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A Study on The Development of The Lipid Based Delivery Systems for The Enhanced Algicidal Effect of Novel Synthetic Agents

조선대학교 대학원

약 학 과

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신규 살조물질의 살조효과 개선을 위한 지질기반 약물전달시스템에 관한 연구

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조선대학교 대학원

약 학 과

조 유 진

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지도교수 지 준 필

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약 학 과

조 유 진

조유진의 석사학위논문을 인준함

위원장 조선대학교 교수 유진철 (인)
위 원 조선대학교 교수 황승림 (인)
위 원 조선대학교 교수 지준필 (인)

2015년 11월

조선대학교 대학원

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신규 살조물질의 살조효과 개선을 위한 지질기반 약물전달시스템에 관한 연구

조 유 진
지 도 교 수 : 지 준 필
조선대학교 대학원 약학과

최근 수십 년 동안 급격하게 증가한 유해조류는 수상생태계, 산업 등에 영향을 미쳐 막대한 경제적 손실을 초래하고 있다. 이러한 문제해결을 위해 초음파 처리나 화학적 응집제 처리 등이 이용되고 있지만 낮은 효율성과 수상생태계 파괴 등의 우려로 인하여 사용이 제한되고 있다. 보다 효율적이고 친환경적이며 안전한 방법을 찾기 위해 새로운 살조물질들을 합성하였다. 그러나 물에 잘 녹지 않은 불용성 특징을 갖는 이 물질들은 수상환경에서 적용하기에는 한계가 있었다.

본 연구에서는 다양한 전하를 띠는 지질기반 약물전달시스템 기술을 개발하여 살조물질을 가용화하고 음전하를 띠는 조류의 세포벽에 대한 반응성을 높여 살조효과를 개선하고자 하였다.

본 연구에 사용한 신규 살조물질인 5-(3,4,5-Trimethoxy-benzylidene)-thiazolidine-2,4-dione(TD10)과 N-[(3,4-dichlorophenyl) methyl] cyclohexanamine(DP92) 에 대하여 프리포뮬레이션 연구를 통해 살조물질의 logP(분배계수)와 용해도를 평가하여 최적의 약물전달시스템을 설계하기 위한 기본적인 정보를 확보하였다. 전달시스템의 입자크기, 표면전하, 봉입효율 등을 기준으로 살조물질의 특성에 맞는 최적의 약물전달시스템을 선정한 후, 유해조류에 적용하여 살조효과를 평가하였다. 또한 광학 현미경을 통하여

유해조류에 대한 살조활성을 비교 확인하고 서로 다른 표면전하를 갖는 전달시스템에 대한 살조활성을 비교 평가하였다.

TD10은 제형 개발 연구 중 온도에 민감한 불안정한 구조로 인하여 장기간 보존 시 분해되는 결과를 보여 더 이상의 실험을 진행하지 않았다.

DP92는 조류 중 적조류에 특이적으로 살조효과를 갖는 살조물질로서 이에 적합한 다양한 표면전하를 갖는 리포좀 제형을 설계하고 이 제형에 DP92을 봉입하여 리포좀의 표면전하에 따른 살조효과를 평가하였다.

다른 lipid 함량을 갖는 리포좀을 제조하여 입자크기, 표면전하에 대한 평가를 진행하였다. 약물: lipid=1: 30 리포좀(3L)에서 입자크기는 150.37±0.49, 120.47±1.04, 133.43 ±0.61 nm, 표면전하는 15.48±1.63, 0.67±0.10, -0.48±0.60 mV 그리고 1: 60 리포좀 (6L) 비율에서 입자크기는 122.67±1.17, 130.74±3.03, 132.20±1.51 nm, 표면전하는 24.64±0.80, 3.61±0.62, 0.77±0.92 mV 를 갖는다). 개발한 리포좀 제형의 살조효과를 평가하기 위하여 총 두 종류의 적조에 살조물질이 봉입된 리포좀을 살포하여 24시간 동안 대조군과 세포성장억제율을 비교 평가하였다.

주로 적조 살조에 이용되는 DP92는 리포좀 제형으로 제조를 하였고, 먼저 실험을 진행하였던 TD10은 온도에 민감하며 불안정한 구조로 장기간 보존 시 약물의 구조 변형으로 인하여 실험을 진행하지 못하였다.

1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane, chloride salt (DOTAP), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC) and L-α-phosphatidylcholine, Egg, Chicken (EggPC) 를 통해 (+), (N), (-) 전하를 띠게 해주었다. 다른 lipid 함량을 갖는 리포좀을 통해 각각 전하에 따른 살조활성을 보았다. 총 두 종류의 적조, 한 종류의 녹조에 살포하여 24h 후 대조군과 비교하여 세포 성장억제율을 관찰하였고 다른 결과를 얻었다.

첫 번째 조류인 Heterocapsa circularisquama (H. circularisquama) 에서는 대조군과 비교하여 3L의 IC50 값은 0.328±0.01, 0.652±0.05 and 0.751±0.10 μM, 대조군 1.815±0.05 μM 에 비해서 3, 3, 2배 (평균 2.7배) 개선의 결과를 보였고

6L에서는 0.328±0.04, 0.612±0.01 and 0.738±0.01 μM 값으로 4, 3, 3배 (평균 6.7배) 높은 값을 보였다. 양전하를 띠는 리포좀은 나머지 리포좀에 비해 가장 좋은 활성을 보였다. 이것은 음전하를 띠는 조류 세포벽과 양전하를 띠는 제형에서 더 높은 정전기적 상호작용으로 인한 영향 때문인 것으로 판단된다. 그러나 양전하의 강도에 따른 차이는 크게 없었다.

두 번째 적조 Heterosigma akashiwo (H. akashiwo)에서도 마찬가지로 3L(+), (N), (-)에서 0.185±0.01, 0.225±0.03 and 0.280±0.01 μM 값을 가지고 대조군 0.629±0.05 μM 에 비해 9, 7, 7배 (평균 7.7배) 로 높은 값을 보였고, 6L에서는 0.157±0.01, 0.192±0.03 and 0.181±0.02 μM 로 6, 3, 2배 (평균 3.7배) 높은 값을 보였다. 그러나 이 적조에서는 DOTAP, DOPC, EggPC 양이 많은 리포좀일 때, 더 낮은 IC50 값을 보였고 지질의 양에 의존하는 살조활성을 보였다.

본 연구를 통하여 우수한 살조효과를 갖고 있으나, 불용성으로 인해 수상환경 적용에 재한이 있는 살조물질에 대해 높은 농도로 가용화하고 음전하를 띠는 조류 세포벽에 대한 반응성을 높여 살조효과를 극대화 할 수 있는 양전하 리포좀 약물전달시스템을 개발하였다

2. Introduction

The harmful algal blooms (HABs), which are so-called ‘red tides’, have an adverse effect on the marine ecosystem and industries throughout the worldwide. The problem of HABs (which is) increased dramatically from the several decades lead to oxygen-depleted water, mass deaths of marine organisms and potential threat to human who consume of seafood (*1, 2*). Also, The HABs have caused serious economic costs of international issues. For instance, the losses have reported to $75 million during the period 1987-2000 in US as well as (approximately) $10 million per year in 1970s in Japan (*3*). The aquaculture industry has losses $60 million in 1995, and $10-20 million for three years from 2000 in Korea (*4*).

The Heterocapsa circularisquama (H. circularisquama) classed as Dinophyceae is occurred due to disturbance of water stratification from western Japan (*5*). It has caused significant damage to shellfish and exhibited the growth of plankton by cell contact (*6*). The Heterosigma akashiwo (H. akashiwo), belonging to Raphidophyceae, have mainly distributed over Japan and occurred fatal damage to farming fishes results from reduction dissolved oxygen levels in water and gas exchange of the gill. It also excretes neurotoxins agglutination of red blood cells or hemolysis from fish blood. (*7*)

Many experts have an effort to resolve the problem of HABs. The treatment approach to manage of HABs has attempted ozonation, chlorination ultrasonic, synthetic or flocculation for algae cohesion and mineral for clay-based flocculant (*8, 9*). However, in this ways have very low efficiency and destroyed the water ecosystem due to nonspecific algae targeting. For that reason, we need to more safety biological strategy for removal of HABs (*10*).

