheon-Si, Korea) with approximately 1 ml total volume per well. Various concentrations of the test compounds were introduced to the cultures during the exponential growth phase. All the microalgae were exposed to the compounds at final concentrations of 50, 20, 10, 5, 2, 1, 0.1 and 0.05 μM. As the non-harmful algal control, drug solutions were applied at concentrations >100 μM. The concentrations of *H. akashiwo*, *C. marina* and *C. polykrikoides* used in this experiment were approximately 3.2×10^5, 1.2×10^4 and 2.4×10^3 cells/ml, respectively, and those of non-harmful algae used in this experiment were approximately $1–3 \times 10^5$ cells/ml. The algal cells were counted 3 days after inoculation using a Burker Turk hemacytometer with Sedgwick–Rafter counting chamber under an Olympus light microscope with 40× and 100× magnification (Olympus Co., Tokyo, Japan).

Algicidal activities of TD53–liposome and TD53 were expressed as the reduction ratio (%) from the number of cell divisions per day. The reduction ratio (%) was determined using the following equation: % algicidal activity = $(1 - Tt/Ct) \times 100$, where T (treatment) and C (control) are the cell densities with and without algicidal compound at different concentrations and t is the inoculation time (day).

2.7. HPLC analysis of TD53

The concentration of TD53 was analyzed by the HPLC assay. Rosiglitazone was used as an internal standard for the assay. The chromatographic system was consisted of a pump (LC-10AD), an automatic injector (SIL-10A) and a UV detector (SPD-10A) (Shimadzu Scientific Instruments, Tokyo, Japan). An octadecylsilane column (Gemini C18, 4.6 mm × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA) was eluted with a mobile phase consisting of 10 mM phosphate buffer:acetonitrile (25:75, v/v%, pH 4.3). The flow rate was 1.0 ml/min with the detection wavelength set at 320 nm. The retention time of TD53 and the internal standard was 20 min and 5.7 min, respectively.

3. Results and discussion

3.1. TD53 loading in liposomes

We first investigated whether the content of DMPC constituting liposomes may affect the TD53 amount loaded in liposomes. When liposomes were prepared with DMPC varying from 10 mg to 30 mg per 1.1 mg TD53, the amount of TD53 loaded in liposomes increased in a DMPC concentration-dependent manner (Fig. 2). The amount of TD53 loaded in liposomes prepared with 20 or 30 mg DMPC was 1.4- and 1.7-fold higher compared with that in liposomes prepared with 10 mg DMPC. Judging from the limited water solubility of TD53, it is likely that liposomes with higher PC content exhibited higher TD53 loading capacity by providing more spaces to embed TD53 between phospholipid molecules constituting liposomal membranes.

3.2. Characterization of liposomes

To investigate the physical properties of TD53-entrapped liposomes, we measured the mean particle size and polydispersity index (PI) of liposomes relative to the amount of DMPC content. The mean particle size and PI of liposomes decreased in a DMPC content-dependent manner (Fig. 2B). Therefore, liposomes prepared with 30 mg DMPC exhibited the smallest mean particle size (234 ± 38 nm) and the smallest PI (0.130 ± 0.027), as well as providing the highest TD53 loading capacity.

To check the stability of TD53-entrapped liposomes in the presence of F/2 media used in the algicidal studies, we further investigated the stability of liposomes by monitoring the time-dependent release of TD53 from liposomes (DMPC 30 mg) incubated in F/2 media. Up to 96 h incubation, the amount of TD53 released from liposomes increased in a time-dependent manner but still remained extremely low (<2% after 96 h incubation at room temperature). Considering that cells were incubated with drugs for 96 h in the algicidal activity tests, these data indicated that the stability of TD53-entrapped liposomes could be maintained in F/2 media during algicidal activity tests.

3.3. Evaluation of algicidal activities

Previously, various TD derivatives have been synthesized and underwent the extensive screening for algicidal activities against harmful algae (Kim et al., 2010). Among those TD derivatives, TD53

