DEVELOPMENT OF LIPID NANOPARTICLES FOR ANTICANCER DRUG DELIVERY SYSTEMS

By

Akhayachatra Chinsriwongkul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

Program of Pharmaceutical Technology

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การพัฒนาระบบนำส่งยารูปแบบลิปิดนาโนพาร์ติเคิลสำหรับนำส่งยาต้านมะเร็ง

โดย

นาง อรรฆยฉัตร ชินศรีวงศ์กูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
สาขาวิชา เทคโนโลยีเภสัชกรรม
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
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The Graduate School, Silpakorn University has approved and accredited the thesis title of "Development of Lipid Nanoparticles for Anticancer Drug Delivery Systems" submitted by Akhayachatra Chinsriwongkul as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Technology.

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The purpose of this research was to formulate lipid nanoparticles (lipid emulsions (LE), nanostructured lipid carriers (NLC) and polymer coated-NLC) for delivery of anticancer drug, all-trans retinoic acid (ATRA). The ATRA was incorporated into lipid nanoparticles by the de novo emulsification method and their particle sizes were reduced by ultrasonicator. The formulation factor i.e. type and oil ratio, initial ATRA concentration on the physicochemical properties (i.e. particle size, size distribution, droplets surface charge, pH, percentage yield, percentage drug release, photostability and stability of lipid nanoparticles) was determined. Moreover, the anticancer efficacy of ATRA-loaded lipid nanoparticles on human acute promyelocytic leukemia cells (HL-60) and human hepatoma cells (HepG2) were also studied. The order of solubility of ATRA in solvent was oleic acid (O) > MCT (M) > soybean oil (S) > water. The physicochemical properties of ATRA-loaded LE, including mean particle diameter and zeta potential, were modulated by changing the initial ATRA concentration as well as the type and mixing ratio of oil and oleic acid as the oil phase. The average particle sizes of LEs were less than 250 nm with negative zeta potential. The addition of oleic acid in LEs resulted in high loading capacity. The photo-degradation rate was found to be dependent on the initial drug concentration but not on the oil used in this study. The release rate did not affect by the initial ATRA concentration but type affected by the type of oil. The formulation containing oleic acid showed the highest release rate of ATRA from LEs. The stability of LEs formulation composed of 30% of four types of liquid lipids (S, M, the mixture of SO=3:1 or MO=3:1) was desirable, therefore, NLC was formulated by using the blend of solid lipid (cetyl palmitate; CP) and four types of liquid lipids (S, M, SO and MO) at the weight ratio of 1:1. The results indicated that oleic acid affected the ATRA loading capacity in NLC. The release of ATRA from NLC was less than LE, but the physical stability of NLC was better than LE. Moreover, the higher loading capacity of ATRA can be achieved by NLC. The physicochemical properties of NLC coated with polymers were not significantly different from uncoated-NLCs. The cytotoxicity results showed that all ATRA loaded lipid nanoparticles had higher cytotoxicity than the free drug and HL-60 cells were more sensitive to ATRA than HepG2 cells.

Program of Pharmaceutical Technology Graduate School, Silpakorn University Academic year 2009
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Thesis Advisors' signature 1 ..... 2..... 3.....

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คำสำคัญ : ออล-ทรานส์ เรติโนอิก แอซิด / ลิปิดนาโนพาร์ติเคิล / ลิปิดอิมัลชัน / นาโนสตรักเจอร์ลิปิดแคริเออร์