Thiazolidinedione (TD) that activitied by binding to peroxisome proliferator activated receptors (PPAR) was appeared as adjunctive treatment for diabetes mellitus (type2) and related diseases (*11-13*). Also, recently TD was found to growth inhibition on the harmful algae. Based on these results, TD derivatives were synthesized. However, it is hard to apply to aqueous environment due to its low solubility. One of TD derivatives was solubilized via liposomal delivery system (*14*).

To treat red tides, new algicidal agent, N-[(3,4-dichlorophenyl) methyl]

cyclohexanamine (DP92) is synthesized. DP92 induced a high degree of selective algicidal effect on red tides. However, DP92 was not soluble in aqueous solution and improvement of its solubility was needed to enhance its algicidal effect and wide application in marine environment.

Liposomes composed with biocompatible and biodegradable materials are considered as good candidate for drug delivery system. The structure of liposome is similar to biological membrane as lipid bilayer structure and, the structure can encapsulate both hydrophilic and hydrophobic drugs (*15*). According to use various phospholipids, the properties of liposome such as zeta potential, encapsulation efficiency and size can be adjusted (*16*). The liposomes are still widely studied in various fields. The surface modified the liposomes by pegylated reduced body clearance of drug. (*17*, *18*). The membrane penetration of liposomes depends on lipid composition, size and charge of surface. Positive surface charged liposomes lead to better interaction due to a higher binding affinity with negatively cell surfaces with comparison of neutral and negative charged liposomes. Furthemore, positive charged liposomes have enhanced drug extension and absorption than their of negative or neutral charged liposomes (*19*).

To develop optimized delivery system for algicidal agent, we were designed surface charged liposomes with positive 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane, chloride salt (DOTAP), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC) and L-α-phosphatidylcholine, Egg, Chicken (EggPC). The properties of liposomes such as mean diameters, zeta potential and encapsulation efficiency (EE) were characterized. To evaluate the algicidal effect of DP92 loaded liposomes with different surfaces charge, two different kinds of red tides (H. circularisquama and H. akashiwo) were employed (Fig. 1).

3. Materials and Methods

3-1. Materials

1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane, chloride salt (DOTAP), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC) and L-α-phosphatidylcholine, Egg, Chicken (EggPC) were purchased from the Avanti polar lipid Inc (Alabaster, AL, USA), cholesterol (≥99%) was purchased from Sigma Aldrich. Methanol and acetonitrile (ACN) of all HPLC grade were purchased from SK Chemicals (Korea). The synthesized algicide were received to lab. Professor Hoon Cho (Fig. 2).

3-2. Preformulation study of algicidal agents

To characterize solubility of algicidal agent, the agent was mixed with water at 5 mg/ml strried at 25℃ and 1000 rpm for 6 hours (n=3). Partition coefficient of the agent was determined with octanol/water method. The agent (50 mg) was mixed with solution composed with 5 ml of octanol and, of water and stirred at 25℃ and 1000 rpm for 6 hours (n=3). One milliliter of each phase was taken and centrifuged at 25℃ and 13527 rcf for 20min. The supernatant was filtered through a 0.45μm filter then diluted 10 times with ACN. The concentration was calculated by HPLC analysis method. Partition coefficient and logP value were calculated according to the equations:

$$\text{Partition coefficient (P)} = \frac{\text{concentration of drug in octanol (μg/ml)}}{\text{concentration of drug in water(μg/ml)}}$$

.

$$\text{logP} = \log \frac{\text{concentration of drug in octanol (μg/ml)}}{\text{concentration of drug in water(μg/ml)}}$$

3-3. Algae culture conditions

The F/2 medium was filtered with 0.45 μm then 0.2 μm membrane filter and sterilizated in autoclave for 15 min at 121℃. The filtered medium was modulated pH 8 under shaking at 23℃ then incubated under shaking before using for cultivation of the cell. The polystyrene cell cultrue flasks were obtained from SPL corporation. To evaluated activity of algicide, two different kind of algae H. akashiwo and H. circularisquama were obtained from Korea marine microalgae culture center (KMMCC). Harmful algaes were grown in a culture flask at 20℃ under constant light cycle 10 light 14 dark, 40 PPFD of Light intensity and 33 psu of salinity in F/2 medium (*20*).

3-4. Preparations of liposomes

To prepare liposomes, thin film hydration method was adopted as the conventional method (*21*). Briefly, appropriate weight of drug and cholesterol were mixed with organic solvent (methanol: chloride = 1: 2, v/v) in a 20ml round-bottomed flask, then phospholipid (DOPC, DOTAP or EggPC) was added (Table 1). The 20 ml round-bottomed flask was connected to a rotary evaporator (RV 8, IKA, China) which rotated for 20 min at 100 rpm under vaccum in thermostatically controlled heating baths (HB 10, IKA, China) at 40℃. After 20 min, organic solvent was evaporated and thin lipid film was obtained inside surface of the round-bottomed flask. Subsequently, the dried lipid film was dispersed with 1 ml of hot distilled water. To remove free drug liposomes were filtered through a 0.45 μm syringe membrane filter. Appropriate liposome size was controlled by filtering liposome extruder (Liposo-Fast, AVESTIN. Inc, Canada) composed of polycarbonate membrane with 100 nm pore diameter and 19 mm diameter. Liposomess suspensions were stored at 4℃ until further studies. Drug free liposomes were prepared for toxicity evaluation of liposomes on algae.

3-5. Quantification of algicidal agents by HPLC analysis

Algicidal agents were determined by HPLC analysis. The chromatographic systems

were consisted of pumps (LC-20AD, LC-10ADVP), an autosampler (SIL-10ADVP) and UV detector (SPD-10VP) (Shimadzu Scientific Instruments, Tokyo, Japan). C18 column (Luna C18, 4.6 mm X 150 mm, 5 μm; Phenomenex, Torrance, CA, USA) was used and was heated at 40℃. TD10 and DP92 were analyzed with different mobile phase and UV wavelength conditions. To analyze TD10, mobile phase was consisted of ACN: 0.1% TFA = 1:1 and detection wavelength was fixed at 340 nm. DP92 was eluted with a mobile phase consisting of 10 mM ammonium acetate: ACN (10:85, v/v%) and detection wavelength was fixed at 237 nm. Injection volume was 20 μl and flow rate was 1.0 ml/min.

3-6. Characterization of liposomes

The size distribution, polydispersity index (PI) of liposomes dispersions were determined by dynamic light scattering method using a Zeta sizer (Zetasizer nano S, Malvern Instruments Ltd., UK). Zeta potential of liposomes dispersionwas measured using a zeta potential analyser (Zetasizer9000, Malvern Instruments Ltd., UK). Liposomes dispersion was diluted 10 times with distilled water or F/2 medium before size or surface charge measurement.

To quantify the algicidal agents loading in liposomes, the HPLC assay was employed. The aliquot of the algicidal agent loading liposomes (0.1 ml) was dilued 10 times with ACN (0.5 ml) and the solution was directly injected to HPLC system. The encapsulation efficiency (EE) was calculated according to the equation:

$$\mathrm{EE}(\%) = \frac{\mathrm{Encapsulated\ drug}(\mu g/ml)}{\mathrm{Total\ drug}(\mu g/ml)} \mathrm{X100}$$

3-7. Algicidal activity study

To evlaulate algicidal activity of developed liposomes, H. akashiwo and H. circularisquama were employed as harmful algae. Liposomes prepared with three different phospholipids at different concentration (Table2). The algicidal activity of drug in DMSO as control and drug-loaded liposomes were evaluated against H. akashiwo or H. circularisquama at various concentration of drug. Each formulation was calibrated using F/2 mediaum to adjust same concentration of drug through the calculated EE% value by HPLC. The harmful algae that was treated 20 μl of liposomesand 180 μl of algae in 96-well. The algae were exposed to liposomess at final concentration 0.1, 0.2, 0.5, 1, 2 and 5 μM on H. circularisquama or 0.05, 0.1, 0.2, 0.4, 0.5 and 1 μM on the H. akashiwo, respectively. The treated algae was incubated in algae culture condition for 24 hours, then evaluated a growth inhibition effect by optical microscope with 400× magnification. Algicidal activity was calculated according to the equation:

$$\text{Algicidal activity } (\%) = \left(1 - \frac{\text{Tt}}{\text{Ct}}\right) \times 100$$

Where T is the number of treated cells and C is the number of control.

