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Development of DH-I-180-3 loaded lipid nanoparticle for photodynamic therapy

조선대학교 대학원

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반 상 준

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광역학 치료를 위한 DH-I-180-3가 봉입된 지질나노입자의 개발

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이 논문을 약학 박사학위신청 논문으로 제출함.

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광 역학 치료 (PDT)는 암 유망한 비 침습적인 치료방법이다. 광감작제와 빛의 특정한 파장은 PDT의 핵심구성 요소이다. 본 연구에서는 동시 형광 이미징 및 표적 치료를 위한 지질 나노 입자를 개발하기 위하여 2세대 광감작제인 DH-I-180-3를 포함하는 나노 입자를 개발하였다. 고체지질 나노 입자(SLN)와 나노구조 지질 캐리어(NLC)는 계면활성제인 poloxamer 188과 공통계면활성제인 레시틴을 가반으로 용매증발법과 고온균질화 기술을 이용하였다. 스테아르산 및 Capmul® MCM C8 은 각각 고체 지질 및 액체 지질로 사용되었다. SLN 및 NLCS 의 입자 크기는 약 200 nm이었으며 스테아린산 부분이 Capmul® MCM C8 로 대체되었을 때 그 크기는 감소되었다. SLN 및 NLCS에서 액체 지질의 비율량이 증가하였을 때 약물 적재 효율이 크게 향상 되었다. SLN / NLCS 모두 분산 지수 가 0.3 미만이었고, 좁은 입도 분포를 나타내었다. 모든 지질 나노입자의 제타 전위는 -30mV 미만으로 충분한 반발력을 유지하고 개선된 물리적 안정성을 보였다. 동결 건조된 SLN / NLCS에서도 입자 크기 및 분산도 지수에 유의한 변화는 관찰되지 않았다. 본 제형의 photocytotoxic 효과를 MCF-7 세포에서 평가 하였을 때, SLN의 GI 50은 DH-I-180-3 용액의 절반 이하였으며 Capmul® MCM C8를 5 또는 15 % 를 함유한 NLC는 SLN 보다 더 높은 세포 독성을 나타내었다. 형광 현미경 이미지 역시 DH-I-180-3이 봉입된 SLN and NLC의 세포내 축적이 향상되었음을 보여주고 있으며 이는 photocytotoxicity 시험 결과와 밀접한 상관관계를 보인다. 따라서 DH-I-180-3의 나노 입자 봉입이 그것의 표적지향성과 photocytotoxicity를 강화 한다는 결론을 내릴 수 있었다.

1. Introduction

Cancer is one of the most devastating diseases worldwide, with an estimated 26.4 million new cases expected per year, globally, by 2030(Boyle and Levin, 2008). Application of conventional cancer treatment strategies such as primarily chemotherapy, radiotherapy and surgery are limited owing to their aggressiveness, systemic toxicity, damage to healthy tissue, as well as functional and aesthetic debilitation(Cho et al., 2008 Wong et al., 2007).

In this regard, photodynamic therapy (PDT) has been considered as a promising treatment for various cancers as well as non-cancer diseases(Cheng and Burda, 2011). Fundamental element of PDT includes photosensitizer, specific source of light to active the photosensitizer and the produced oxygen. Upon activation, photosensitizer transforms the drug from its ground state into an excited singlet state. Emitting fluorescence, excited singlet state can decay back to ground state or undergo electron spin conversion to its triplet state. In addition, simultaneous type I or type II reaction takes place leading to the formation of radicals or radical ions, or singlet oxygen. Half-life of the singlet oxygen is $< 0.4\ \mu s$, and, therefore, the radius of the singlet oxygen is $< 0.02\ \mu m$(Dolmans et al., 2003 Hopper, 2000). Eventually, the produced reactive species causes significant oxidative damage to the surrounding cells resulting direct cell death and tumor ablation by apoptosis or necrosis, damaging the tumor associated vasculature and activates the immune response against tumor cells. The specific damaging portion includes mitochondria, plasma membrane, Golgi apparatus and endoplasmic reticulum, DNA(Castano et al., 2004 Dolmans et al., 2003 Hopper, 2000 Sharman et al., 1999 Triesscheijn et al., 2006). Because PDT is a cold photochemical process, there is no tissue heating, and connective tissues such as collagen and elastin are largely unaffected. Therefore, much less risk to the integrity of underlying structures than with thermal laser techniques and surgery(Dougherty et al., 1998b).

Photosensitizers are compounds that are capable of absorbing a light of

specific wavelength and transforming it into useful energy. Several researches have demonstrated the ideal characteristics of photosensitizer in molecular and clinical aspects. There are many properties which an ideal photosensitizer should possess. One of them is high absorption of the PS between 650 nm and 850 nm where the tissue penetration is quite high. Human tissue transmits light most effectively in the red part of the visible spectrum, and the newer photosensitizer can absorb 630 nm or above can be activated to a depth of 1 cm(Hopper, 2000). Theoretically, wavelengths approaching 700-800 nm will penetrate tissue about 1 cm while wavelength closer to 600 nm penetrates to about 0.5 cm(Allison and Sibata, 2010 Castano et al., 2004). An ideal photosensitizers should be selective to the target tissue, be safe, i.e., should not result in side effects such as mutagenic, carcinogenic or allergic effects, should not be toxic in therapeutic doses and not damage normal healthy cells. Moreover, metabolism and excretion after administration should be as rapid as possible to minimize photosensitivity. In addition, it should be inexpensive, easy to synthesize and commercially available(Paszko et al., 2011 Sharman et al., 1999). Furthermore, it should have high singlet oxygen quantum yield for the photochemical reaction, be a single and pure compound with a stable composition, and be soluble in water and should not form aggregates. In addition, developing a clinically effective photosensitizer should possess characteristics, for instance, initiation of type II photodynamic reaction (PDR), localization in target cell, amphiphilicity, capable in fluorescence, dissymmetry(Allison and Sibata, 2010). In addition, it should exhibit the following criterion: stable composition, easily synthesized or readily available, minimal self-aggregation tendency, not highly hydrophobic or encapsulated inside appropriate carriers, non-toxic in the absence of light exposure, photo-stable, absorbance in the red region of spectrum with high extinction molar coefficient, target specificity, can be quickly cleared form the body(Bechet et al., 2008).

Since the discovery from Niels Finsen, a Nobel laureate in 1903, PDT has grown into a Food and Drug Administration (FDA)-approved therapy for

several malignancies(Cheng and Burda, 2011 Dougherty et al., 1998a Morris et al., 2003 Weishaupt et al., 1976). PDT is widely used to treat versatile human diseases including cancer, age related molecular degradation, skin disease.PDT is applied in various tissue and organ, including esophagus, stomach, skin head and neck(Dolmans et al., 2003). Besides, PDT is currently used in the clinic as an adjunctive therapy for the treatment of a variety of solid tumors including inoperable esophageal tumors, head and neck cancers, and microinvasive endobronchial nonsmall cell lung carcinoma(Hopper, 2000). In addition, PDT is being investigated for the treatment of several other cancer types including breast and prostate cancers(Dolmans et al., 2003). Moreover, Bladder cancer, Barrett's esophagus, endobronchial cancer, Actinic keratosis, basal cell carcinoma, cervical cancer, endobronchial cancer, gastric cancer, papillary bladder cancer(Triesscheijn et al., 2006). Clinical trial is ongoing in case of lung cancer, esophageal carcinoma, gastric cancer, head, neck and oral cavity and some cutaneous malignant disease(Hopper, 2000). Available approved drug in cancer type: basal cell carcinoma, cervical cancer, endobroncheal cancer, esophageal cancer, gastric cancer, head and neck cancer, papillary bladder cancer(Dolmans et al., 2003).