อรรฆยฉัตร ชินศรีวงศ์กูล : การพัฒนาระบบนำส่งยารูปแบบลิปิดนาโนพาร์ติเคิลสำหรับนำส่งยาต้านมะเร็ง. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : รศ.ดร.ปราณีต โอปณะโสภิต, รศ.ดร.ธนะเศรษฐ์ ง้าวหิรัญพัฒน์ และ ดร.อุรชา รักษ์ตานนท์ชัย. 199 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาตำรับลิปิดนาโนพาร์ติเคิล (ลิปิดอิมัลชัน (LE), นาโนสตรักเจอร์ลิปิดแคริเออร์ (NLC), และ NLC ที่เคลือบด้วยพอลิเมอร์) เพื่อใช้สำหรับนำส่งยาต้านมะเร็งคือ ออล-ทรานส์ เรติโนอิก แอซิด (ATRA) โดยใช้วิธีเตรียมแบบ เดอ โนโว อิมัลซิฟิเคชัน จากนั้นลดขนาดอนุภาคด้วยเครื่องอัลตราโซนิค ศึกษาปัจจัยต่างๆ ได้แก่ ชนิดและสัดส่วนของน้ำมัน ความเข้มข้นเริ่มต้นของยา ATRA ที่มีผลต่อคุณสมบัติทางเคมีกายภาพ (ขนาดของอนุภาค ค่าการกระจายขนาดอนุภาค ประจุที่พื้นผิวอนุภาค ค่าความเป็นกรดด่าง ปริมาณยาที่ถูกกักเก็บในสูตรตำรับ เปอร์เซ็นต์การปลดปล่อยยาออกจากตำรับ ความคงตัวต่อแสง และความคงตัวของลิปิดนาโนพาร์ติเคิล) ตลอดจนศึกษาประสิทธิภาพในการต้านมะเร็งของลิปิดนาโนพาร์ติเคิลที่บรรจุยา ATRA กับเซลล์มะเร็งเม็ดเลือดขาว (HL-60) และเซลล์มะเร็งตับ (HepG2) จากการศึกษาความสามารถของน้ำมันในการละลายยา ATRA พบว่า โอเลอิค แอซิด (O) > มีเดียมเชนไตรกลีเซอไรด์ (M) > น้ำมันถั่วเหลือง (S) > น้ำ คุณสมบัติทางเคมีกายภาพของ LE ที่บรรจุ ATRA ได้แก่ขนาดของอนุภาค และค่าความเป็นประจุที่ผิวอนุภาค สามารถเปลี่ยนแปลงโดยการเปลี่ยนความเข้มข้นเริ่มต้นของ ATRA และชนิดของน้ำมัน รวมถึงสัดส่วนของน้ำมันกับโอเลอิค แอซิด ที่ใช้เป็นวัตภาคน้ำมัน ระบบ LE มีขนาดของอนุภาคเล็กกว่า 250 นาโนเมตร และประจุที่พื้นผิวอนุภาคมีค่าเป็นลบ การใช้โอเลอิค แอซิดทำให้ LE สามารถกักเก็บยาได้มากขึ้น ในการศึกษานี้พบว่าอัตราการสลายตัวด้วยแสงขึ้นกับความเข้มข้นเริ่มต้นของยา แต่ไม่ขึ้นกับชนิดของน้ำมันที่ใช้ อัตราการปลดปล่อยยาไม่ขึ้นกับความเข้มข้นเริ่มต้นของ ATRA แต่ขึ้นกับชนิดของน้ำมันที่ใช้ ซึ่งโอเลอิค แอซิดให้อัตราการปลดปล่อย ATRA จาก LE ได้สูงสุด ผลการศึกษาแสดงให้เห็นว่า LE ซึ่งประกอบด้วยน้ำมัน 4 ชนิด (S, M, สารผสม SO และ MO ในอัตราส่วน 3:1) ในปริมาณ 30% เป็นระบบที่มีความคงตัวสูง ดังนั้นจึงตั้งตำรับ NLC โดยผสมลิปิดชนิดแข็ง (ซิทิลปาล์มิเตท, CP) ใช้ร่วมกับน้ำมันทั้ง 4 ชนิด (S, M, SO, และ MO) ในอัตราส่วน 1:1 ผลการศึกษาชี้ว่าโอเลอิค แอซิดส่งผลต่อความสามารถในการกักเก็บยาของ NLC และในทางตรงข้ามกับ LE การปลดปล่อยตัวยาออกจาก NLC น้อยกว่า LE NLC มีคงความคงตัวทางกายภาพสูงกว่า LE นอกจากนี้ NLC ยังสามารถกักเก็บยาไว้ได้สูงกว่า LE พบว่าคุณสมบัติทางเคมีกายภาพของ NLC ที่เคลือบด้วยพอลิเมอร์ ไม่แตกต่างจาก NLC ที่ไม่ได้เคลือบด้วยพอลิเมอร์ ผลการศึกษาความเป็นพิษต่อเซลล์มะเร็งของลิปิดนาโนพาร์ติเคิลพบว่าตัวยาที่บรรจุในระบบ LE มีความเป็นพิษต่อเซลล์มะเร็งสูงกว่าตัวยาที่ไม่ได้บรรจุในระบบใดๆ และเซลล์ HL-60 ตอบสนองต่อ ATRA สูงกว่า HepG2

สาขาวิชาเทคโนโลยีเภสัชกรรม บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2552
ลายมือชื่อนักศึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1..... 2..... 3.....

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CHAPTER 1
INTRODUCTION

All-trans retinoic acid (ATRA) is a physiologically active form of a metabolic product of vitamin A. It is a poorly water soluble substance and sensitive to light, heat and air. Retinoids are potent agents for control of both cellular differentiation and cellular proliferation. Several studies have shown that retinoids can suppress the process of carcinogenesis both *in vitro* and *in vivo*, especially, in the treatment of acute promyelocytic leukemia (APL). ATRA is not a cytolytic agent but instead induces cytodifferentiation and decreases proliferation of malignant cells. Although the oral ATRA dosage form has been demonstrated to be effective against a range of cancers in clinical trials, the important drawback of using oral ATRA is its poor bioavailabilty. ATRA is almost insoluble in aqueous solutions and its intestinal absorption is affected by the pH and fatty acid composition of intraluminal bile. Therefore, plasma concentrations of orally administered ATRA are highly variable (Ozpolat and Berestein 2003 : 293). In addition, the oral ATRA administration in patients who cannot swallow capsules may be another concerned. Therefore, an attempt to find other routes of administrations that might increase therapeutic efficacy and reduce side effect of the drug has been made.