3-8. Statistical analysis

IC50 values were calculated through nonlinear regression, data were fitted to a sigmoidal dose-response relation using the program Graphpad Prism (ver. 5.01; GraphPad Software, Inc., San Diego, CA), according to the equation:

$$\text{Y} = \frac{100}{(1 + 10\text{^}((\text{LogIC50} - \text{X}) * \text{HillSlope}))}$$

where X is log of dose or concentration and Y is normalized response, 0 to 100%, increasing as X increases. The logIC50 is same log units as X and Hillslope is slope factor or hill slope, unitless. 95% confidence intervals of ID50 were also calculated

using the program Graphpad Prism (ver. 5.01; GraphPad Software, Inc., San Diego, CA).

Statistical analysis was performed using Student's t-test and analysis of variance (ANOVA). A p-value of less than 0.05 was considered significant.

4. Results and discussion

4-1. Preformulation study of algicidal agents.

In order to characterize physicochemical properties of the algicidal agnets, preformulation studies, especially solubility, partition coefficient and logP were performed. The results of preformulation studies were listed in Table 2. As shown in Figure 2, TD10 has three carboxyl groups those are polar and gift hydrophilic property to TD10. Thereby, TD10 has the higher water solubility as 62.18± 0.30 μg/ml compared to that of DP92. Furthermore, partition coefficient and logP of TD10 were lower than those of DP92 as expected. However, the results represented that not only DP92 but also TD10 were not enough soluble to water as algicidal agent. The preformulation results and the property that we consider the charge interaction between delivery system and algae lead to liposomes as an adequate candidate for further study. Liposomes have double layers structure that encapsulates not only hydrophobic materials but also hydrophilic materials (*22*). Phospholipids employed for liposome preparation are biodegradable and biocompatible, furthermore, they can be modified with various chemical moieties for different surface charge in aqueous environment (*23*, *24*).

4-2. Preparation and characterization of TD10 loaded liposomes

To prepare TD10 loaded liposomes, three different kinds of phospholipids were employed. DOTAP, DOPC and EggPC were used for positively charged (L(+)), neutally charged (L(N)) and negatively charged (L(-)) surface of liposomes, respectively. The composition of the liposomes was listed in Table 1. The diameters of liposomes were under 250 nm with narrow distribution regardless of the kinds and amount of phospholipids as presented on Fig. 3. Encapsulation amount of TD10 into liposomes increased in proportion to amount of phospholipid (Fig.4).

The liposomes prepared with 30% of phospholipids (1:30) ca. 80 μg/ml of TD10, and the value was 13 times higher than the solubility of TD10. Furthermore, EE (%) of the

liposomes prepared with 30% of phospholipids (1:30) was the highest value as 79%. Therefore, we selected the liposomes prepared with 1:30 phospholipids as the optimized liposomes.

However, TD10 was unstable during storage although it was stored at 4℃. As shown in Fig.5, HPLC chromatograph was shown that TD10 was degraded and the physicochemical properties were changed. The degradation of TD10 was due to the reactive moieties of TD10 such as carboxyl groups. Therefore, we stopped further study with TD10.

4-3. Preparation and characterization of DP92 loaded liposomes

Another novel synthesized algicidal agent- DP92 was employed for further study. The development study of liposomes for DP92 was started from the optimized liposomes composition for TD10. As shown in Fig.6, the diameters of the DP92 loaded liposomes with 1:30 DOTAP (3L(+)), DOPC (3L(N)) and EggPC (3L(-)) were 150.37±0.49, 120.47±1.04 and 133.43±0.61 nm, respectively. The DP92 loaded liposomes prepared with 1:60 DOTAP (6L(+)), DOPC (6L(N)) and EggPC (6L(-)) had similar diameters with those of 3L liposomes. The amount of phospholipids did not effect on the diameter because three milligram of phospholipids was enough amount to stabilized and minimized double layers of liposomes. The zeta potential value of the 3L (+), (N) and (-) were 15.48±1.63, 0.67±1.14 and -0.48±0.86 mV. By addition of phospholipids, the zeta potential of 6L (+), (N) and (-) increased as 24.64±0.80, 3.61±0.62 and 0.77±0.92 mV, sequentially (Fig.7). DP92 was loaded into the liposomes and encapsulation efficiency (EE) of liposomes were ca. 70 to 90% (Fig. 8, Table 3). Liposomes composoed with DOPC loaded the lowest concentration of DP92 in both two different concentration of DOPC (3 and 6 mg). The exact mechanism was uncertain, however, DP92 and DOPC were not compatible compared to DOTAP or eggPC. The amount of phospholipids did not increase EE of DP92 and the results represented that the liposomes stably formed with over 3 mg of phospholipids.

4-4. Evaluation of algicidal activity of DP92 loaded liposomes

H. akashiwo and H. circularisquama which are are swimming marine algaes were employed as HABs models to evaluate algicidal activity of DP92 loaded liposomes (Fig. 9). Firstly, cytotoxicity of the liposomes without DP92 was evaluated with various concentrations. The cytotoxicity of the control group- DMSO increased in proportion to the concentration of DMSO, especially, 1 μM of DMSO killed 100% of both algaes. However, the developed liposomes without DP92 showed the algicidal activity on 20% and less of both algaes at whole concentrations and it seemed that the liposomes were not cytotoxic (Fig. 10,11).

Two species of HABs were treated with DP92 loaded liposomes at different concentration ranges of DP92 and DP92 in DMSO was used as control group. Firstly, H. circularisquama was treated with DP92 loaded liposomes and DP92 in DMSO. IC50 value of control group was 1.815±0.05 μM. As presented in Fig. 12, IC 50 value of 3L(+), (N) and (-) were 0.328±0.01, 0.652±0.05 and 0.752±0.10 μM, respectively. Similar IC50 value were gained with 6L (+), (N) and (-) as 0.328±0.04, 0.612±0.01 and 0.738±0.01 μM, respectively. The positive charged liposomes with DP92 brought the strongest algicidal acitivity, but, there was no significant difference in the strength of positive charge with two different DOTAP concentrations. The algicidal activity of the DP92 loaded liposomes were investigated with another HABs- H. akashiwo. As presented in Fig. 13, IC50 value of control group was 0.629±0.05 μM. IC 50 value of 3L(+), (N) and (-) were 0.185±0.01, 0.225±0.03 and 0.280±0.01 μM, and those of 6L(+), (N) and (-) were 0.157±0.01, 0.192±0.03 and 0.181±0.02 μM, resepctively. When DP92 was loaded into the positive charged liposomes, the strongest algicidal activity was induced similar with the results of H. circularisquama. Furthermore, as presented in Fig. 14 and 15, the aligicidal activity induced with different ratio when same concentration of DP92 in DMSO or DP92 loaded positively charged liposomes was treated with HABs. DP92 loaded positively charged liposomes destroyed the structures of H. circularisquama and H. akashiwo within 2 hours, however, same concentration DP92 in

DMSO did not induce any change.

The strongest algicidal activity of DP92 loaded positively charged liposomes was due to solubilization of DP92 that increased 90 times than that of DP92 and the electrostatic interaction between negatively charged wall of HABs and positively charged surface of liposomes (*25*). Especially, H. circularisquama and H. akashiwo have a flagellum and swim in aqueous environment. DP92 can induce algicidal activity on H. circularisquama and H. akashiwo in culture environment, however, it is hard to interact with them due to their fast movement- swimming in real aqueous environment. Therefore, the charge interaction between the positively charged surface of liposomes and the wall of H. circularisquama or H. akashiwo can efficiently deliver DP92 to algaes and plays very important role in algicidal activity.

