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The Aggregation State and Hemolytic Activity of Nystatin

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니스타틴의 응집 특성 및 용혈 활성

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ABSTRACT–The aggregation behavior of nystatin (NYS) in the presence of pluronic F127, triblock copolymer of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO), was measured and correlated with hemolytic activity. Antifungal activity was also studied using Saccharomyces cerevisiae as a model strain. The critical aggregation concentrations (CAC) of the drug were 50.1, 108.0, 134.2, 154.3, and 217.9 μM at 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% pluronic F127 solution, respectively. The levels of NYS required to start lysis of erythrocytes were about 80, 100, 125, 150, and 200 μM at 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% pluronic F127 solution, respectively. It was 50 μM in the absence of the polymer. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of NYS-pluronic F127 lyophilizate were same at 3 μg/ml, while MIC and MFC of pure NYS are 3 μg/ml and 12 μg/ml, respectively. By modulating the aggregation behavior of NYS, pluronic F127 was able to reduce the toxicity of the drug without compromising the MIC and MFC.

Keywords–Nystatin, Critical aggregation concentration, Minimal inhibitory concentration, Pluronic, Hemolysis

The number of the systemic fungal disease patients is rising due to the increasing number of immunocompromised patients such as AIDS (1-2). Moreover, the long term use of immunosuppressive drugs after organ transplantation also contributes to this increasing trend. Polyene macrolides such as Amphotericin B (AmB) is the drug of choice for the treatment of systemic fungal diseases. It acts at a membrane level, binding sterols and forming pores that can lead to cell death. Its selectivity towards fungal cells is a result of its preference for binding of ergosterol in fungal cell membranes, over cholesterol in mammalian cells (3-4). However, the selectivity of the drugs is so poor that they are highly toxic to host cells.

Several lipid-based formulations were developed for toxicity reduction and were recently approved by FDA for use in U. S. (5-7). However, their use is restricted only when the symptom is refractory to standard formulation of AmB (Fungizone ^{TM}), a mixture of AmB with deoxycholate in phosphate buffer. There are also controversies about the cost-effectiveness due to exceedingly high costs for lipid products. Therefore, many researches are still ongoing to find less toxic and less expensive formulations.

Lipid formulation of nystatin (NYS) is also developed and currently on the Phase III clinical trial in U. S. NYS and AmB have a very similar chemical structure composed of hydrophilic and hydrophobic domains in their molecules. Owing to their amphiphilicities, they readily form self-aggregation in aqueous milieu which is responsible for toxicity to mammalian cells. We found that poly (ethylene oxide) (PEO) impacted the self-aggregation behavior of AmB molecules, leading to less aggregated and less toxic form (8). We have hypothesized that triblock copolymer of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO) which is widely used pharmaceutical excipient may also impact the self-aggregation behavior of AmB and NYS molecules.

In this paper, we measured critical aggregation concentration (CAC) and hemolytic activity of NYS in the presence of varied concentrations of pluronic F127, triblock copolymer of PEO and PPO. Also, we prepared the NYS-pluronic F127 lyophilizates and measured the hemolytic and antifungal activities of the lyophilizates using *Saccharomyces cereviciae* as a model strain.

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Experimental

Materials

NYS was obtained from Sigma. Pluronic F127 was kindly donated by BASF Corporation. Bacto™ YPD agar and Bacto™ YPD broth were purchased from DIFCO Laboratories. *Saccharomyces cereviciae* (ATCC 4921) was purchased from ATCC. All other chemicals were analytical grade.

Determination of critical aggregation concentration (CAC)

NYS (16 mg) was dissolved into 0.3 ml of dimethyl sulfoxide (DMSO) under light protection and was added into 15 ml of pluronic F127 solution (0.1%-2.0%w/v in distilled water), making 1100 μM NYS. It was then two-fold diluted with the pluronic F127 solution until 4.0 μM of the drug was obtained. After incubation at 37 °C for 30 min, it was subjected to light scattering measurement using fluorescence spectro - photometer (F-3010, Hitachi) with fixed excitation and emission wavelengths at 450 nm. Bandwidths for excitation and emission were set at 3 nm and 1.5 nm, respectively. The increase of light scattering intensity of the sample compared to the same concentration of pluronic F127 solution was plotted as a function of logarithmic concentration of NYS. It showed abrupt increase at certain critical point. This point was obtained by the intercept of the two lines and defined as CAC where self-aggregation of NYS molecules began. The detailed procedure is described elsewhere (9). All tests were repeated at least three times.