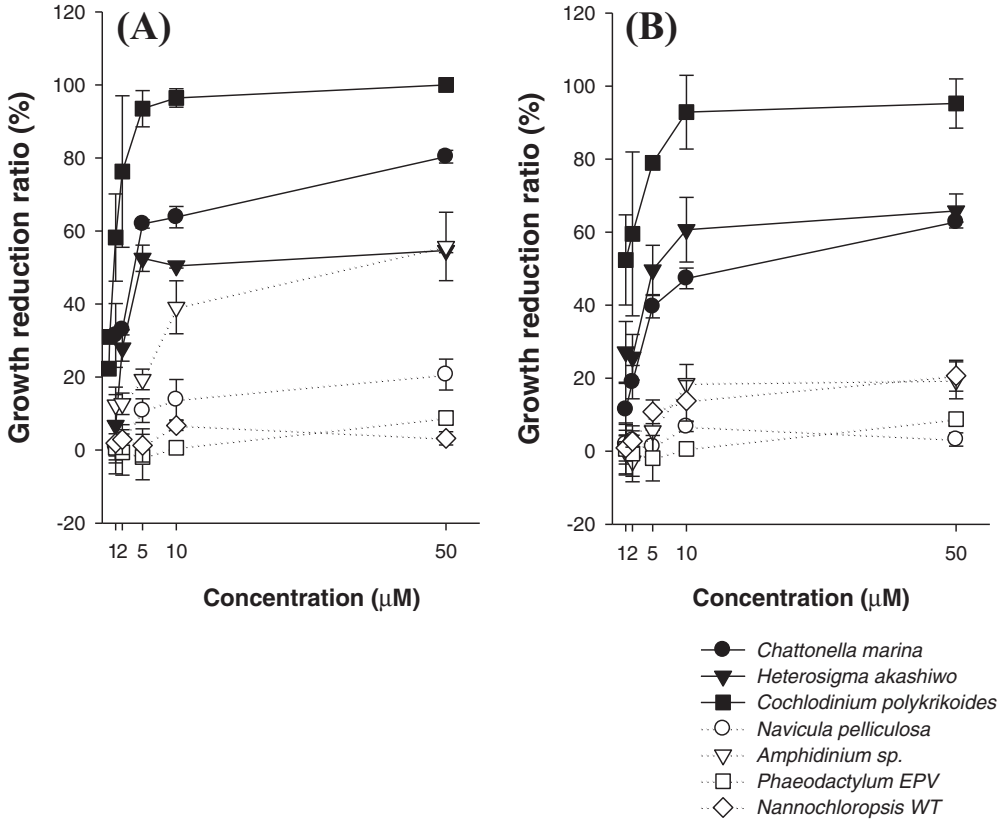

Fig. 3. Algicidal efficiency of liposomal TD53 (A) and free TD 53(B) against several harmful (closed symbols) and non-harmful (open symbols) algal bloom species (means ± S.D., $n = 3$).

exhibited selective algicidal activity against three kinds of common harmful algae, *Heterosigma akashiwo* (*H. akashiwo*), *Cocholodinium polykrikoides* (*C. polykrikoides*) and *Chattonella marina* (*C. marina*), while it was insensitive to all the non-HAB species tested (Fig. 3). TD53 displayed varying degree of algicidal activity for the tested HAB species. Especially, *C. polykrikoides* showed high sensitivity to TD53 with IC_{50} value of 0.949 μM, while moderate algicidal activity was observed with *C. marina* and *H. akashiwo* with IC_{50} values of 4.221 μM and 5.596 μM, respectively (Table 1). While TD53 appeared to be a potent and selective inhibitor against harmful algae, TD53 is poorly water soluble and it is necessary to improve its solubility to ensure further safety evaluation at high doses and also to maximize its effectiveness as a novel algicidal agent.

Liposomes have gained extensive attention as a drug delivery system for many years owing to its capability to provide advantage over conventional formulations. The advantage includes the improvement in the poor aqueous solubility, poor cellular uptake and undesirable in vivo biodistribution. Furthermore, liposomes are regarded as one of very promising targeted drug carriers since phospholipids can able to be attached to specific molecules such as ligands or antibodies. Therefore, as a formulation aid and also as a carrier for enhanced drug delivery to the cells, we developed the liposomal delivery system for TD53 and evaluated its effectiveness in improving the algicidal activity of TD53.

As illustrated in Fig. 3, the algicidal activity of TD53 in different formulations (liposome vs. non-liposome) was examined in harmful and non-harmful algae. The IC_{50} values of TD53 in liposome against *C. marina* and *H. akashiwo* and *C. polykrikoides* were 2.675, 2.029, and 0.480 μM, respectively. Compared to those obtained from free TD53, the IC_{50} values of TD53 in liposome against harmful algae were reduced by approximately 50%, indicating that growth reduction effect on HABs of TD53 could be significantly promoted by liposomal delivery systems (Table 1). In contrast, the algicidal activity of TD53 in liposome against non-harmful algae species, *N. pelliculosa*, *Nannochloropsis oculata* and *Phaeodactylum* EPV did not exceed 20% within the concentration range tested and the IC_{50} values appeared to be beyond the range of concentration tested ($\gg$100 μM). On the other hand, one of non-harmful algae, *Amphidinium* sp. showed relatively higher sensitivity to TD53 in liposome, however, its IC_{50} value was still 20–100 folds higher than those for harmful algae. The effect of empty liposome against *Amphidinium* sp. was also monitored to check the toxicity of liposome itself

Table 1
Comparison of IC_{50} (μM) values of TD53 and liposomal TD53 against the harmful and nonharmful algal species.