5. Conclusion

Liposome systems were used to increase the solubilization of the drug with low solubility. We were prepared liposomes with charge, the result have enhanced effect of drug through accused electrostatic interactions between negatively charged cell and positively charged formulation. Overall, the drug-loaded liposomes were able to see that the effect appears higher. However, although the formulation of liposomes charge were a different effect depending on the type of red tide. In conculsion, the drug delivery system is an effective means to compensate the limitations of drugs, as well as for loading the drug to enhance the membrane permeability of the cells in the formulation.

6. Reference

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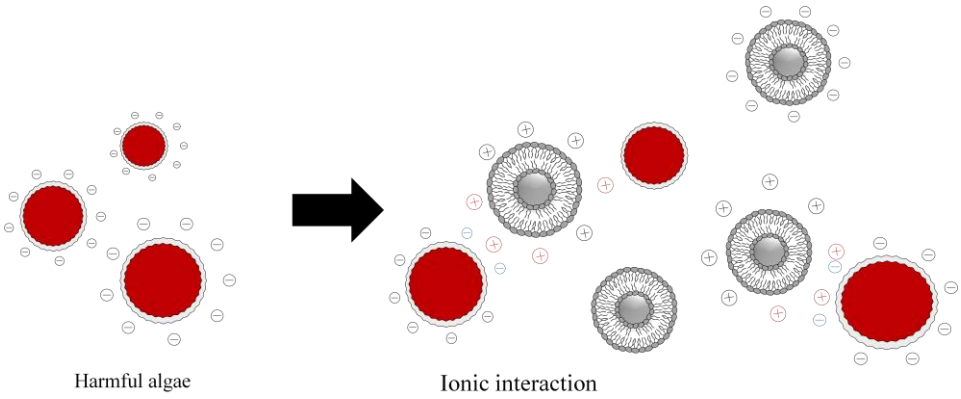
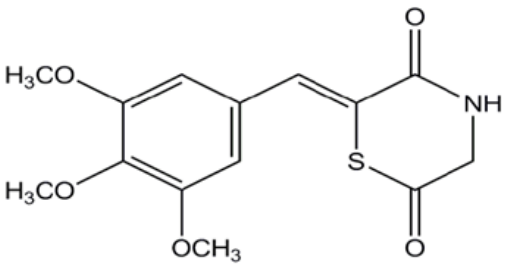


Fig. 1. Schematic representation for ionic charge interaction between the positive charged liposomes and negative charged wall of harmful algae.

(a)



(b)

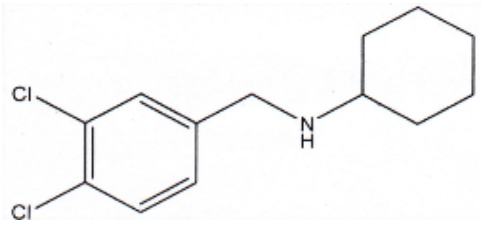


Fig. 2. The chemical structure of (a) TD10 (5-(3,4,5-Trimethoxy-benzylidene)-thiazolidine-2,4-dione) (b) DP92 (N-[(3,4-dichlorophenyl) methyl] cyclohexanamine).

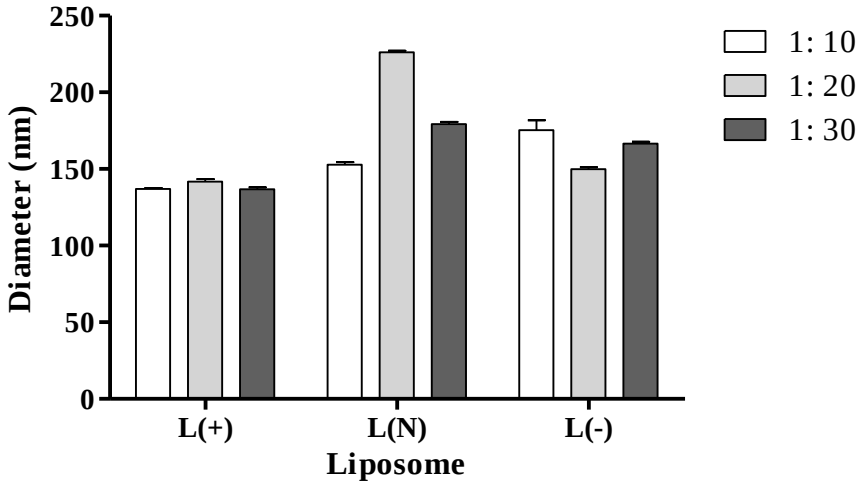


Fig. 3. Diameter of TD10 loaded liposomes with positive and neutral and negative charge (drug: lipid= 1: 10, 20 and 30) by zeta sizer. Data are presented as means± S. D (n=3).

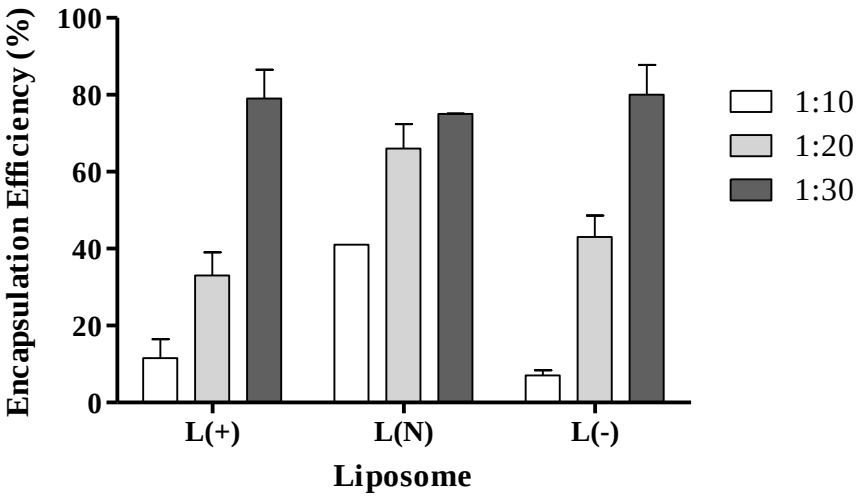


Fig. 4. Encapsulation efficiency of TD10 loaded liposomes with positive and neutral and negative charge (drug: lipid= 1: 10, 20 and 30) by HPLC. Data are presented as means± S.D (n=3).

(a)

(b)

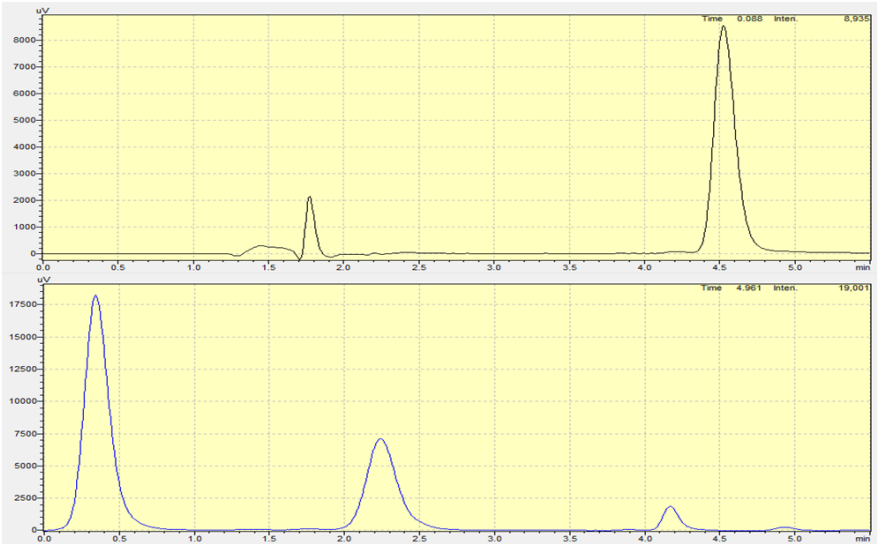


Fig. 5. Encapsulation efficiency of TD10 loaded liposomes by HPLC (a) initial (b) after one month.