The first photosensitizer approved for clinical use was Photofrin®, a hematoporphyrin derivative. It has been approved in most countries as a treatment for a variety of cancers including esophageal cancer, superficial bladder cancer, early and late stage lung cancer and malignant/non-malignant skin disease(Henderson and Dougherty, 1992 Paszko et al., 2011). The clinical success of Photofrin® has inspired the development of new PDT photosensitizers. A number of second generation photosensitizers have developed to improve some limitation of Photofrin® such as poor water solubility, prolonged photosensitivity due to low clearance rate, and lack of absorption in long wavelength(Bin et al., 2005 Leung et al., 2002 Paszko et al., 2011).

DH-I-180-3 is one of recently developed second generation photosensitizers.It is a chlorine derivative from silkworm excreta. Superiority of DH-I-180-3 to Photofrin® against Lewis Lung Carcinoma (LLC1) cells was demonstrated in

vitro and in vivo(Lee et al., 2003). It showed longer wavelength absorption (660 nm), shorter body clearance and higher potency at same dose than Photofrin®. Moreover, PDT with DH-I-180-3 have demonstrated cytotoxicity in 3T3-L1 pre-adipocyte and adipocyte cells, which has opened possibility to apply PDT in obesity treatment(Choi et al., 2005).

However, still DH-I-180-3 had drawbacks such as poor water solubility and low selectivity to their desired targets just like other photosensitizers(Konan et al., 2002 van Nostrum, 2004). Even though the hydrophobicity allows photosensitizers to penetrate cell membrane, it makes them incompatible for systemic administration(Konan et al., 2002 Oleinick et al., 2002). Hydrophobic photosensitizers tend to form aggregation under physiological conditions, which could affect negatively photophysical properties (i.e., 1O2 generation) and photodamage activity(Chatterjee et al., 2008). On the other hand, nonselective distribution of photosensitizers in body could affect the PDT outcome even with specific delivery of light to the target(Kessel, 2004 van Nostrum, 2004).

To overcome common shortcomings of photosensitizers including DH-I-180-3, several approaches loading the photosensitizers on nanoparticles have been reported. Nanoparticle are submicron sized, colloidal particles, with sizes ranges from 10-1000 nm in diameter(Parveen et al., 2012). In recent years, research in the area of targeted cancer therapy has led to the development of nanoparticulate delivery vehicles that can carry optimum photosensitizerpayloads selectively to the tumor site, while minimizing nonspecific drug uptake and systemic side effects. Active research in this area is evident from reports of (a) encapsulation of photosensitizermolecules in liposomes, dendrimers, micelles and polymeric nanoparticles, quantum dots, (b) conjugation of photosensitizer molecules to gold nanoparticles and other inorganic nanoparticles, and (c) engineering of a cell-selective delivery strategy of the nanoparticles or photosensitizer molecules themselves using tumor-homing motifs like albumin, transferrin, LDL, monoclonal antibodies and folic acid(Bechet et al., 2008 Konan et al., 2002 Paszko et al., 2011 Wang et al., 2004).

Nanoparticles represent a relatively new trend in drug delivery(Petros and

DeSimone, 2010). Large particles (>200 nm) are usually captured by macrophages and typically exhibit a more rapid rate of clearance than particles with radii under 200 nm. This can help to maintain higher circulating levels of the therapeutic agents once administered(Petros and DeSimone, 2010). It is called 'enhanced permeability and retention (EPR) effect'(Cheng and Burda, 2011 Iyer et al., 2006 Matsumura and Maeda, 1986). Nanomaterials can satisfy all the requirements for an ideal PDT agent. Most prominent among the features of successful agents is their photosensitizing ability, wherein targeted mitochondria absorb visible light; however, this feature often has been found serendipitously or empirically. Nanotechnology platforms potentially can deliver large numbers of photosensitizer and/or imaging agents. Nanoparticles (NPs) are uniquely promising in that (1) their hydrophilicity and charge can be altered; (2) they possess enormous surface area that can be modified with functional groups possessing a diverse array of chemical and biochemical properties, including tumor-selective ligands; (3) owing to their sub-cellular and sub-micron size, they can penetrate deep into tissues and are generally taken up efficiently by cells; (4) because numerous universal strategies for the preparation of nanomaterials are already in place, PS-loaded NPs can be made by numerous methods, such as covalent linkages and self-assembly(Wang et al., 2004).

Lipid nanoparticles were developed in the last decade of the last century as alternative carrier system to emulsions, liposomes and polymeric nanoparticles. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) are the two main types of lipid nanoparticles. The research activities in SLN and NLC in the last two decades focused mainly on pharmaceutical nondermal administration routes, i.e. parenteral, peroral, ocular and pulmonary administration.(Müller et al., 2002c)

Solid lipid nanopaprticless (SLN) were developed at the beginning of the 1990s as an alternative carrier system to emulsions, liposomes and polymeric nanoparticles asa colloidal carrier system for controlled drug delivery. In recent years, the study of SLN has markedly increased, especially with the method of

high pressure homogenization. SLN have been developed and investigated for parenteral, pulmonary and dermal application routes. Because of their small size, SLN may be injected intravenously and used to target drugs to particular organs. The particles together with all intravenously injected and colloidal particulates are cleared from the circulation by the liver and spleen(Parveen et al., 2012 Wong et al., 2007). Solid lipid nanoparticles (SLN) offer an attractive means ofdrug delivery, particularly for poorly water-soluble drugs. They combine the advantages of polymeric nanoparticles, fat emulsions and liposomes(Dingler et al., 1996 Schwarz et al., 1994). SLN consist of drug trapped in biocompatible lipid core and surfactant at the outer shell, offering a good alternative to polymeric systems in terms of lower toxicity(Dingler et al., 1996 Müller et al., 2000a). Moreover, the production process can be modulated for desired drug release, protection of drug degradation and avoidance of organic solvents. This flexibility in large scale may have a paramount importance in commercialization of new products(Wissing et al., 2004b). Aforementioned characteristics make SLN an interesting carrier system for optimized delivery of drugs.

Among the lipid-based formulations, the nanostructured lipid carriers (NLCs), regarded as the second-generation of lipid nanoparticles, are attracting foremost consideration as alternative colloidal drug carriers. NLCs have evolved from solid lipid nanoparticles (SLN) and are composed of a mixture of spatially different lipid molecules, i.e., solid lipid is blended with liquid lipid to overcome the disadvantages of SLNs such as limiteddrug loading, larger particle size, risk of gelation and drug leakage during storage caused by lipid polymorphism. In view of this NLC may be regarded as an alternative to SLN and can be exploited as a novel approach for the delivery of photosenzitizer DH-I-180-3 (Müller, 2007 Müller et al., 2007 Müller et al., 2002c).

In this study, we developed SLN and NLC formulations loading DH-I-180-3in order to overcome the drawbacks of DH-I-180-3. Solid and liquid lipids were screened for the selection of lipid matrix of SLN and NLCs whereby high drug loading and low particle size are achieved simultaneously. The

characteristics,such as particle size, polydispersity index, zeta potential, drug loading, entrapment efficiency, drug release profile in media and storage stability, of prepared nanoparticles were evaluated. Then, the photocytotoxic effect as well as cellular accumulation of DH-I-18-3 loaded on nanoparticles was investigated in comparison with DH-I-180-3 in solution. Moreover, all the parameters of NLCs were compared with the SLN.

2. Materials and methods

2.1. Materials

DH-I-180-3 was a generous gift from LitePharmTech (Seoul, Korea). Stearic acid and sucralose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Lutrol® F68 (Poloxamer 188) were obtained from BASF (Ludwigshafen, Germany). Tween® 80 and lecithin, from soybean were purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). Capmul® MCM C8 (Glyceryl Monocaprylate) was obtained from Abitec (Columbus, Ohio). Antibodies against PARP (Poly (ADP-ribose) polymerase) were purchased from Cell Signaling Technology (Beverly, MA, USA); β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Methods

2.2.1. Screening of lipid for nanoparticle matrix

Solubility of DH-I-180-3 in various solid lipids and liquid lipids were determined by visual evaluation.Briefly, 100 mg of each lipid was taken. In case of solid lipids, they were separately heated above their melting points. 5 mg of DH-I-180-3 was gradually added to molten or liquid lipid with continuous agitation. The dissolved amount of DH-I-180-3 was noted visually.