	IC_{50} (μM)[a]						
	Harmful algal species			Non-harmful algal species			
	Chattonella marina	*Heterosigma akashiwo*	*Cocholodium polyckrikoides*	*Amphidinium* sp.	*Navicula pelliculosa*	*Phaeodactlum* EPV	*Nannochlopsis* sp.
Liposomal TD53	2.675 ± 0.027	2.029 ± 0.206	0.480 ± 0.001	47.002 ± 1.020	100>	100>	100>
TD53	4.221 ± 0.317	5.596 ± 0.002	0.949 ± 0.000	100>	100>	100>	100>

[a] Values are the means ± S.D. of three experiments.

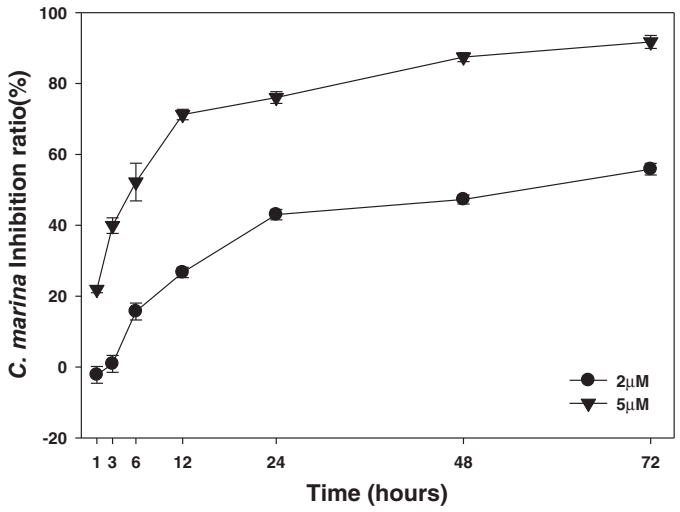

Fig. 4. Time dependent response in the growth reduction of liposomal TD53 on harmful alga, *Chattonella marina*. The control group was considered as no growth reduction ratio (means ± S.D., $n = 3$).

but the growth inhibition effect of empty liposome was insignificant.

At 2 and 5 μM concentrations, TD53 in liposome showed time dependent manner of algicidal activity against *C. marina*. The growth reduction ratios of TD53 in liposome on *C. marina* increased with the incubation time during 6–12 h and then reached a plateau (Fig. 4). Time dependent reduction of growth of other HABs species by TD53 in liposome showed similar pattern as *C. marina* (data now shown). Drugs loaded in liposomes may be taken up by cells as either free drug after being released out from liposomes or as liposome-loaded drugs. Judging from the present data showing that less than 2% TD53 was released from liposomes during incubation with F/2 media used in algicidal activity tests, it is unlikely that most TD53 was taken up by HAB species after being released from liposomes. Rather it suggests that TD53 loaded in liposomes may be taken up by HAB species as liposome-loaded forms. Furthermore, although not measured here, it is presumable that because the cellular uptake of liposomal TD53 is higher than that of free TD53, liposomal TD53 can exhibit increased growth reduction capability in HAB species compared with free TD53.

Given that (i) *C. polykrikoides* blooms have caused heavy damage to fish farms in the Republic of Korea, Japan and other countries (Kim et al., 2007; Onoue and Nozawa, 1989), (ii) liposomal TD53 showed strong algicidal activity against *C. polykrikoides* while it was nontoxic against non-harmful algae including *Nannochlopsis* sp. and *Phaeodactylum* EPV used in animal feed in the aqua industry (at least hundreds fold of selectivity), the use of liposomal TD53 may be a promising way to manage the harmful algal blooms.

4. Conclusion

The liposomal delivery system for TD53, a novel algicial drug was prepared by using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) with narrow size distribution and appeared to be effective to enhance the efficacy of TD53 without any loss of selectivity to harmful algae.

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