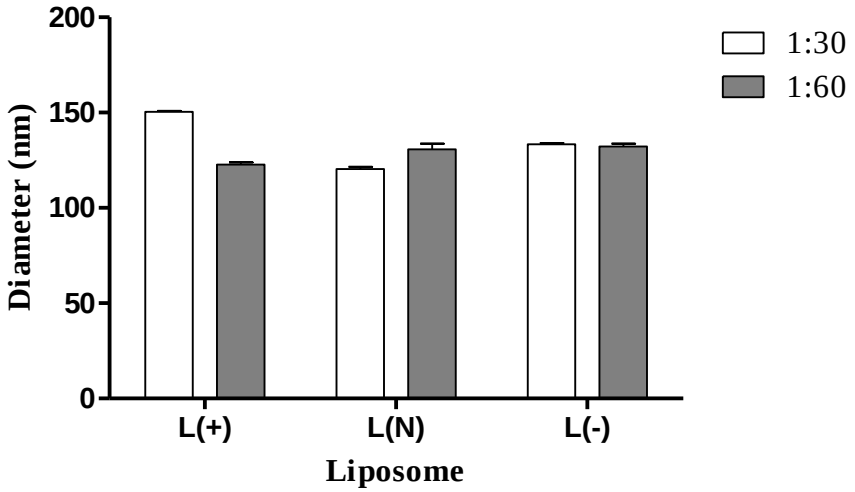


Fig. 6. Diameter of DP92 loaded liposomes with positive and neutral and negative charge (drug: lipid= 1: 30 and 60). Data are presented as means± S.D (n=3).

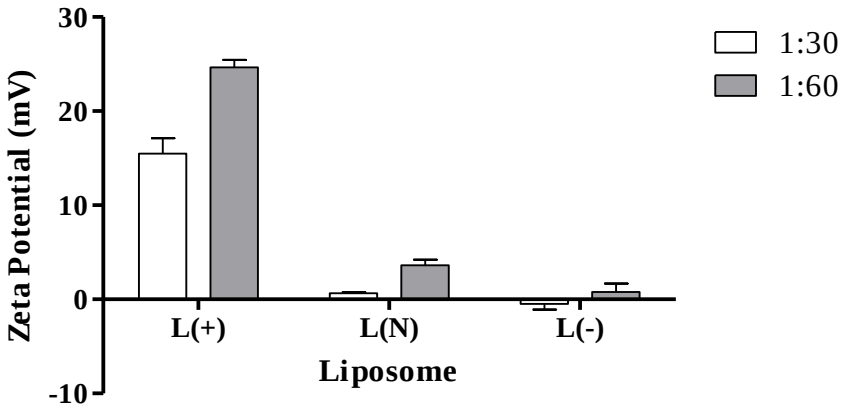


Fig. 7. Zeta potential of liposomes with positive and neutral and negative charge (drug: lipid= 1: 30 and 60) Data are presented as means± S.D (n=3).

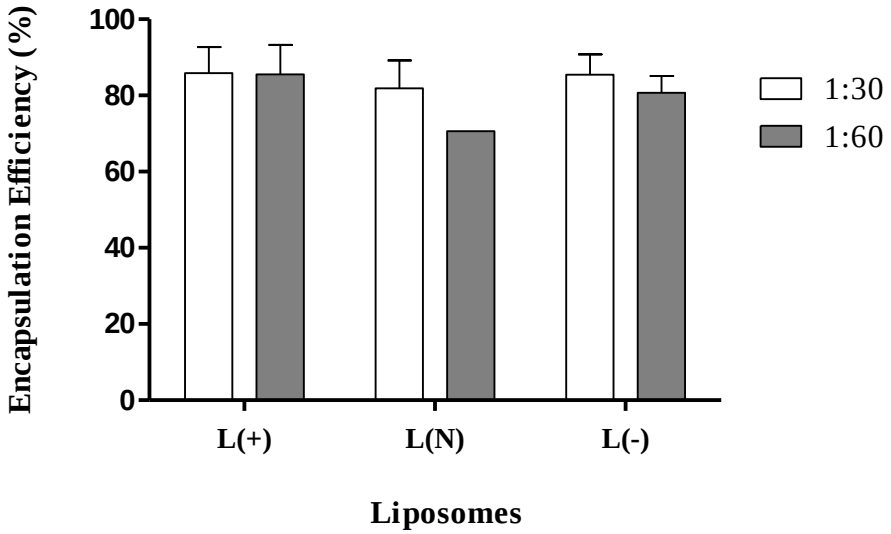
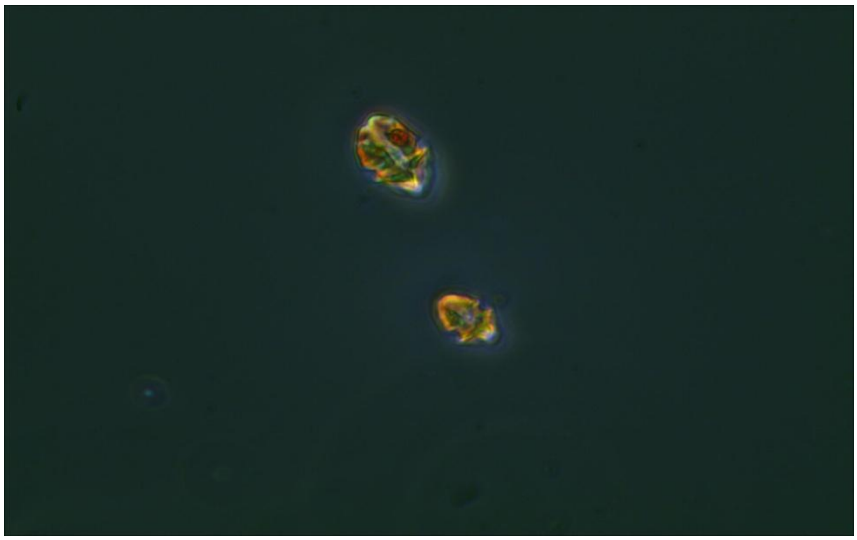


Fig. 8. Encapsulation efficiency of DP92 loaded liposomes with positive and neutral and negative charge (drug: lipid= 1: 30 and 60) by HPLC. Statistically no significant differences: $P < 0.05$. Data are presented as means± S.D (n=3).

(a)



(b)

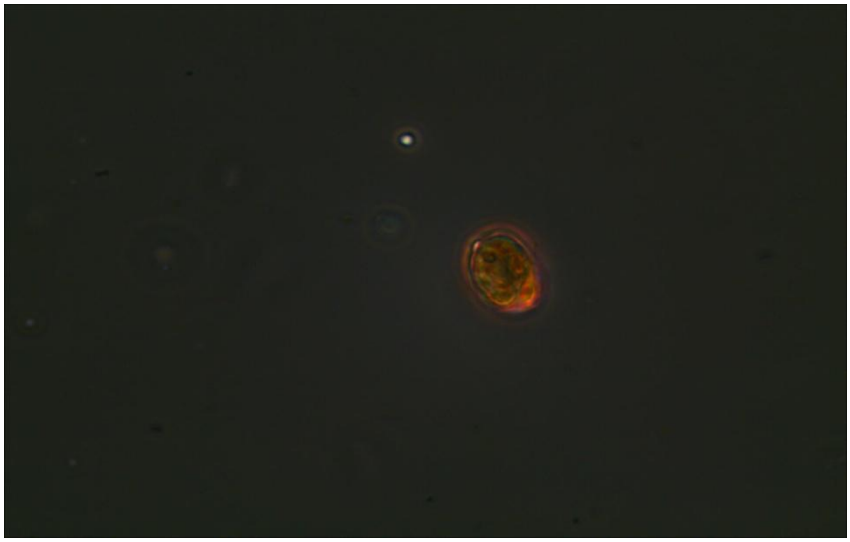
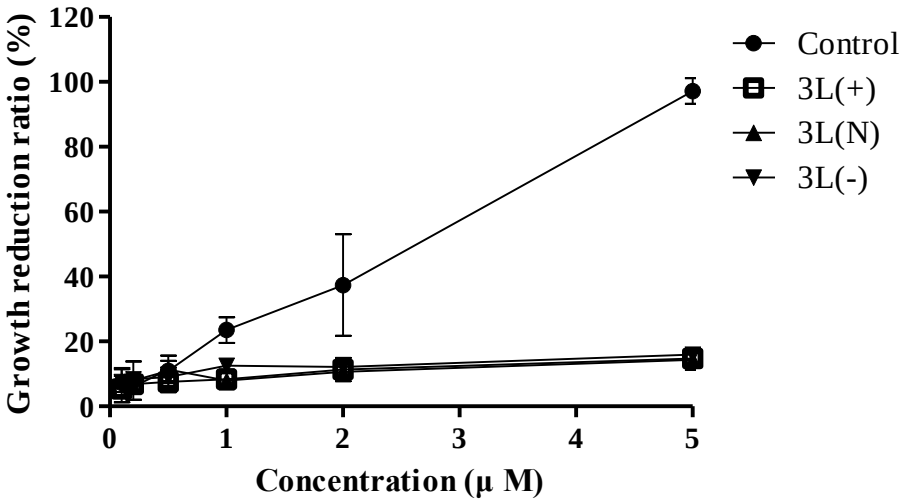