2.2.2. Preparation of SLN/NLCs

SLN/NLCs were prepared by solvent evaporation and hot homogenization method. Briefly, 25 mg of stearic acid, 6.25 mg of lecithin, and 5 mg of DH-I-180-3 were dissolved in 600 μl of dichloromethane/methanol (1:1) under heated condition to dissolve stearic acid completely. Capmul® MCM C8 was substituted for allocated part of stearic acid in the preparation of NLCs. Meanwhile, in a separate vial, 18.75 mg of poloxamer 188 or other surfactants

was dissolved in 10 mL of purified water and heated to 80 °C. Organic solution was slowly dropped in aqueous solution under homogenization (20,500 rpm) using Ultra-Turrax T10 basic (IKA, Germany) in 80 °C. The whole mixture was further homogenized until the organic solvents evaporated completely. The o/w nanoemulsion, then, was homogenized in room temperature for 10 min and kept in 4 °C for 30 min to solidify the emulsion. In order to remove large particles and un-entrapped drug, the dispersion was centrifuged at 9500 rpm for 10 min. After centrifugation, the supernatant, which contains nanoparticles, was carefully collected.

2.2.3. Determination of particle size, polydispersity index (PI), and zeta potential (ZP)

The particle size, polydispersity index, and zeta potential of SLN/NLCs were measured by photon correlation spectroscopy (PCS) using Zetasizer 3000 (Malvern Instruments, UK) at 25 °C. Nano-dispersion was suitably diluted with purified water before the measurement.

2.2.4. Differential scanning calorimetry (DSC) analysis

DSC analysis wasperformed using Pyris 6 DSC (PerkinElmer, Netherlands). For DSC measurement, 5 mg of stearic acid or lipid blends of stearic acid and Capmul® MCM C8 were put in aluminum pans. A scan rate of 10 °C/min was employed in the 25-90 °C temperature range.

2.2.5. Drug loading and entrapment efficiency

Generally, entrapment efficiency was determined from the percentage by subtracting the amount of free drug in aqueous phase from that of the initial added drug(Souto et al., 2004). However, the freedrug, in spite of having a low

molecular weight of 724.86, was not able to be separated from the nanoparticles dispersion by using ultrafiltration device having a molecular weight cutoff of 30,000 Da (Ultracel®-30k, Millipore Corporation, Billeria, MA). Besides, the amount of free DH-I-180-3 in aqueous phase was assumed to be negligible due to its significantly low aqueous solubility (1.29±0.41 μg/mL). Thus, the entrapment efficiency of SLN/NLCs was determined from the percentage by subtracting the amount of drug in the aforementioned precipitate during the preparation of SLN/NLCs from that of the initial added drug.

$$\text{Entrapment efficiency (\%)} = \left(1 - \frac{amout\ of\ drug\ in\ the\ precipitate}{amount\ of\ initial\ added\ drug}\right) \times 100$$

The drug loading of the SLN/NLCs was calculated by measuring the amount of DH-I-180-3 in lyophilized SLN/NLCs. The obtained SLN/NLCs dispersions were lyophilized for 24 h. After dissolving 3 mg of the lyophilized SLN/NLCs in 10 mL of acetone, the amount of DH-I-180-3 was analyzed by HPLC.

$$\text{Drug loading (\%)} = \frac{amount\ of\ loaded\ drug\ in\ SLN/NLCs}{amount\ of\ lyophilized\ SLN/NLCs} \times 100$$

2.2.6. HPLC analysis

DH-I-180-3 was determined by using a HPLC system, consisting of a UV detector (SPD-10A),a pump (LC-10AD) and an automatic injector (SIL-10A). The mobile phase consisted of water and acetonitrile (10:90) and the flow rate was 1.0 mL/min. The wavelength of the UV detector was set at 409 nm and a reversed-phase column (Gemini 5μC18 110A, Phenomenex, USA) was used. The column temperature was maintained at 30 °C.

2.2.7. Lyophilization

The SLN/NLCs were lyophilized using a programmable freeze-dryer (Shin PVTFD 10R, Shinil Lab, Seoul, Korea). 10 w/v% of cryoprotectant was added to the each SLN/NLCs dispersion and kept in -80 °C for 2 h. Slow freezing,

then, was carried out on the shelves in the freeze-dryer (shelf temperature -40 °C). The SLN/NLCs dispersions were lyophilized for 24 h from -40 °C to 25 °C.

2.2.8. Stability studies

Physical stability studies of the SLN/NLCs were carried out for 6 month period. Lyophilized SLN/NLCs were stored protected from light at 4 ºC. At the predetermined time interval, 1, 2, 3, and 6 month period, lyophilized nanoparticles were dispersed well in deionized water by gently vortexing. Particle size and polydispersity index of SLN/NLCs were measured by photon correlation spectroscopy (PCS) using Zetasizer 3000 (Malvern Instruments, UK) at 25 °C.

2.2.9. *In vitro* drug release

To investigate the release rate of DH-I-180-3 from the nanoparticles, in vitrodrug release study was conducted. SLN/NLCs, equivalent of 0.25 mg DH-I-180-3, were dispersed to 10 mL of aqueous dissolution medium (2%w/v poloxamer 188 solution). They were kept stirring at 100 rpm in 37 ºC. At predetermined time interval, 2 mL of samples were withdrawn and compensated with fresh medium. Samples were centrifuged at 24000g for 20 min, Supernatant was collected and filtered through 0.02 μm Whatman® filter unit and analyzed by HPLC.

2.2.10. Transmission Electron Microscopy (TEM)

The morphology of SLN was observed by transmission electron microscopy (H-7600, Hitachi, Japan). The sample was prepared by placing a drop of SLN dispersion that was previously diluted 10-folds with double-distilled water onto

a Formvar, 200 mesh copper grid (TED PELLA, INC., USA). The sample was dried in the air before TEM observation.

2.2.11. Cytotoxicity assay by photodynamic therapy

Cell proliferation assay MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/ mL streptomycin at 37 ºC in a humidified atmosphere containing 5% CO2. The cells (1 x 105cells/well) were cultured in 12 well plates overnight, and treated with various concentrations of DH-I-180-3 either loaded in SLN/NLCs or dissolved in N,N-dimethylformamide (DMF). After 1 h incubation with DH-I-180-3, the cells were exposed to light dose of 1.25 J/cm2 from a red light-emitting diode (LED). This source emitted light at 613 - 645 nm wavelengths. The peak at 635 nm (Philips Luxeon Lumileds, San Jose, CA) was characterized by 35 mW/cm2as measured with a Delta Ohm DO 9721 quantum photo-radiometer and thermometer data logger (Model DO9721, Padua, Italy). Immediately after PDT, the cells were exposed to fresh medium followed by incubation for 24 h. Cell viability was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium Bromide (MTT) assays. Briefly, the cells were washed twice with ice-cold PBS, and incubated with MTT reagent (5 mg/mL in PBS) for 3 hours at 37 °C. The medium was, then, removed and 250 µl acid isopropanol (0.04 M HCl in isopropanol) was added. The optical density was then measured by a Microplate Autoreader ELISA (Bio-Tek Instruments, Inc., Winooski, VT) at a wavelength of 570 nm.

Western blotting MCF-7 cells seeded in 6-well plates (2 x 105 cells/well) were treated with photodynamic therapyfor 24 h as previously described. The cells were then washed with PBS and harvested in lysis buffer. Samples containing equal amounts of protein were loaded onto each lane of an SDS-polyacrylamide gel for electrophoresis and subsequently transferred onto a

polyvinylidene difluoride membrane. The membranes were blocked and then incubated with antibodies.