Hemolysis

Erythrocytes were diluted with isotonic PBS, pH 7.0, conta - ining each percent of pluronic F127. Dilution was adjusted so that the absorbance at 576 nm was 0.4 when completely lysed in the presence of 20 μg/ml AmB. NYS was dissolved separately into DMSO and diluted with pluronic F127 solution in isotonic PBS. It was incubated at 37 °C for 5 min, and appropriate volumes were added into diluted erythrocytes to get varied levels of NYS. Aliquots (2.5 ml) of diluted erythrocytes containing varied levels of NYS were incubated at 37°C for 30 min with gentle shaking. Unlyzed erythrocytes were removed by centrifugation. The supernatant was collec - ted and analyzed for hemoglobin by absorption spectroscopy at 576 nm. The percent lyzed erythrocytes was determined by the following equation: % hemolysis=100(Abs-Abs $_0$)/(Abs$_{100}$-Abs$_0$), where Abs, Abs$_0$, and Abs$_{100}$ are the absorbances for the sample, control with no NYS and control in the presence of 20 μg/ml AmB, respectively.

Preparation of NYS-pluronic F127 lyophilizate

Appropriate amounts of NYS were dissolved into 0.5 ml of dimethyl acetamide under light protection, and an aliquot (0.35 ml) of the solution is added into 10 ml of 5.0%w/v pluronic F127 solution. Molar ratios of NYS to pluronic F127 varied from 2:1 to 1:4. After mixing homogeneously, it was incubated at 37°C for 30 min and frozen quickly by using liquid nitrogen and freeze-dried for 24 h. The NYS content of the freeze-dried product was measured as follows. About 1 mg of the product was weighed accurately and dissolved into 0.1 ml of distilled water. Dimethyl formamide (0.9 ml) was added and vigorously stirred for 5 min. It was further diluted and assayed for the drug content by absorption spectroscopy at 307 nm after filtering through 0.22 μm syringe filter.

Antifungal activity of NYS-pluronic F127 lyophilizate

Samples were dissolved into DMSO and diluted with Bacto™ YPD broth medium, giving a NYS level of 8 μg/ml and filtered through sterile syringe filter (0.22 μm) for sterilization. An aliquot (800 μl) was put into microcentri - fuge tube and two-fold diluted with broth medium until the NYS level of 0.1 μg/ml was obtained. To each tube, 50 μl of the inoculum which contained 5×10^3 cfu/ml of *Saccharomyces cerevisiae* in sterile distilled water was added, giving a total volume of 850 μl per tube, and incubated at 30 °C for 24 h with vigorous agitation. A solvent control, pluronic F127 control, and medium control were performed simultaneously to check the growth inhibiting activities of DMSO, pluronic F127, and sterility of broth medium, respectively. The minimal inhibitory concentration (MIC) was defined as the minimum concentration of NYS that showed a full inhibition of growth of *Saccharomyces cerevisiae* in the tube, determined by measuring the optical density at 600 nm. For measurement of minimal fungicidal concentration (MFC), the same procedure as MIC measurement was employed before incubation step. Tubes containing *Saccharomyces cerevisiae* were placed in an incubator at 37 °C, so that the cells were killed. After 2 h incubation, the survived cells were precipitated on the bottom of the tube by centrifugation (12000 g, 10 sec) and washed with sterile distilled water one time and collected. This collected portion was spread evenly on the YPD agar plate and incubated at 30 °C for 24 h. Thereafter, the colony on the agar plate was observed and initial concentration of the drug that killed *Saccharomyces cerevisiae* completely was defined as MFC. All experiments were repeated three times.

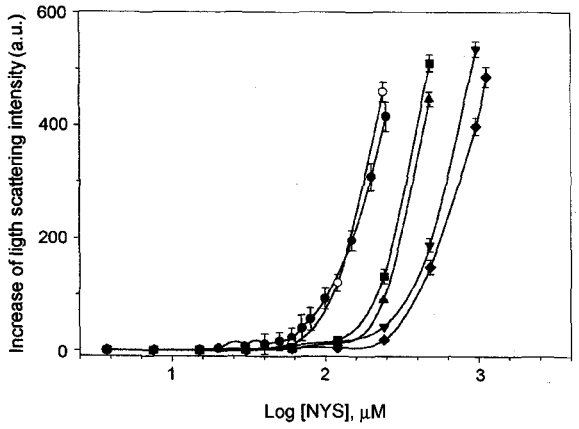

Figure 1–Light scattering of NYS in the presence of varied levels of pluronic F127. ●; 0%, ○; 0.1%, ■; 0.5%, ▲; 1.0%, ▼; 1.5%, ◆; 2.0%.