Fig. 9. Harmful algae by electro-microscopy at 400X (a) H. circularisquama (b) H. akashiwo.

(a)



(b)

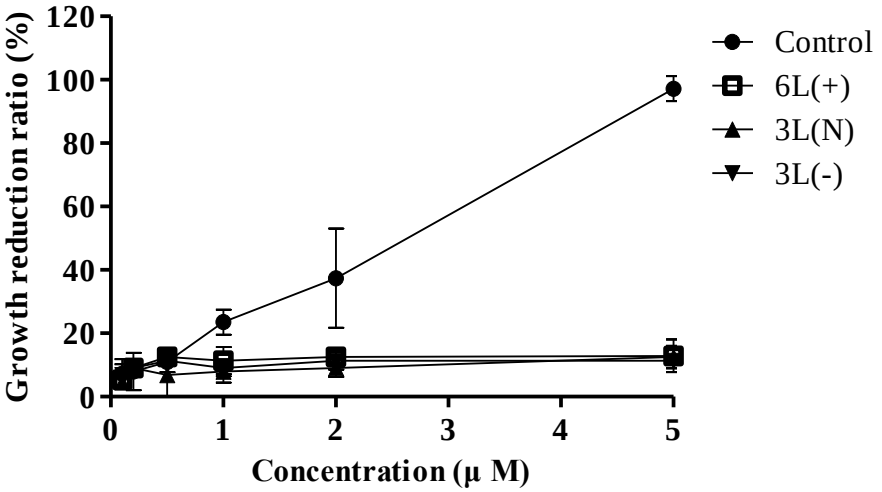
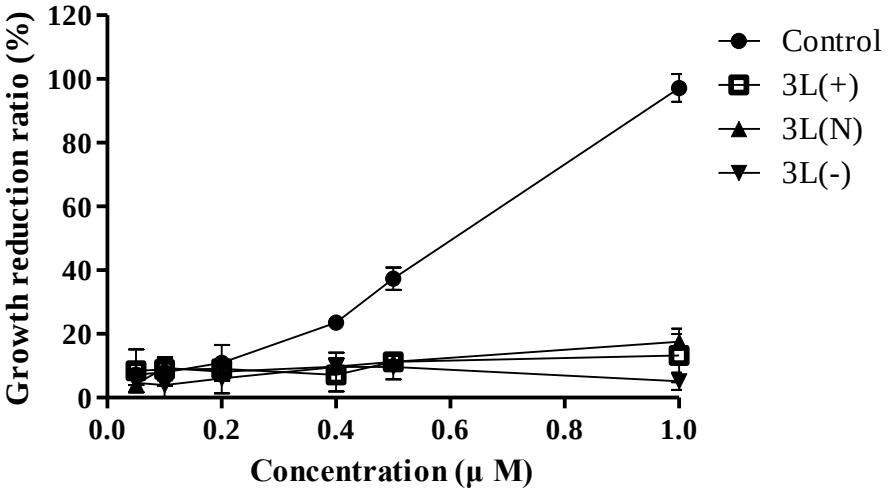


Fig. 10. The toxicity evaluation of blank liposomes on H. akashiwo (a) drug: lipid = 1: 30 and (b) drug: lipid = 1: 60. Data are presented as means± S.D (n=3).

(a)



(b)

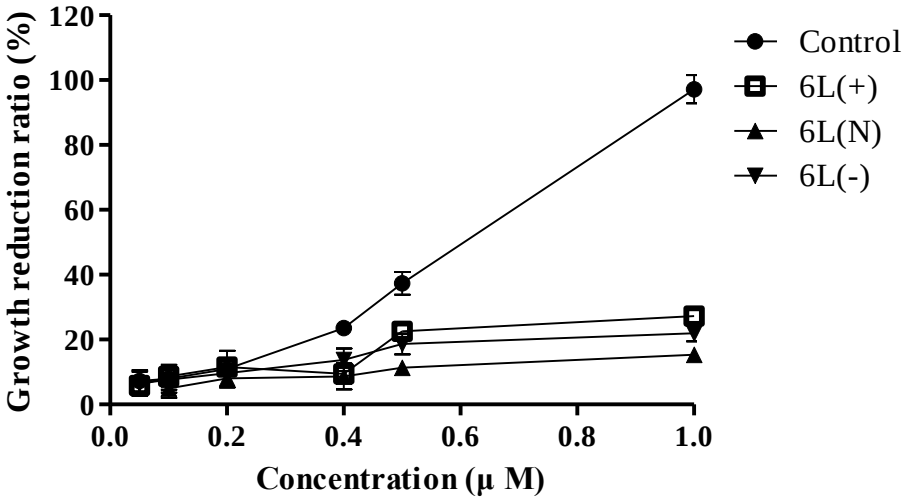
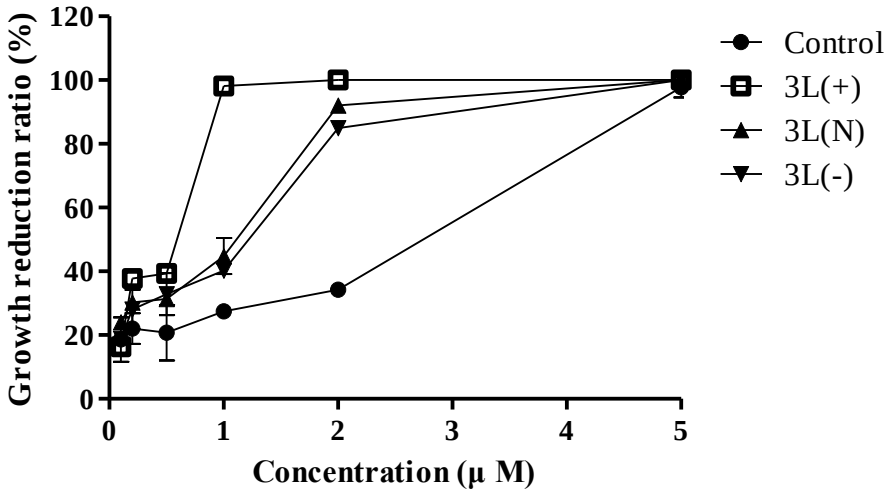


Fig. 11. The toxicity evaluation of blank liposomes on H. akashiwo (a) drug: lipid = 1: 30 and (b) drug: lipid = 1: 60. Data are presented as means± S.D (n=3).