2.2.12. Cellular accumulation study

Fluorescent microscopy MCF-7 cells (2 x 104 cells/well) were cultured in a 4-chamber slide. To examine the accumulation of various formulations in cells, MCF-7 cells were incubated in culture media containing 0.05 μg/mL of DH-I-180-3 either loaded in SLN/NLCs or dissolved in DMF for 1 hin darkness. The fluorescence of DH-I-180-3 in the cells was observed with a conventional fluorescent microscope (Olympus IX 71, Olympus America, Inc., Melville, NY) and Olympus microimage software.

Flow cytometric analysis MCF-7 cells (5 x 105 cells/well) were seeded in 6 cm2 plates. To examine the accumulation of various formulations in cells, MCF-7 cells were incubated in culture media containing 0.05 μg/mL of DH-I-180-3 either loaded in SLN/NLCs or dissolved in DMF for 1 h in darkness. The cells were removed from the plate with trypsin-EDTA and collected in phenol red-free growth medium. The fluorescence of DH-I-180-3 in the cells was observed with a FACSCalibur using CellQuest software (Becton-Dickinson).

2.2.13. Statistical analysis

Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Student T-test was conducted for multiple comparison test was used to compare the significance of the difference between the groups, a p-value < 0.05 was accepted as significant. Data were expressed as mean and standard deviation of separate experiments ($n = 10$).

3. Results and discussion

3.1. Determination of nanoparticle matrix

Suitable solubilityof drug in lipid matrix is a prerequisite for the well-fabricated SLN/NLCs(Müller et al., 2000b). One of the most important factors to evaluate the suitability of lipid matrix is its loading capacity. Generally, the loading capacity is highly related to the solubility of drug in lipid matrix. In order to select the suitable lipid matrix for SLN, approximate solubility of DH-I-180-3 in various solid lipids was determined by visual evaluation (Table 1). The solubilization of DH-I-180-3 in lipid matrices could notto be analyzed by DSC via melting peak due to its amorphous state (Figure 1). Table 1 shows that the highest amount of DH-I-180-3 could be dissolved in stearic acid among solid lipids tested. Stearic acid is one of the most common solid lipids which were used as matrix for lipid nanoparticles(Bocca et al., 1998 Hu et al., 2005 Mehnert and Mäder, 2001a) and was used in a parenteral formulation(Cavalli et al., 1997 Fundarò et al., 2000 Zara et al., 1999). Moreover, it is expected to have a good biocompatibility and low toxicity since it is a main composition of the body fat(Zhang et al., 2000). These advantages coupled with high solubilizing potential have favored its selection for the matrix of SLN.

To develop NLC formulation, the solubility of DH-I-180-3 in various liquid lipids was also determined. The liquid lipid is expected to satisfy two major factors when it is used as a lipid blend matrix for NLC. One is higher solubilizing potential of drug than solid lipid. Generally it is known that liquid lipids have better solubilizing effect than solid lipids(Müller et al., 2002a Wissing et al., 2004a). However, in this case, only Capmul® MCM C8 showed better solubilizing potential than stearic acid (Table 1). Second is a good miscibility with solid lipid. Otherwise oil droplet can be formed during the cooling down processdue to separation from solid lipid(Müller et al., 2002b Pardeike et al., 2011). Stearic acid and Capmul® MCM C8 were well mixable at

various ratios and no oil expulsion from the solid lipid was observed. High solubilizing potential, good miscibility with stearic acid, GRAS (generally regarded as safe) status (21 CFR § 184.1505), and the past use in parenteral application(Subedi et al., 2009)favored the use of Capmul® MCM C8 as a part of lipid blend matrix for NLC.

DSC analysis was performed to investigate the characteristics of lipid blend forSLN/NLCs. As shown in Figure 2, all of the thermograms showed the melting point of stearic acid and it decreased with increasing Capmul® MCM C8 content in the lipid blends. In addition, Table 2 shows thatmelting parameters, i.e., melting point, onset temperature, and enthalpy, were reversely proportional to the content of Capmul® MCM C8 (r2=0.9512, 0.9920, 0.9323 for melting point, onset temperature, and enthalpy, respectively). The decreasing trend of these parameters indicatesdistortion of crystal lattice of stearic acid and subsequent reduction in crystallinity owing to partial substitution of stearic acid with Capmul® MCM C8(Jenning et al., 2000). A higher load of drug can be achieved as a result of creating less ordered lipid matrix(Pardeike et al., 2009). Large distance between the fatty acid chains and imperfect crystal allow space for more drug accommodation within lipid matrix(Müller et al., 2002a). Based on the result from DSC analysis, lipid blends containing up to 30 % of Capmul® MCM C8 were selected as matrix for NLC. Capmul® MCM C8 could not be used due to physical stability. All lipid blends may exist in solid state at body temperature.

It has been reported that nanoparticles stabilized with binary surfactants tend to have smaller particle size and higher storage stability compared to formulations with only one surfactant(Mehnert and Mäder, 2001a). Several combinations of surfactants which have different HLB values were screened as components for stabilizerincluding lecithin/tween 80, lecithin/Cremophor ELP, lecithin/Solutol HS15, lecithin/Poloxamer 407, and lecithin/Poloxamer 188. A combination of Poloxamer 188 and lecithin (with a HLB of 29 and 4, respectively) was selected considering particle size, polydispersity index and zeta potential. Both lecithin and Poloxamer 188are known to have no significan

toxicity and acceptability for parenteral injection(Müller et al., 2000b). The SLN, stabilized with poloxamer 188/lecithin (3/1) mixtureshowed smaller particle size as well as higher drug loading compared to the SLNs with single surfactant.

3.2. Characterization of SLN/NLCs

SLN and NLCs containing various contents of Capmul® MCM C8, stabilized with poloxamer 188/lecithin mixture (3/1), were prepared by solvent evaporation and hot homogenization technique. It has been reported that nanoparticles stabilized with binary surfactants have better properties i.e., lower particle size and higher storage stability compared to formulations with only one surfactant (Mehnert and Mäder, 2001b). Poloxamer 188 and lecithin (with a hydrophile-lipophile balance (HLB) of 29 and 4, respectively) were selected as stabilizers due to their lack of toxicity and acceptability for parenteral injection (Müller et al., 2000b). Moreover, Poloxamer 188, a polymeric surfactant, is well known to its lack of toxicity, including its nonthrombogenic properties (Schmolka, 1977). Lecithin is an acetone insoluble phosphatides, components of cell membranes and are thus consumed as a normal part of diet. It is widely used in pharmaceutical application as dispersing, emulsifying, and stabilizing agents. Furthermore, it was included in intramuscular and intravenous injectables, parenteral nutrition formulations, as well as topical products. It is widely used in nanoparticles delivery system for instance, liposome, DNA delivery(Cui et al., 2006 Şenyiğit et al., 2010).

As per the TEM image of SLN (Figure 3), the prepared nanoparticle was spherical in shape. Characteristics of DH-I-180-3 loaded SLN and NLCs are summarized in Table 3. As shown in Table 3, the particle size and polydispersity index (PI) of SLN/NLCs ranged from 200 to 222 nm and from 0.25 to 0.29, respectively. The analyzed particle size of SLN by PCS measurement was larger than the particle size determined by TEM image. Slight decrease in particle size was observed in NLCs compared to SLN. The

effectsof partial substitution of solid lipid with liquid lipid on the particle size of the lipid nanoparticles are controversial to some extent. In general, low oil content up to 15 %w/w did not have significant influence on the particle size(Ying et al., 2008 You et al., 2007), while the particle size decreased significantly above 30 w/w% of the oil content(Fang et al., 2008 Hu et al., 2005 Huang et al., 2008 Jores et al., 2004). On the contrary, Souza et al.(Souza et al., 2011)and Kovacevic et al.(Kovacevic et al., 2011b) have observed marginal effect of oil incorporation on the particle size even at high oil content (50 to 60 w/w%). Similar results were obtained from this study. It was found that the incorporation of liquid oil did not have significant impact on the particle size and its distribution. It seems that the conflicting results are due to the different preparation procedure and surfactant system employed(Souza et al., 2011).