A parenteral administration has shown to be a good alternative for ATRA administration to increase the reliable potency and duration of it's activity in cancer patients. However, the major obstacle for drug reaching the adequate biological compartments is the limitation of ATRA water solubility. Therefore, various approaches have been examined in order to improve its aqueous solubility such as cyclodextrin complexes (Lin et al. 2000 : 265), liposomes (Shimizu et al. 2003 : 45), niosomes (Manconi et al. 2002 : 237), polymeric micelles (Zuccari et al. 2005 : 369), solid lipid nanoparticles (SLN) (Lim et al. 2004 : 53), and based nanocarrier systems including liposome, lipid emulsions, solid lipid lipid emulsions (Hwang et al. 2004 :

175). Among these approaches, the use of solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) seems to be attractive since ATRA is a lipophillic compound and it is well documented that these systems could reduce toxicity associated with drug administration. Moreover, lipid-based nanocarriers i.e. lipid emulsions and SLN could incorporate many lipophilic-cytotoxic drugs due to their ability to solubilize large quantities of these drugs in their matrix systems.

There is a parenteral formulation of ATRA commercially available, ATRA-IV® (formerly known as ATRAGEN®, Antigenics Inc.), which is an intravenous lyophilized ATRA liposome for injection. The incorporation of ATRA into liposomes has significantly improved the potency and duration of it's activity with reducing side effect associated with the drug. Most importantly in preclinical and clinical models when the drug administered intravenously, liposomal encapsulated-ATRA, it can maintain stable plasma concentrations over a prolonged time after multiple dosing (Ozpolat and Berestein 2003 : 293). Although liposomal encapsulated ATRA has more advantages than the oral formulation, the capacity of drug loading is still limited. From this reason, other drug delivery system giving a large drug loading for ATRA must be searched.

Lipid emulsions are heterogeneous systems composed of oil phase dispersed as droplets in the aqueous phase and stabilized by phospholipids resulting in an oil in water (O/W) emulsions (Lucks and Müller, in Nielloud and Marti-Mestres, eds. 2000 : 231). They have been developed on the model of the intestinal chylomicron since after World War II as a parenteral nutrition. The first safe intravenous lipid emulsion introduced as Intralipid® in the 1960's consisted of an O/W emulsion of 10 or 20% soybean oil droplets (70-400 nm in size) stabilized by a monolayer of 1.2% egg yolk mixed phospholipids and 2.25% glycerol (Benita, in Benita, ed. 1998 : 1). The wide and clinical well accepted usage of emulsion for parenteral nutrition has raised the possibility of using the internal oil phase of this O/W emulsion for solubilizing water-insoluble drugs which are often difficult to deliver (Kland and Benita, in Benita, ed. 1998 : 119). There are major differences between O/W emulsions and liposomes. Liposomes contain an outer bilayer of amphipathic molecules such as phospholipids with an aqueous compartment inside

and are superior carriers to deliver hydrophilic drugs into target tissues, however incorporation of a lipophilic drug into bilayer membrane changes the properties of the particles and results in loss of control of delivery. Emulsions, in contrast, have only one layer of amphipathic molecules such as phosphatidylcholine (PC) on the surface, and the inside core is filled with highly lipophilic oil. Lipid emulsion systems therefore have more potential as drug carriers for highly lipophilic molecules solubilized in the core oil (Hodoshima et al. 1997 : 81). Since the lipid emulsions are purposing for parenteral applications, it is necessary to meet pharmacopoeial requirements. The emulsions must be sterile, isotonic, non-pyrogenic, non-toxic, biodegradable and physically and chemically stable. Furthermore, no droplets larger than the diameter of the finest capillaries (about 5 μm) are available. The size needs to be below 1 μm, and generally ranges between 100-500 nm. With larger droplet size, potential oil embolism may occur (Klang and Benita, in Benita, ed. 1998 : 119). Due to many favorable properties of lipid emulsions (i.e. low toxicity, biocompatible, biodegradable, high loading capacity, easy to prepare-handle and possible to increase in batch size or production capacity (Müller and Runge, in Benita, ed. 1998 : 219). Drug delivery and targeting research using lipid emulsions as carriers of poorly water soluble drugs have been explored (Brisaert and Plaizier-Vercammen 2000 : 49). However, due to the liquid state of the oil droplets, a prolonged drug release cannot be achieved. After intravenous injection, partitioning of the drug within milliseconds or seconds between the liquid oil phase and the aqueous phase of the blood normally occurs. Even lipophilic drugs exhibit a burst release due to the relatively large volume of the water phase compared to the few amount of the oil phase of the emulsion. To overcome these disadvantages associated with the liquid stage of the oil droplet of lipid emulsions as previously mentioned, the use of solid lipid, which remains in solid state at room temperature and body temperature, instead of liquid oils is very attractive to achieve controlled drug release, leading to the formation of solid lipid nanoparticles (SLN) at the beginning of the 1990's.