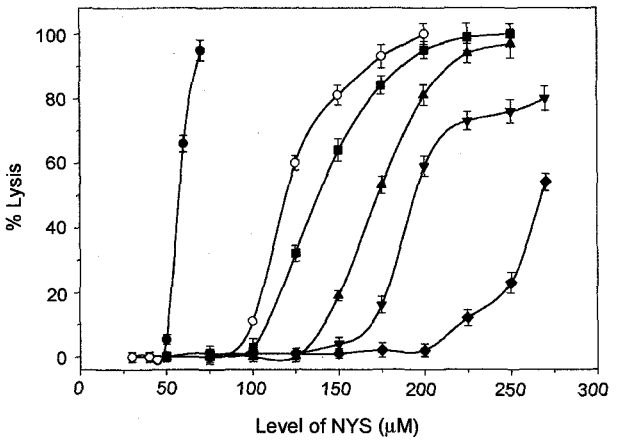

Figure 2–Hemolytic activity of NYS in isotonic PBS containing varied levels of pluronic F127. ●; 0%, ○; 0.1%, ■; 0.5%, ▲; 1.0%, ▼; 1.5%, ◆; 2.0%.

Table I–*Critical Aggregation Concentrations (CAC)[a] of NYS in Distilled Water Containing Varied Levels of Pluronic F127*

% pluronic F127	0.1 %	0.5 %	1.0 %	1.5 %	2.0 %
CAC (μM)	50.1	108.0	134.2	154.3	217.9

[a]in the absence of pluronic F127 is 52.8 μM. r^2=0.9536

Results and Discussion

Light scattering intensity increased abruptly at a critical level of NYS and showed two distinct linear lines when plotted as a function of logarithmic concentration of NYS (Fig ure 1). The abrupt increase of light scattering is attributed to the emergence of colloidal particles, i.e., self-aggregated species of NYS molecules. Table I summarized the CAC's of NYS in pluronic F127 solution. They were 50.1, 108.0, 134.2, 154.3, and 217.9 μM in the presence of 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% pluronic F127 solution, respectively. CAC increased proportionally as a function of the concentration of pluronic F127 solution (r^2=0.9536). The mechanism of CAC increase appears to be owing to Winnik and Regismond's finding that amphiphilic molecules interact with the hydrophobic domain of water-soluble polymer chain (10). NYS is an amphiphilic drug that forms micelle-like self-aggregates. The toxicity of polyene macrolide drug such as NYS and AmB has been repeatedly correlated to their self-aggregation state (3-4, 11). Given the similarity in structure to AmB and the effect of pluronic on the CAC, it is reasonable to hypothesize that interaction with the pluronic reduces the toxicity of the NYS through deaggregation.

Figure 2 shows percent hemolysis versus level of NYS in the presence of pluronic F127. The levels of NYS required to start lysis of erythrocytes were about 80, 100, 125, 150, and 200 μM at 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% pluronic F127 solution, respectively. In contrast, it was 50 μM in the absence of pluronic F127. Lysis onset levels are in good correlation with CACs and increase according to the increase of pluronic F127 concentration. This result clearly suggests that it is the deaggregated form of NYS that is nontoxic to mammalian cells, similar to the previously referenced results observed using another member of the polyene macrolide family, AmB.

We prepared the NYS-pluronic F127 lyophilizates having varied molar ratios of NYS to pluronic F127 and measured their hemolytic activities in isotonic PBS. The levels of NYS required to start hemolysis were about 75 μM for molar ratios of 2:1 and 1:1 and about 100 μM for molar ratios of 1:2 and 1:4, respectively (Figure 3). Physical mixture (NYS:pluronic F127=1 mole:2 moles) did not attenuate the toxicity, showing the same lysis onset level of 50 μM as pure NYS. The