Zeta potential (ZP) is one of the parameters which predictthe physical stability of emulsion and suspensions(Freitas and Müller, 1998). The particle charge is one of the factorsthat determine the physical stability of emulsion and suspensions. Physical stability of particles increases proportionally as in an increase in the electrostatic repulsion between particles. Zeta potential, determinant of particle charge, indicates the physical stability of NPs. It is assumed that a minimum zeta potential of higher than −60.0 mV is required for excellent physical stability and of higher than −30.0 mV for good physical stability(Kovacevic et al., 2011a). As can be observed from Table 3, prepared formulation containing SLN orNLC possesses zeta potential between -49.86mV to -36.17 mV. SLN and NLC 5% showed the maximum zeta potential value compared with the NLC10% to NLC 30%. Even though there is no specific trend; however, increasing the amount of liquid lipid in the formulation decreased the zeta potential value. Actual mechanism for the slight decrease of zeta potential upon addition of Capmul® MCM C8 in not clear. However, several researchers have investigated the mechanism of zeta potential value depending upon the properties of the lipid blends used. A. Kovacevic et al, 2011, showed that the zeta potential of the nanoparticles increased with increasing oil content from -32 mV (no oil) to -50 mV (60% oil). Assuming an unchanged situation

for the stabilizer layer on the surface, the increase in the zeta potential can only originate form an increase of the surface potential. In addition, there are more charges on the surface, potential source are free fatty acids contained in the solid lipid but also in the oil. The oil nano-compartments of NLC can be embedded into the solid lipid matrix or to be localized at the surface of solid plates and the surfactant layer. Therefore, this increase in zeta potential can be the result of changes on the NLC surface(Kovacevic et al., 2011a). In contrary, our study revealed that zeta potential slightly decreased upon incorporation of oil in solid lipid. InNLC formulations the surface of the particle changes to less ordered shape. This was attained from the DSC data as mentioned earlier (Fig.2). However, the change in the surface morphology might have changed the embedded charge creating component of the formulations. In addition, Capmul® MCM C8 is a non inonic lipid carrier, consequently, reduces the net surface charge. As Capmul® MCM C8 increases, anionic surfactant lecithin's net charge might have decreased relatively. Thus, total charge decreases upon increase of Capmul® MCM C8. However, without additional investigations, discussing potential reasons would be speculative. Further mechanistic investigation are necessary for a better understanding of this effect The understanding ofsuch influences of oil in lipid blend would allow a more controlled selection of optimum stabilizers form lipid nanoparticles dispersion in the future.

An important parameter for evaluating SLN and NLC formulation drug loading and entrapment efficiency (EE) are two fundamental elements. As can be seen from Table 3, SLN and NLC 5% showed almost similar drug loading capacity. However, the entrapment efficiency of SLN and NLC 5% increased from 89.35 to 92.27 %, respectively. In addition, increasing the liquid lipid in NLC formulation increased the DL as well as EE. It is observed from table 3 that formulation NLC10% to NLC 30%, drug loading ranges from 9.19 to 9.68 %, whereas, EE ranges from 95.90 to 96.69 %. In contrast with the SLN formulation DL increase as well as the EE. This can be explained owing to the presence of liquid lipid in NLC formulations. It is well known phenomenon that

liquid lipids cause numerous crystal defects in solid lipid and provide imperfections in highly ordered crystal. In this space more drug molecules get entrapped(Müller et al., 2002c Souto et al., 2004). Thus, incorporation of Capmul® MCM C8 in solid lipid increased drug loading and EE. Similar results were observed by radheshyam and kamla, 2011. They have showed that incorporation of olic acid (liquid lipid) in the Glycerol monostearate (solid lipid), improved the EE from 68.16 % to 93.33 %(Tiwari and Pathak, 2011a).

SLN are prepared from solid lipids or blends of solid lipids. After hot high pressure homogenization the lipid recrystallizes, at least partially. In our study DSC data shows the crystallinity of SLN formulation higher than the NLC formulations (Fig.2). Drug expulsion in SLN can occur owing to its highly ordered crystal lattice(Müller et al., 2002c). Moreover, lipid matrix transforms from high energy modifications, characterized by the presence of many imperfections, to the modification forming a perfect crystal with no room for guest molecules. This can lead to the less drug loading in the SLN formulation. In contrary, the lipid matrix of NLC particles is constituted by solid lipids and liquid lipids, which can be mixed in such a combination that the particle solidifies upon cooling but does not recrystallize and remains in the amorphous state. A second type of NLC is formed when the lipid molecules are chemically very different, resulting in a structure with many imperfections to accommodate the drug and thus higher loading capacity (Müller et al., 2002c Souto et al., 2004).

NLC formulations are responsible for higher entrapment efficiency in comparison to SLN formulation. This result is due to the binary mixture of liquid and solid lipids, resulting in only a very weak crystallization. It can also be observed from the DSC data which shows less crystallinity in case of NLC formulation then SLN.

3.3. *in vitro* drug release

in vitrodug release study was conducted to observe the release trend of DH-I-180-3 from the nanoparticles. The release pattern was investigated in 2 %w/v poloxamer 188 aqueous solution for 24 h. As depicted in Figure 4, the release pattern of DH-I-180-3 from all formulations (SLN/NLCs) showed initial burst release followed by a prolonged release. At low oil content, the extent of release from NLC was similar to that from SLN. However, it increased when the oil content of NLC was greater than 15%. The increased release rate of the drug from the nanoparticle may result from the increased mobility of drug owing to the reduced crystallinity of lipid matrix(Tiwari and Pathak, 2011b zur Mühlen et al., 1998). Nonetheless, the release of drug from all the nanoparticles tested was significantly low, releasing less than 20 % after 24 h. This indicated that most of DH-I-180-3 may remain within the nanoparticles even after long circulation within the blood. The release of the drug in the blood need to be minimized since the SLN/NLC should be internalized into the cell with high content of the drug maintained. It has been shown that the nanoparticles were able to be internalized into thecell through active process such as endocytosis(Chavanpatil et al., 2007).Moreover it has been reported that photodynamic therapy can also be accomplished without the release of photosensitizer from nanoparticles(Cheng and Burda, 2011). Thus, high efficiency in photodynamic therapy could be expected when the release of the drug is minimized prior to the internalization into the cell.

3.4. Lyophilization and long term stability

Freeze dying of the nanoparticle is a prerequisite to improve their long term stability(Abdelwahed et al., 2006). It helps to avoid particles aggregation and leakage of encapsulated drug out of nanoparticles. However, stress of freezing and dehydration during freeze dying process may destabilize the colloidal suspension of nanoparticles. In addition, the crystallization of ice may exerta

mechanical stress on nanoparticles leading to their destabilization in lyophilization process. Thus, It is necessary to add a cryoprotectant to the nanoparticle suspension before lyophilization to prevent these fragile property(Abdelwahed et al., 2006 Schwarz and Mehnert, 1997). Cryoprotecting effects of several sugars on the prepared SLN were investigated. The cryoprotectants tested included mannose, maltose, mannitol, sucrose, sucralose, sorbitol, and trehalose. Each sugar (10% w/v) was added to SLN suspension and lyophilized for 24 h. Only Sucralose was effective in maintaining particle size and polydispersity index (PI)of SLN after lyophilization (data not shown). Using all other cryoprotectants resulted in increase in both particle size and PI. Figure 5 compares the particle size of SLN/NLCs before and after lyophilization. The particle size of NLC 30% slightly increased by about 10 %. All others showed slight decrease in particle size after the lyophilization. Moreover, PI of all nanoparticles were also slightly decreased after lyophilization (data not shown), indicating that the physical characteristics of SLN/NLCs were not damaged by the hash condition during the lyophilization process.