The SLN combine advantages of solid particles, emulsions and liposomes. The advantages of solid particles are a protection of incorporated active compounds against chemical degradation and more flexibility in modulating the release of the

compound. The advantages of liposomes and emulsions are that they are composed of well tolerated excipients and they can easily be produced on a large scale, the pre-requisite for a carrier to be introduced to the market (Müller, Radtke and Wissing 2002 : S131). More important, SLN formulations carrying cytotoxic drugs such as doxorubicin and paclitaxel have been studied and demonstrated the favorable results (Wong et al. 2007 : 491). Although SLN shows many valid advantages but potential problems associated with SLN such as insufficient loading capacity, drug expulsion after polymorphic transition during storage and relatively high water content of the dispersions (70-99.9%) have been observed (Rossi et al. 2007 : 329).

Nanostructured lipid carriers (NLC) have been then introduced at the end of the 1990s in order to overcome the potential difficulties of SLN described above. Since drug loaded in SLN is limited due to the formation of the lipid crystal and drug expulsion is caused by the ongoing crystallization process towards a perfect crystal. Thus, the basic idea to reduce crystallization process is to give the lipid matrix a certain nanostructure by mixing solid lipids with liquid lipids (oils). The resulting matrix of these lipid particles shows a melting point depression compared to the original solid lipid but the matrix is still solid at body temperature. Depending on the way of production and the composition of the lipid blend, three different types of NLC are obtained i.e. imperfect type, amorphous type, and multiple types (Müller et al. 2002 : S132).

When colloidal drug carrier is administered intravenously, it can be cleared rapidly from blood stream circulation by the reticuloendothelial system (RES) governed by macrophages through a process called phagocytosis. This is initiated by an adsorption of certain serum proteins, opsonins, so-called opsonisation at the carrier surface, then accumulated in RES-related tissues, such as liver, spleen, and bone marrow. However, the drug accumulation in RES-related tissues may be favorable in certain instances e.g., when the site of action of the drug is in one or several of these tissues. It has been found that the uptake of colloidal drug carriers by the RES depends on a range of factors, e.g., the size and surface properties of the carrier, hydrophobicity, charge, and chemical functionality. In particular, the uptake with an increasing particle size, hydrophobicity (Malmsten 2002 : 104). Modifying the colloid

surface with a hydrophilic and flexible polymer such as poly(ethylene glycol) (PEG), so called PEGylated colloids is widely used to prolong circulation time. The longevity of PEGylated colloids is attributed to the highly hydrated and flexible PEG chains, which reduce interactions with plasma proteins and cell surfaces. Incorporating sphingomyelin (SM) at the interface is another approach that has shown to enhance the circulation longevity of emulsions and liposomes (Rossi et al. 2007 : 330).

Therefore, the objective of this research was to develop lipid nanoparticles including lipid emulsion and NLC formulation of anticancer drug, all-trans retinoic acid (ATRA) with high loading and high stability. The physical properties of ATRA such as aqueous and oil solubility (soybean oil, medium chain triglyceride (MCT), oleic acid and thereof mixture in various ratio, were determined. The formulation factors such as type of lipids, content of drug, on the physicochemical property (particle size, size distribution, droplets surface charge, pH), percentage yield, percent drug release, photoprotective efficacy, formulation stability and anticancer activity of these lipid nanoparticles were evaluated. Since, modification of colloidal surfaces by PEG-polymer coating could prevent opsonization consequently prolongation in the blood circulation time leading to drug targeting by EPR effect. Thus the lipid nanoparticles formulations which posses better physicochemical properties and stability were subsequently selected to be coated with PEG-polymers. The physicochemical property (particle size, size distribution, droplets surface charge, pH), percent entrapment efficiency, percent drug release, photoprotective efficacy, formulation stability and anticancer activity of the coated-lipid nanoparticles were also examined in the same manner of those uncoated-lipid nanoparticles.

CHAPTER 2
LITERATURE REVIEWS

All-trans retinoic acid and drug delivery system

Retinoids are natural and synthetic compounds of similar structure and the term retinoids refers to entire compounds including both naturally occur and synthetic retinol (vitamin A) metabolites and analogs. Retinoids have long been established as crucial compounds for several essential life processes including healthy growth, vision, maintenance of tissues, reproduction, metabolism, tissue differentiation (normal, premalignant cells, and malignant cells), haemopoiesis, bone development, spermatogenesis, embryogenesis, and overall survival (Abu et al. 2005 : 712). To date, three generations of retinoids have been developed. All-trans retinoic acid (ATRA, tretinoin or vitamin A acid) is a physiologically active form of a metabolic product of vitamin A. It belongs to the first generation retinoids. It is a yellow or light orange crystalline powder with a molecular weight of 300.44. Storage in airtight containers at a temperature below 25ºC is highly recommended, preferably under an atmosphere of inert gas because it can be destroyed in the presence of light and oxidants. Moreover, it is poorly water soluble substance. The structure formula is shown in Figure1:

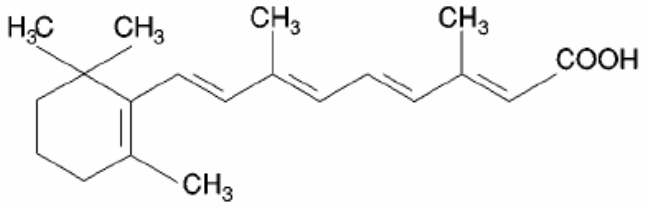


Figure 1 Structure formula of all-trans retinoic acid (ATRA).