(a)



(b)

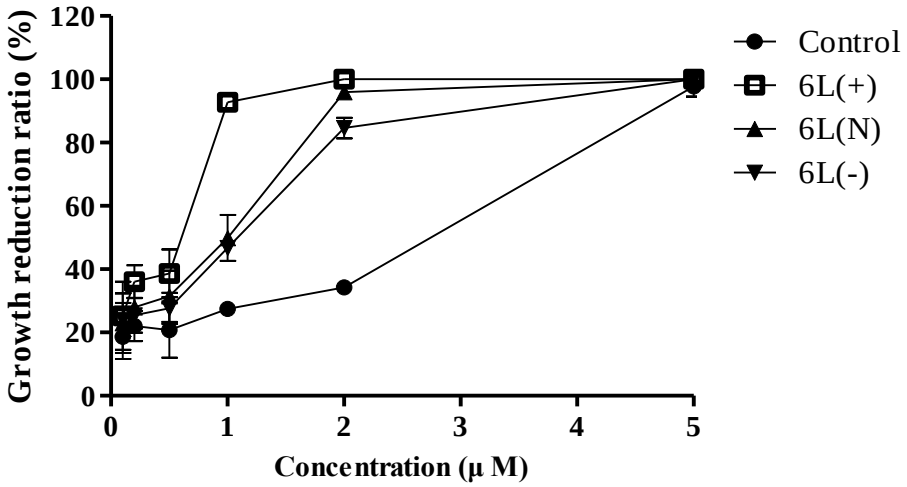
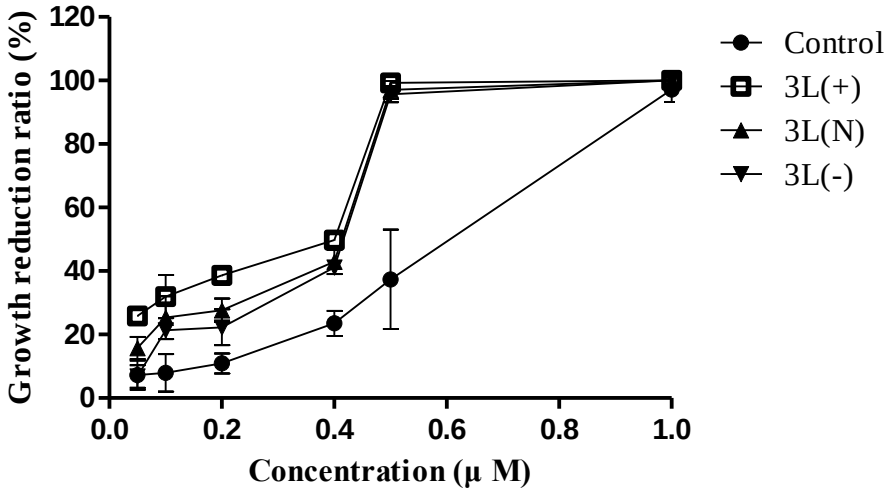


Fig. 12. The algicidal activity of liposomes with positive, neutral and negative charge on H. circularisquama (a) drug: lipid = 1: 30 and (b) drug: lipid = 1: 60. Data are presented as means± S.D (n=3).

(a)



(b)

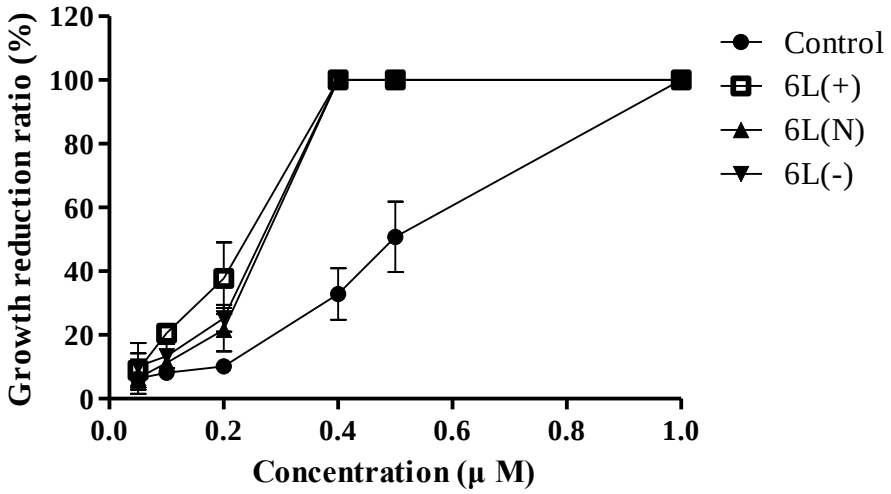


Fig. 13. The algicidal activity of liposomes with positive, neutral and negative charge on H. akashiwo (a) drug: lipid = 1: 30 and (b) drug: lipid = 1: 60. Data are presented as means± S.D (n=3).

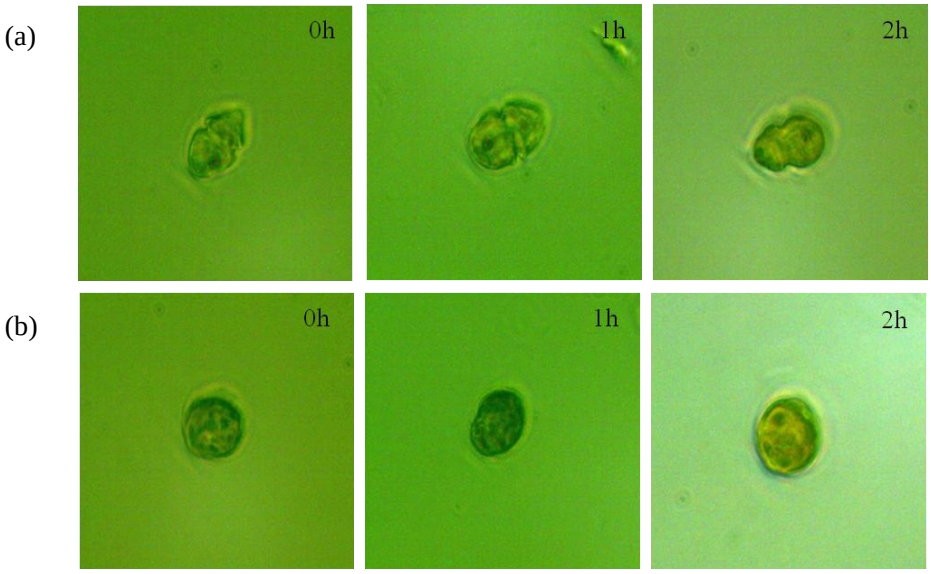


Fig. 14. Algicidal activity of DMSO on harmful algae at 0, 1, 2h by electromicroscopy at 400X (a) H. circularisquama at 5 μM (b) H. akashiwo at 1 μM.

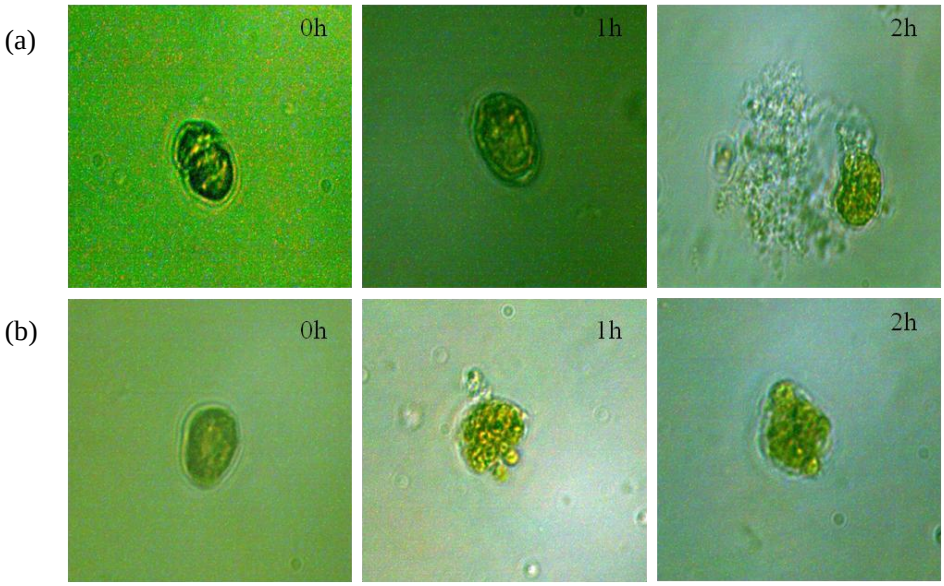


Fig. 15. Algicidal activity of DP92 loaded liposomes with positive charge on harmful algae at 0, 1, 2h by electromicroscopy at 400X (a) H. circularisquama at 5 μM (b) H. akashiwo at 1 μM.