The lyophilized SLN/NLCs were, then, stored at 4 ℃ up to 6 month for stability study. At predetermined time intervals of 1, 2, 3, and 6 months, particle size and PI of the nanoparticles were evaluated. Figure 6 shows time dependent change in particle size of SLN/NLCs. The particle size of all the nanoparticles did not change significantly after storage up to 6 months when compared to the freshly prepared ones. PI of SLN/NLCs was also well maintained. All the PI values during storage period were below 0.3, indicating narrow size distribution and monodispersity(Dong et al., 2009) except NLC 30%. NLC 30% once showed PI of 0.32 at 3 months storage. The slight increase in PI in case of NLC 30% might be related to high level of liquid lipid. These results indicated good long term stability of the lyophilized SLN/NLCs. It was further confirmed by the absence of visible phase separation.

3.5. *In vitro* photocytotoxicity studies

Photocytotoxicity of DH-I-180-3 in solution and loaded in SLN/NLCs was evaluated using MCF-7 cells. NLC 5 and 15% formulations were selected for this study. The cells were incubated with each formulation for 1 h and exposed to the light after washing out the photosensitizer with fresh media. The cell viability was assessed using MTT in 24 h after photodynamic therapy. The cell proliferation of DH-I-180-3 was examined as a function of concentration from 0.05 to 0.4 μg/mL. Cells incubated with NLC 15% at the concentration of 0.4 μg/mL of DH-I-180-3, without the light exposure, were used as dark control. In addition, the viability of cells exposed to the light only without any treatment with DH-I-180-3 was assessed to observe the influence of the light irradiation. As illustrated in Figure 7A, light irradiation itself did not affect the cell viability. Moreover, DH-I-180-3 as well as its carrier, NLC matrix, did not show any significant toxicity in the absence of light exposure. This indicated that DH-I-180-3 and its carrier have minimal dark toxicity which is a prerequisite for the ideal photosensitizer. It is obvious that until the cells are exposed to both the light irradiation and the drug, photocytotoxic effect should not be observed. All the screened formulations showed concentration dependent photocytotoxic effect in the concentration range of 0.05 to 0.4 μg/mL. The 50% growth inhibitory concentration (IC50) of DH-I-180-3 solution, DH-I-180-3 loaded SLN, NLC 5%, and NLC 15% were 0.171, 0.082, 0.069, and 0.072 μg/mL, respectively. In comparison with DH-I-180-3 solution, nanoparticles loaded with DH-I-180-3 showed significantly improved cytotoxic effect. When the photocytotoxicity was compared amongst nanoparticles loaded with same concentration of DH-I-180-3, NLC formulations showed slightly higher toxicity than SLN. However, the extent of liquid lipid incorporation in the matrix did not show significant effect on photocytotoxicity. In addition, the cleaved form of PARP was increased by photodynamic therapy in MCF-7 cells state (Figure 7B). These results suggest that photodynamic therapy also leads to photocytotoxicity by apoptosis in MCF-7 cells. To confirm the cell death

mechanism followed by photodynamic therapy, PARP cleavage was assessed by Western blot analysis. The cleaved form of PARP was increased after photodynamic therapy with DH-I-180-3 (Figure 7B), which was consistent with previous experimental result(Lim et al., 2006). This indicates that the photocytotoxicity of MCF-7 cell was induced by apoptosis.

To evaluate the extent of intracellular uptake of DH-I-180-3, the cells incubated with DH-I-180-3, DH-I-180-3 loaded SLN, or NLCs were observed using a fluorescence microscope and flow cytometry. Figure 8A shows cellular fluorescence images of DH-I-180-3 after the cells were incubated for 1 h with various formulations. Accumulation of DH-I-180-3 in the cells well correlated with the in vitro photocytotoxicity study results. Consistent with the in vitrophotocytotoxicity results, stronger fluorescence was observed in the cells which were incubated with DH-I-180-3 loaded nanoparticles than the cells with DH-I-180-3 solution. NLCs showed higher accumulation of DH-I-180-3 in the cells than SLN and no significant difference between NLCs was observed in fluorescence images. This result is supported by the quantification of DH-I-180-3 byflow cytometric analysis. The quantity of DH-I-180-3 solution, DH-I-180-3 loaded SLN, NLC 5%, and NLC 15%-accumulation cells were 37.62, 53.38, 85.34, and 67.84 %, respectively. (Figure 8B). Based on these results, it could be concluded that the enhanced photocytotoxic effect of SLN/NLCs loaded with DH-I-180-3 resulted from the improved cellular accumulation. Whereas drug in solution can be internalized to the cells by diffusion only, drug loaded in nanoparticles could be internalized to the cells not only by diffusion subsequent to release from nanoparticles, but also by endocytosis of drug loaded nanoparticle itself. Recently, Martins et. al. found that the mechanism of SLN endocytic internalization was mainly through a clathrin-dependent endocytic pathway (Martins et al., 2012).

4. Conclusions

In this study, we have prepared SLN and NLC formulations loaded with DH-I-180-3, a second generation photosensitizer, by solvent evaporation and hot homogenization technique. Decrease in particle size and improvement in drug loading were observed in NLC formulations compared to SLN. All the prepared SLN/NLCs formulations were stable during 6 month storage at 4 ℃. When the photocytotoxic effect was evaluated in MCF-7 cells, DH-I-180-3 loaded in SLN/NLCs showed significant improvement when compared to DH-I-180-3 in solution. Based on the image of cellular fluorescence microscope, it was concludedthat the increased cellular accumulation was due to the incorporation of DH-I-180-3 into the nanoparticles, which resulted in improvement in photocytotoxicity.

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Table 1. Approximate solubility of DH-I-180-3 in various solid lipids and liquid lipids

State	Lipids	Solubility
Solid	**Stearic acid**	**10-15 w/w%**
	Stearic alcohol	**5-10 w/w%**
	Cetyl alcohol	
	Dynasan® 114	**< 5 w/w%**
	Witepsol®H15	
	Cetyl palmitate	
Lipid	**Capmul® MCM C8**	**> 30 w/w%**
	Oleic acid	**5-10 w/w%**
	Sesame seed oil	
	Castor oil	**< 5 w/w%**
	Cotton seed oil	
	Olive oil	

Table 2. Melting parameters of lipid blends of stearic acid and Capmul® MCM C8

Capmul® MCM C8 content (w/w% Capmul® MCM C8)	Melting point (℃)	Onset Temperature (℃)	Enthalpy (J/g)	Crystallinity (%)
0	**71.91**	**67.94**	**211.88**	**100**
5	**71.35**	**65.57**	**213.57**	**101**
10	**70.76**	**65.01**	**196.48**	**92**
15	**69.24**	**63.05**	**174.84**	**89**
30	**67.90**	**59.51**	**140.45**	**80**

Table 3. Particle size, polydispersity index (PI), zeta potential (ZP), drug loading 62, and entrapment efficiency (EE) for DH-I-180-3 loaded SLN and NLCs.