Source: P. Opanasopit et al., "N-Phthaloylchitosan-g-PPEG design for all-trans retinoic acid-loaded polymeric micelles," European Journal of Pharmaceutical Sciences 30 (2007) : 427.

Several experimental studies have shown the antiproliferative activity of retinoids both *in vitro* and *in vivo* (Kim et al. 2006 : 455). Many researchers have examined the ability of retinoids to treat various diseases such as acute promyelocytic leukemia (APL), Kaposi's sarcoma, head and neck squamous cell carcinoma, ovarian carcinoma, bladder cancer, and neuroblastoma (Zuccari et al. 2005 : 370). The mechanism of action of retinoids in chemoprevention and therapy of cancers involves modulation of cell proliferation and differentiation (Kuo et al. 2006 : 80). The effects of retinoid are mainly mediated by nuclear retinoid receptors which are members of the steroid hormone receptor superfamily. There are two types of retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each composed of three members, α-β-γ, and several isoforms. It has been postulated that nuclear RARs are the final mediators of ATRA action on gene expression that may lead to cell differentiation, inhibited growth, and ultimately cell death (Vieson and Moffitt 1995 : 1).

Differentiation therapies in oncology are broadly defined as those that induced malignant reversion (i.e. the malignant phenotype becomes benign) (Spira and Carducci 2003 : 338). Retinoid compounds are known to be involved in chemoprevention and differentiation therapy of some cancers, resulting particularly effective in the management of APL. Clinically, the differentiation therapy have been most successful for APL with the use of ATRA (Toma et al. 2005 : 27).

Vesanoid® is an oral ATRA in a 10 mg soft gelatin capsule, which is available in market by Roche. The indication of this dosage form is for the induction of remission in patients with APL. Although the oral ATRA dosage form has been demonstrated to be effective against a range of cancers in clinical trials, the important drawback of using oral ATRA is its poor bioavailabilty. ATRA is almost insoluble in aqueous solutions and its intestinal absorption is affected by the pH and fatty acid composition of intraluminal bile. Therefore, plasma concentrations of orally administered ATRA are highly variable (Ozpolat and Berestein 2003 : 293). Moreover, long-term oral treatment with ATRA (usually within 1-6 weeks) has shown a progressive decline in plasma drug levels, probably due to the induced cytochrome P-450-dependent metabolism of ATRA (Hwang et al. 2004 : 175-176). In addition,

the orally ATRA administration in patients who unconscious or cannot swallow capsules may be concerned. Therefore, an attempt to find other routes of administrations and alternative method for delivery drug that might increase the therapeutic efficacy and reduce side effect of free form ATRA such as liver function abnormality, dryness of skin, constipation and conjunctivitis of the drug is needed.

An appropriate choice to overcome these problems is the parenteral administration of ATRA, but from the fact that a parenterally administered drug is injected directly into blood, which is typically only possible in form of a drug solution or emulsion. After administration, the drug will be presented as molecules in solution (i.e. blood). It distributes in the body according to its physicochemical properties, e.g. its partitioning coeffiecient. The presence as molecular solution turns these molecules to be easily accessible to degrading factors (eg. water, enzymes), and then they cannot be directed (targeted) to their desired site of action. One approach to overcome these problems would be the entrapment of those drugs into a particulate carrier system. An incorporation is not only to protect them against degradation both *in vitro* and *in vivo*, but also the release rate can be modified, offering as well targeting approaches (Martins et al. 2007 : 595). Among various kinds of drug delivery systems, lipid-based nanocarrier systems including liposome, lipid emulsions, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) might help to solve these problems. Since ATRA is a poorly water soluble compound. It is well documented that colloidal drug delivery system could increase drug loading and could reduce toxicity associated with drug administration.

There is an intravenous lyophilized ATRA liposome for injection (ATRA-IV®, Antigenics Inc., or formerly known as ATRAGEN®, Aronex Pharmaceuticals, Inc.). When incorporated in liposomes, ATRA-associated toxicity is markedly reduced whereas anti-tumor properties (growth inhibition and differentiation induction) of the drug remain unchanged. Less toxic form of liposomal-ATRA allows the use at higher doses and may potentially lead to higher tissue concentrations and long duration of action at target-site (Ozpolat et al. 2003 : 293). Although liposomal encapsulated ATRA have more advantages than oral formulation as described above, the limitation of ATRA loading capacity of liposome should be considered. Since

liposomes are lipid membranes, thus, the lipophillic drugs could only deposit on these contain bilayer membranes. From this reason, other lipid-based carriers that giving higher drug loading for ATRA should be considered .