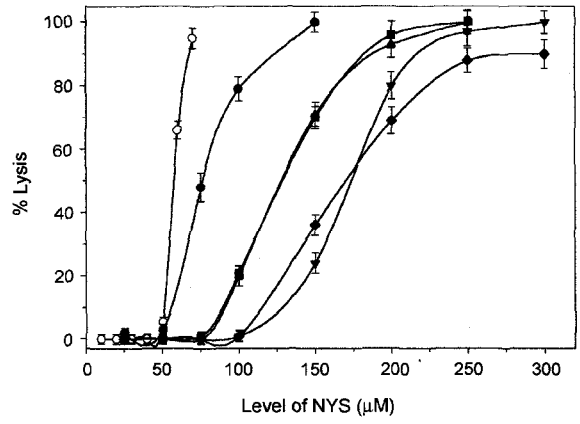

Figure 3–Hemolytic activity of NYS-pluronic F127 lyophilizates and physical mixture in isotonic PBS. ○; pure NYS, ■; NF (2:1), ▲; NF (1:1), ▼; NF (1:2), ◆; NF (1:4), ●; physical mixture (NYS:pluronic F127=1 mole:2 mole).

Table II–*Properties of NYS-Pluronic F127 Lyophilizates*

molar ratio (NYS:pluronic)	NYS content (%w/w)	input NYS content (%w/w)	yield[a] (%)	solubility (mg/ml)	50 % lysis inducing level of NYS (μM)
NF (2:1)	9.49	12.81	74.1	1.3	130
NF (1:1)	6.00	6.85	87.6	0.9	130
NF (1:2)	3.43	3.54	96.9	1.4	175
NF (1:4)	1.77	1.80	98.3	>2.0	170
pure NYS	-	-	-	0.54	60

[a] yield = 100×NYS content / input NYS content

significant difference between physical mixture and lyophi - lizates indicates that there is an interaction of the drug and the polymer during incubation and freeze-drying processes for lyophilization. Based upon Winnik and Regismond's report, this interaction appears to be hydrophobic binding that may be dissociated after administration into blood circulation. Polyene macrolides are also known to strongly bind to serum proteins. Therefore, the lyophilizate should further be evaluated in relation with other competing processes such as protein binding and hydrogen bond. The properties of NYS-pluronic F127 lyophilizates were summarized in Table II. The yield increased as mole fraction of pluronic F127 increased, reaching 98.3% at molar ratio of 1:4. Solubilities of the lyophilizates in distilled water are about two to four times greater than pure NYS. The 50% lysis inducing levels of the lyophilizates were above 130 μM, compared to 60 μM of pure NYS.

Finally, we measured the efficacy of NYS-pluronic F127 lyophilizate in terms of MIC and MFC. MICs of both pure NYS and the lyophilizate were same at 3 μg/ml. MFC's of pure NYS and the lyophilizate were 12 μg/ml and 3 μg/ml, respectively. It is not unusual for antifungal drugs that MIC and MFC are often different. Actually, MFC's of most antifungal drugs are higher than their MIC. Interestingly, however, MFC of NYS-pluronic F127 lyophilizate is exactly same as it's MIC. The background of this enhanced efficacy is unclear at this point. From this and hemolysis results, NYS-pluronic F127 lyophilizate may be a less toxic formulation, maintaining or even potentiating the activity against fungal cells. The model microorganism we used in this report is not pathogenic to humans but is similar to *Candida* that is responsible to many systemic fungal diseases. Antifungal test with several pathogenic strains is currently in progress.

Conclusion

Pluronic F127, triblock copolymer of PEO and PPO, impacted the self-aggregation behavior of NYS molecules and

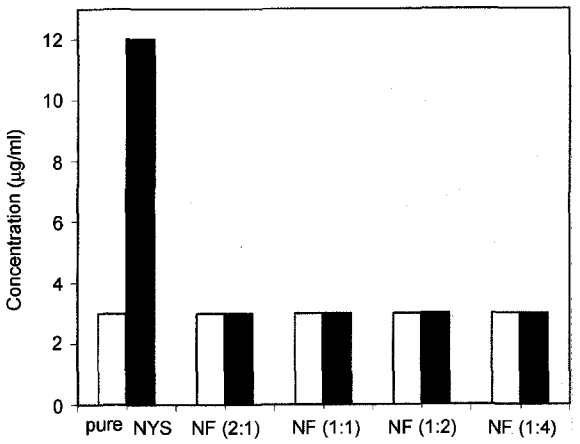

Figure 4–Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of NYS-pluronic F127 lyophilizates. Open bar; MIC, shaded bar; MFC.

significantly reduced the toxicity of the drug. Antifungal activity against *Saccharomyces cerevisiae* was not compromised, showing three times greater efficacy than pure NYS in terms of MFC. It may be worthwhile to develop a formulation of NYS-pluronic F127 lyophilizate for the treatment of systemic fungal diseases.

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