	Particle size (nm) (Cohen et al.)	P.I.	ZP (mV)	DL (w/w%)	EE (w/w%)
SLN	221.53±3.67	0.27±0.02	-49.86±5.35	7.66±0.30	89.35±2.81
NLC 5 %	**201.00±9.40**	**0.25±0.04**	**-49.97±5.49**	**7.53±0.87**	**92.77±0.27**
NLC 10 %	**207.47±11.09**	**0.29±0.02**	**-36.17±1.33**	**9.14±0.54**	**95.90±0.40**
NLC 15 %	**206.00±11.04**	**0.28±0.04**	**-38.36±4.16**	**9.65±0.65**	**96.14±2.30**
NLC 30 %	**201.96±13.81**	**0.29±0.02**	**-40.12±1.88**	**9.90±0.81**	**96.69±0.34**

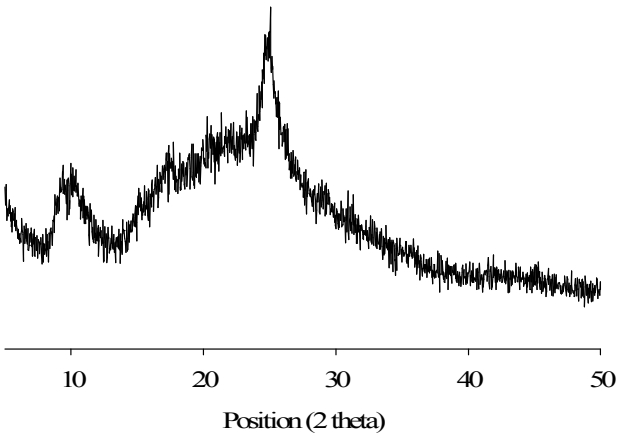

Figure 1. XRD pattern of DH-I-180-3

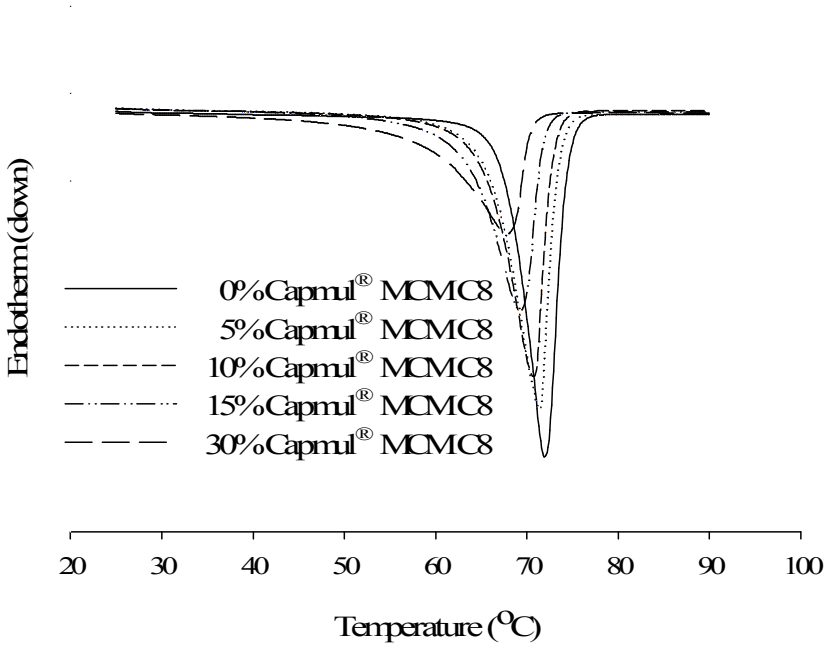

Figure 2. DSC thermograms of lipid blends of stearic acid and Capmul® MCM C8

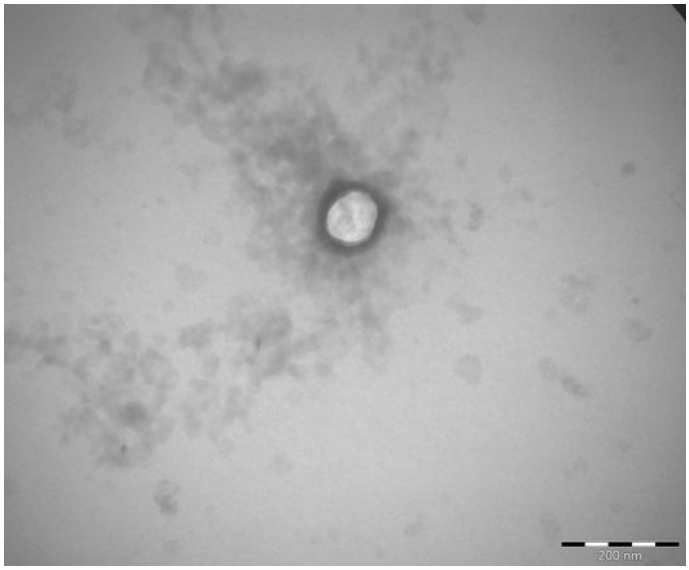

Figure 3. TEM image of prepared SLN formulation without cryoprotectant

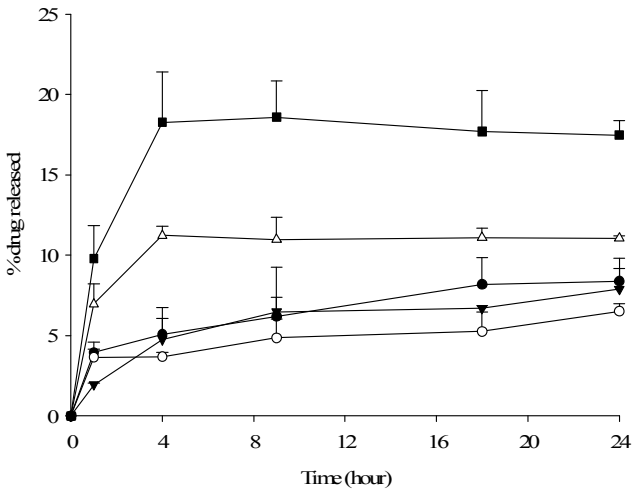

Figure 4. In vitro release profile of DH-I-180-3 from SLN (●, NLC 5% (○, NLC 10% (▼, NLC 15% (△, and NLC 30% (■) formulations in 2 %w/v poloxamer 188 solution. (n = 3)

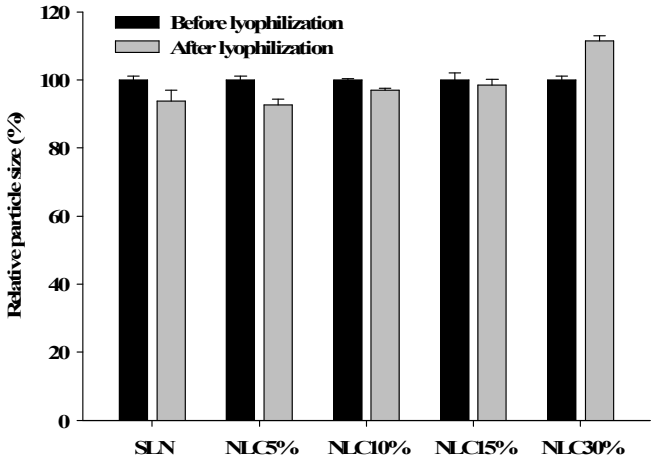

Figure 5. Comparison of particle size of SLN/NLCs between before and after lyophilization

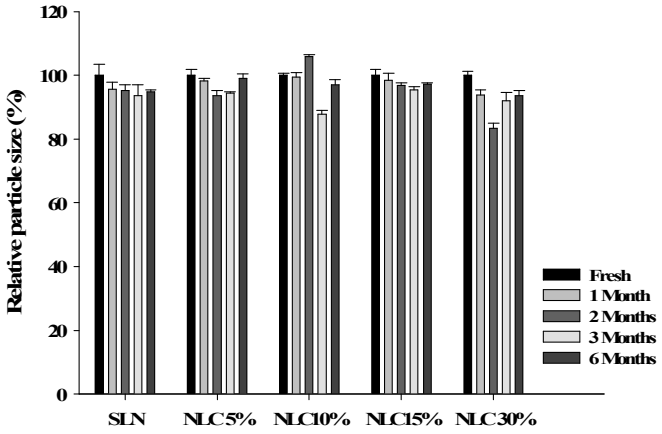

Figure 6. Physical stability of SLN/NLCs in particle size in storage at 4 ºC up to 6 month