Lipid nanoparticles

1. Lipid emulsions

1.1 Definitions and Physical structure of lipid emulsions

Emulsions are heterogeneous system in which one immiscible liquid is dispersed as droplet in another liquid and stabilized by emulsifier. Two common types of emulsions are found in parenteral drug delivery systems, water in oil (W/O) emulsions and oil in water (O/W) emulsions. The W/O emulsions are used in sustained release of steroids and vaccines by intramuscular injection, whereas O/W emulsions or lipid emulsions can be administered by a variety of parenteral routes e.g. subcutaneous, intramuscular and intra-arterial, but are predominantly injected intravenously for parenteral nutrition applications.

Lipid emulsions can be defined as a heterogeneous system, in which oil is dispersed as droplets in aqueous phase and stabilized by phospholipids to prevent coalescence by reducing interfacial tension or creating a physical repulsion between the droplets. The structure of lipid emulsion is shown in Figure 2.

Oil droplets or triglyceride core (diameter 250-400 nm) of lipid emulsions are stabilized with a phospholipids monolayer (diameter 60-90 nm). If lipid emulsions are prepared with an excess of phospholipids, liposomes may occur concurrently as dispersed unilamellar or multilamellar liposomes. However, lipid emulsions differ clearly from the liposomes. In the case of liposomes, phospholipids bilayer separates an aqueous core from a hydrophilic external phase. Whereas phospholipids separate oil phase or lipophilic interior from the external aqueous phase of lipid emulsions.

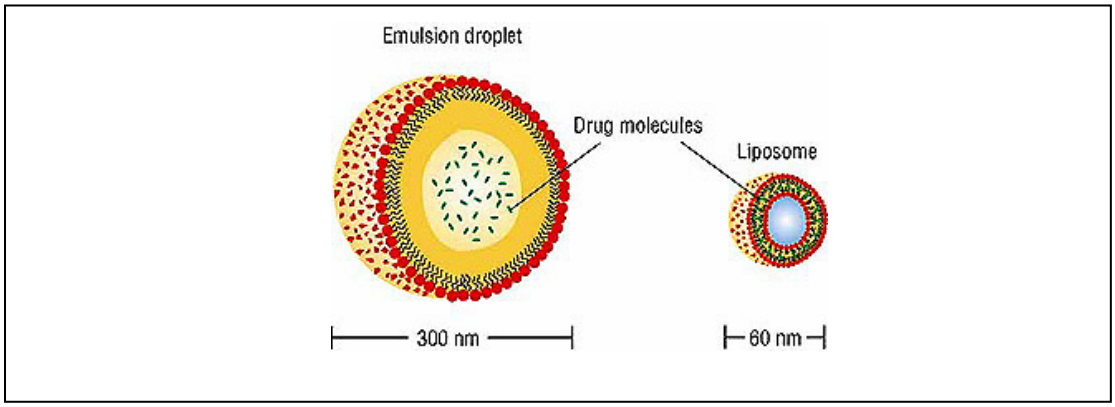


Figure 2 Oil droplets of lipid emulsions are surrounded by a single phospholipids layer and the excess phospholipids show free unilamellar or multilamellar liposomes.

Source: L. Collins-Gold, N.Feichtinger, and T. Wärnheim, "Are lipid emulsions the drug delivery solution?," Modern Drug Discovery 3,3 (2000) : 44.

Once the lipid emulsions are purposing for parenteral applications, it is necessary to meet pharmacopoeial requirements. The emulsions must be sterile, isotonic, non-pyrogenic, non-toxic, biodegradable and both physically and chemically stable. Furthermore, no droplet larger than the diameter of the finest capillaries is available (about 5 µm). The size needs to be below 1 µm, and generally ranges between 100-500 nm. With larger droplet size, potential oil embolism may occur. According to the transportation of fat in the blood stream as very small-emulsified lipid particles called chylomicrons, they are remarkably uniform in size, being about 0.5 to 1.0 µm spheres and are consisting of triglyceride in a central core surrounded by phospholipids. Exogenous or artificial lipid emulsion particles should mimic the chylomicrons as closely in biological properties as possible in respect to their transport in the blood and distribution in the body.

1.2 Advantages and disadvantages of lipid emulsions

Lipid emulsions can be administered by a variety of parenteral routes e.g. subcutaneous, intramuscular and intra-arterial, but are predominantly injected intravenously in parenteral nutrition applications (Steven, Mims, and Coles 2003 : 1). Parenteral emulsions have been in clinical use since after World War II.

Until now, more than 30 years they are best known as an excellence source of calories and essential fatty acids for nonambulatory patients who cannot orally consume or metabolize food properly, with no osmotic effect or as a vehicle for the administration. According to their composition, lipid emulsion are excellent carriers for lipophilic drugs.