Table 1. The composition of prepared liposome with different phospholipid (DOTAP, DOPC and EggPC). The dispersion used purified water.

Lipid / Type	No.	Drug (mg)	Lipid (%)	Cholesterol (%)	Dispersion(ml)
DOTAP	1L(+)	0.1	1	0.1	1
	2L(+)		2		
	3L(+)		3		
	6L(+)		6		
DOPC	1L(+)		1		
	2L(+)		2		
	3L(+)		3		
	6L(+)		6		
EggPC	1L(+)		1		
	2L(+)		2		
	3L(+)		3		
	6L(+)		6		

Table 2. The preformulation of TD10 and DP92 (n=3).

Drug \ Type	Solubility in water (ug/ml)	Partition coefficient	LogP
TD10	62.18±0.30	3.57	0.55
DP92	39.84±0.95	1651.19	3.22

Table 3. Diameter, Zeta potential and Encapsulation Efficiency of DP92-loaded liposomes with positive and neutral and negative charge (drug: lipid= 1: 30 and 60) by zeta sizer and HPLC. Data are presented as means± S.D (n=3).

Type	Z-average (nm)	PI	Zeta potential (mV)	EE%
3L (+)	150.37±0.49	0.11±0.01	15.48 ± 1.63	85.91 ± 6.81
3L (N)	120.47±1.04	0.29±0.04	0.67 ± 0.10	83.74 ± 5.82
3L (-)	133.43 ±0.61	0.10±0.00	-0.48 ± 0.60	85.49 ± 5.26
6L (+)	122.67±1.17	0.36±0.01	24.64 ± 0.80	85.54 ± 7.72
6L (N)	130.74±3.03	0.12 ± 0.03	3.61 ± 0.62	70.66 ± 0.00
6L (-)	132.20±1.51	0.17 ± 0.02	0.77 ± 0.92	80.68 ± 4.41

ABSTRACT

A Study on Development of Lipid Based Delivery System for the Enhanced Algicidal Effect of Novel Synthetic Agents

Yu Jin Cho

Advisor: Prof. Jun-Pil Jee

Department of Pharmacy

Graduate School of Chosun University

HABs (harmful algae blooms) have caused serious problems on the aquatic ecosystem and fishery industry, and led to tremendous economic losses in recent decades. The ultrasonication or chemical coagulant has treated in order to solve the problems but the application has been restricted due to low efficiency and destruction of aquatic ecosystem.

The new algicidal agents were synthesized to find more efficient, eco-friendly and safe manner by our research team. However, the application of the agents have been limited in water environment due to their low water solubility.

In this study, we developed and evaluated the lipid-based drug delivery systems modified with various surface charges in order to solubilize algae agents and enhance the algicidal activity against HABs structured with negative surface charge. Firstly, to characterize the properties of the new algicidal agensts (5-(3,4,5-Trimethoxy-benzylidene)-thiazolidine-2,4-dione (TD10) and N-[(3,4-dichlorophenyl) methyl] cyclohexanamine (DP92)), their solubility, partition coefficient and logP were evaluated by preformation studies. On the basis of the characterized properties, the proper drug delivery system was designed and evaluated for our algicidal agents. Especially, the effect of surface charge of delivery systems on algicidal activity was evaluated by preparation of three different surface charged delivery systems.

TD10 turned into sensitive against external stimulus such as temperature or vacuum and was degraded during storage. We stopped further study with TD10 due to instability of TD10.

We choose liposomes as the proper delivery system for DP92 due to its simplicity to prepare surface charged form, solubilization capacity and biodegradable /biocompatible properties. Three different phospholipids were employed to design various surface charged liposomes. The positively charged liposomes (+) were prepared with 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane, chloride salt (DOTAP). Neutrally charged liposomes (N) were prepared with 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (DOPC) and L-α-phosphatidylcholine (EggPC) was employed for negatively charge liposomes (-). The liposomes were dispersed as around 150 to 200 nm diameter. The diameters of the liposomes with 3 mg (3L) of DOTAP, DOPC and EggPC were 150.37±0.49, 120.47±1.04, 133.43 ±0.61 nm, respectively, and surface charges were 15.48±1.63, 0.67±0.10, -0.48±0.60 mV, respectively. The diameters of the liposomes with 6 mg (6L) of DOTAP, DOPC and EggPC were 122.67±1.17, 130.74±3.03, 132.20±1.51 nm, respectively, and surface charges were 24.64±0.80, 3.61±0.62, 0.77±0.92 mV, respectively. The algicidal activity of the developed liposomes was evaluated by monitoring the inhibition of HABs (Heterosigma circularisquama and Heterosigma akashiwo) growth ratio. The IC50 value of control group composed with DP92 in DMSO was 1.815±0.03 μM. The IC50 values of 3L with (+), (N) and (-) were 0.328±0.01, 0.652±0.05 and 0.751±0.10 μM, respectively, and 6L with (+), (N) and (-) were 0.328±0.04, 0.612±0.01 and 0.738±0.01 μM, respectively, on H. circularisquama. The algicidal activity of the liposomes was two to four times stronger compared to that of control group. Especially, the positively charged liposomes showed the strongest algicidal activity. The solubilization of DP92 and charge interaction between the negative charge wall of H. circularisquama and positive charge of liposomes enhanced algicidal activity of DP92. However, the strength of surface charge did not affect much on algicidal activity. In the case of H. akashiwo, the IC50 value of control group was 0.629±0.05 μM. The IC50 values of 3L with (+), (N) and (-) were 0.185±0.01, 0.225±0.03 and 0.280±0.01 μM, respectively, and 6L with (+), (N) and (-) were 0.157±0.01, 0.192±0.03 and 0.181±0.02 μM, respectively. The algicidal activity

of the liposomes was two to six times stronger compared to that of control group. The algicidal activity of DP92 on H. akashiwo was affected by the phospholipids concentration and surface charge of liposomes. Increase of the phospholipids concentration enhanced the algicidal activity, and the positive surface charge of liposomes led to the highest algicidal activity of DP92. However, the phospholipids concentration played more important role in algicidal acticity than surface charge of liposomes on H. akashiwo.

The aligicidal agents were successfully solubilized with the developed liposomes, and the encapsulation of the agents into proper delivery system enhanced their algicidal activity up to 6 times stronger. Furthermore, the surface charge of the liposomes affected on algicidal activity and, especially, positively charged liposomes showed the strongest algicidal effect compared with neutrally or negatively charged liposomes as we expected.

Keywords: Harmful algae, Liposome, Solubilization, Positive surface charge, Algicidal activity, Delivery system

감사의 글

가장 먼저, 처음 실험실 생활을 하여 모든 것이 서툴렀던 저를 직접 가르쳐주시고 2년 간 실험실을 넓혀가며 지금의 제가 되기까지 이끌어주신 지도교수님 지준필 교수님께 무한한 감사와 사랑을 드립니다. 교수님의 많은 가르침 덕분에 무사히 석사학위 논문을 제출하고 졸업을 하게 되었습니다.

또한 제가 졸업할 때까지 항상 지켜보고 도움을 주신 유진철 교수님, 황승림 교수님께 감사를 드립니다.

대학원 생활 동안 실험실 생활을 하며 힘든 일이 있을 때 항상 웃음과 에너지를 주었던 친구 김미리, 서로의 말동무가 되어주며 외로움을 같이 나눈 유기화학실험실의 김세연 언니 그리고 잠깐이지만 같이 실험실 생활을 하며 저의 활력소가 되어준 정근숙 언니, 박태성 오빠에게도 진심으로 감사드립니다.

마지막으로 언제나 저를 믿어주는 저의 가족, 지금까지 키워주신 부모님께 감사드리며 제가 가장 아끼는 동생들과 기쁨을 함께 나누고 싶습니다.