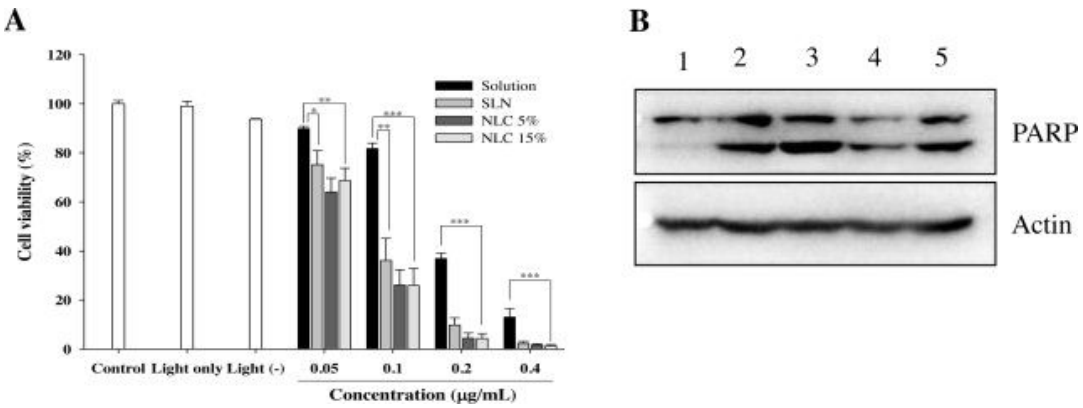

Figure 7. Cytotoxicity induced by photodynamic therapy(A) Cell proliferation of DH-I-180-3 solution, DH-I-180-3 loaded SLN and NLC formulations at various concentration in MCF-7 cell lines (n = 10). *: $p < 0.05$ and **: $p < 0.01$, ***: $p < 0.001$. (B) Western blot analysis of PARP activation after photodynamic therapy. 1) control: cells treated with DMSO; 2)DH- I-180-3 solution; 3) SLN; 4) NLC 5% and 5) NLC 15%

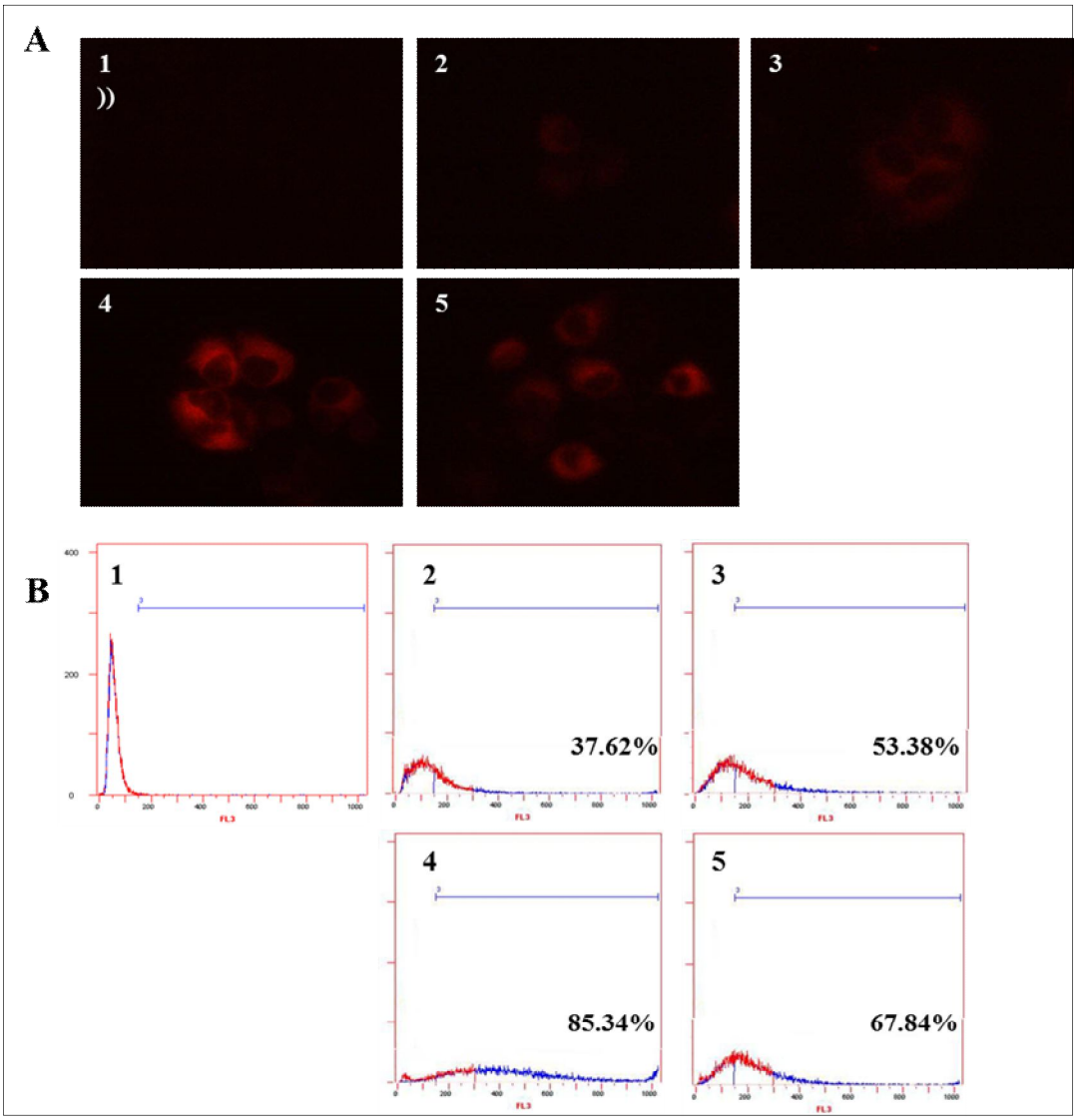

Figure 8. Accumulation of DH-I-180-3 loaded SNL and NLC formulations in MCF-7. (A) Photographs obtained by fluorescent microscopy. (B) The quantification by flow cytometric analysis. 1) Control (cells treated with DMSO) 2) DH- I-180-3 solution; 3) SLN; 4) NLC 5% and 5) NLC 15%

감사의 글

오랜 기간 동안 힘들었지만 이제야 작은 결실을 맺고 감사의 글을 적을 수 있게 되었습니다.

두려움으로 시작했던 박사과정동안 수많은 어려움이 있었지만 많은 분들의 도움으로 마침표를 찍게 되었습니다.

가장 먼저 부족한 저를 제자로 받아주시고 연구기간동안 지도해주신 최후균교수님께 진심으로 머리 숙여 감사드립니다. 앞으로도 교수님의 가르침 잊지 않고 열심히 연구하고 일하는 약사의 길을 걸어가겠습니다. 바쁘신 중에도 논문심사를 해주신 이용복교수님, 한효경교수님, 강건욱교수님, 최홍석교수님께도 깊은 감사를 드립니다.

대학생활부터 석, 박사과정 동안 한결같이 저를 믿고 응원하고 기도해주신 제 인생의 가장 소중한 부모님, 제가 학위과정을 마무리 할 수 있었던 원천이 되주셨습니다.

감사의 글을 올리기에는 눈물이 먼저 납니다. 아버지, 어머니 감사하고 사랑합니다.

멀리에서 학위과정에 충실 할 수 있도록 우리 가정을 항상 행복하고 평탄하게 지켜주시고 저에게 무한한 힘이 되주신 큰형님, 작은형님께도 감사와 영광을 돌립니다.

부족한 저를 도와 바쁜 시간을 쪼개어 연구를 도와준 United 장재상연구원, SK chemical 생명과학연구소 박준형연구원에게 감사드립니다.

마지막으로 작은 기쁨을 함께하고 싶은 인생의 벗들이 있습니다.

학부 때부터 항상 같이해준 가족과도 같은 동생들 최승혁박사, 장재상박사, 양진원박사, 정현철약사, 조성희약사, 응원해줘서 감사하고 평생 같이하자.

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2015년 12월

반 상 준