In the early 1960's, Wretlind met the challenge of developing a safe, metabolizable intravenous fat emulsion was met, this "prototype" formulation was first described latter in 1961 by Schuberth and Wretlind, and it continues to be marketed as Intralipid®, a 10% or 20% soybean oil droplets (70-400 nm in size) stabilized by monolayer of 1.2% egg phosphatides and 2.25% glycerol as an osmotic agent. For the past decade and based on the basis knowledge including formula of Intralipid®, intensive research for poorly water soluble drugs have been concentrated on the design of intravenous lipid emulsion formulations that led to successful marketed products such as the emulsion of 1% Propofol (Diprivan®, Zeneca, UK). In addition, their biodegradability including biocompatibility, physical stability and relatively easiness to produce on large scale, have led submicron lipid emulsions as drug carriers for lipophilic drugs. There are several products currently available on the market using lipid emulsion e.g. Diazepam-Lipuro®, Braun, Germany), Alprostadil (PEG1) (Liple®, Green Cross, Japan), Perfluorodecalin and Perfluorotripropylamine (Fluosol-DA®, Green Cross and Alpha Therapeutics, Japan), Vitamin A, D2, E and K1 (Vitalipid®, Kabi-Pharmacia, Sweden), Propofol (Diprivan®, Zeneca Pharmaceuticals, UK), Dexamethasone palmitate (Limethason®, Green Cross, Japan), Flurbiprofen axetil (Lipo-NSAID® and Ropion®, Kaken Pharmaceuticals, japan), Etomidate (Etomidate Lipuro®, Braun, Germany). Moreover, there are many drugs containing lipid emulsions under clinical and preclinical evaluation e.g. antifungal agents (amphotericin B, miconazole), anaesthetic agents (pregnanolone, halothane, isoflurane), and cytotoxic agents (rhizoxin, taxol, penclomedine, nitrosourea).

The advantages of using lipid emulsions can be described as following:

1.2.1 Solubilization of low water-solubility drugs

For many drugs, insufficient aqueous solubility and/or water hydrolysis are their major formulation challenges. The most commonly way is by pH control for drug with a suitable ionizing group, or by the use of co-solvents such as ethanol, glycols, dimethylacetamide or dimethylsulfoxide. The use of these conventional methods provides undesirable effects in precipitation of the drug at the injection site, leading to reducing drug bioavailability. Besides, the limitations of these approaches include the possibility of pain or phlebitis and deleterious effects on drug stability. Because most drugs with low water solubility have much higher solubility in lipids, the use of an O/W emulsions can reduce or overcome these problems by incorporating the drug into the interior oil phase (Prankerd and Stella 1990 : 139).

1.2.2 Stabilization of hydrolytically susceptible compounds

Drugs intended for intravenous use are often unstable in aqueous or partially aqueous solution. The most commonly used method for stabilization of such a drug is by lyophilization of a sterile and particle-free solution prior to storage. Reconstitution of the freeze-dried powder with an appropriate sterile solvent (immediately before administration) will usually result in a product, which is chemically stable for the time required for administration. Lyophilization has the disadvantage, however, of greater cost and complexity than other methods for the preparation of parenteral products. It has previously been shown that the rate of hydrolytic loss of a drug can be markedly reduced by formulation in an O/W emulsion (Prankerd and Stella 1990 : 142)**.**

1.2.3 Reduction of irritation or toxicity of intravenously administered drugs

Some cytotoxic compounds, e.g., mustine, ethoglucid, and triazine antifol, are quite irritant when given intravenously, although this may not necessarily result from precipitation of the drug at injection site. Triazine antifol is very unstable in aqueous solution, causing severe necrosis on injection site into the mouse-tail vein. This occurs when the drug is given as an aqueous solution of the ethanesulfonate of L-lactate salts. Dilution of an aqueous solution of the L-lactate salt

with Intralipid® resulted in a marked increase in its chemical stability. Moreover, i.v. administration to mice via the tail vein did not result in the necrosis as previously observed with aqueous solution.

Intravenous administration of drugs such as diazepam dissolved in cosolvent mixtures is exacerbated by the injection vehicle. However, anticonvulsant activity of diazepam was not reduced when O/W vehicle was utilized and the acute toxicity was less than that in the cosolvent vehicle.

Intravenous emulsion formulations are therefore an alternative injection vehicle with considerable reduced systemic toxicity, compared with conventional cosolvent mixtures. Precipitation of the drug following injection of an emulsion formulation is unlikely to occur in the same way as after injection and dilution of a cosolvent-based formulation. Hence, local irritation due to precipitated drug should also be reduced or eliminated (Prankerd and Stella 1990 : 143).

1.2.4 Use for parenteral nutrition

Lipid emulsions which used for parenteral administration, are usually named as submicron emulsions or lipid emulsions. They are oil in water emulsions and used for nutrient therapy when patients are unable to intake food or have undergone major surgery. These emulsions are based on vegetable oils and stabilized by lecithin providing adequate source of calories and essential fatty acids. Lipid is the most caloric substrate having the density of calorie more than twice of carbohydrate and protein. Lipid emulsions, therefore, have a practical advantage of providing more calories per volume in a small volume of isotonic fluid via a peripheral vein. As a source of essential fatty acids, lipid emulsions provide vary amount of linoleic and linoleic acid sufficient to prevent or treat essential fatty acid deficiency. Arachidonic acid, which is also essential in humans, can be synthesized from linoleic acid. Moreover, fatty acids corporate in numerous metabolic processes besides energy production. They act as precursors for many important biologically active compounds such as prostaglandins and corticosteroids and are the structural integrity of cell membranes and lipoproteins. Lipid emulsions can be use to treat essential fatty acid deficiency, especially for the pediatric patient whom essential fatty acids are needed during growth and development. Lipid emulsions are also indicated

as a source of fat calories for achieving energy requirements. Lipid has a particular metabolic advantage over carbohydrate in patients with glucose intolerance such as the patient with diabetes mellitus or stress-induced glucose intolerance. In addition, for the ventilator-dependent patient in whom carbon dioxide retention is a problem, lipid emulsions in which fat contributes to the caloric intake will be potential benefit because carbon dioxide produced upon oxidation of fat is less than upon oxidation of glucose. However, there are exceptions to the dairy use of lipid as a caloric source, in the patients with altered lipid metabolism or in whom an adverse reaction to lipid emulsions has occurred. The alteration of lipid metabolism has been found in patients with severe hepatic failure, severe renal failure and sepsis. In general, most of fat adsorbed from the gastrointestinal tract is incorporated in chylomicron and transported in this forms into the lymph and blood circulation. The chylomicron are stable emulsion (diameter about 0.5-1.0 μm) consisting of triglyceride (96%), phospholipids (0.8%) and small amounts of cholesterol (1.7%) and proteins (1.7%). The triglycerides are located in the center. The phospholipids are covered on the surface acting as an emulsifier. Lipid emulsions for parenteral nutrition mimic the natural chylomicron, by mean of the emulsion droplets possess a core of triglyceride (soybean oil) which is stabilized by phospholipids layer (egg or soybean lecithin). Metabolism of lipid emulsion is similar to that of chylomicron.

1.2.5 Potential for sustained release dosage forms

An injectable formulation of a drug does not normally have a prolonged action, except where the drug is formulated either as a suspension (e.g., various zinc insulin injections), or as an oil injection for subcutaneous (s.c.) or intramuscular (i.m.) use (e.g., fluphenazine or haloperidol esters). The rate of absorption of the drug from the oil injection is a function of tissue perfusion. Other factors which may also cause variation in the rate of drug release from oil solution are the diffusivity of the drug, the partition coefficient of the drug, the viscosity of the oil phase, and the interfacial area between the injected oil and the surrounding biological fluids.

Parenteral emulsion formulations of barbiturates have shown a significantly longer duration of action than aqueous solutions of the corresponding

sodium salts and to have increased apparent half-lives. In most cases, the duration of effect was significantly longer when the emulsion was given intravenously, compared to an i.v. injection of a solution of the sodium salt. The time of onset of action was prolonged, compared to the sodium salt solution. Similar behavior was occurred in comparison with an emulsion formulation of diazepam, as the free base, with the more commonly used cosolvent formulation of hydrochloride salt (Prankerd and Stella 1990 : 144).

1.2.6 Possibility to use as drug carriers

The dispersed oil droplets in parenteral O/W emulsions are structurally similar to chylomicrons, the form in which lipoidal materials are transported in the lymph and blood. Chylomicrons are cleared from the blood stream by deposition in liver, adipose tissue, cardiac tissue, cardiac muscle, and lactating mammary gland. The clearance of artificial oil droplets from the blood stream depends on the size of the droplets (small particles are cleared more rapidly than large particles), surface charge (charged particles are cleared faster than neutral particles) and the exact composition of the surfactants used to stabilize the emulsion. The adsorption of blood components, particularly serum proteins (opsonin), appears to be of importance in controlling the interaction of artificial lipid materials with cellular blood components (macrophages). It has been shown that fat particles behave like chylomicrons after exposure to blood (Prankerd and Stella 1990 : 144-145).

It has previously been pointed out that directed drug delivery may be either passive or active (Prankerd and Stella 1990 : 145). Passive targeting used natural physiological processes to achieve its result, e.g., the accumulation of particles of diameter > 6-7 μm in the capillaries of the lung (physical filtration), or tendency of the recticuloendothelial system (RES) to take up particles of colloidal size (recognition of particles as foreign to the body and subsequent uptake by macrophages). Conversely, active targeting implies the modification of the surface of the drug carrier so that the carrier is not recognized as foreign and will thus evade the RES barrier, and the carrier will have an affinity for a particular target tissue, either healthy or not.

The normal affinity of certain tissues for emulsified fat droplets has been exploited in some passive directed drug delivery studies. This is particularly the case for transport of cytotoxic agents to the lymphatic system, e.g., mitomycin C, bleomycin, and 5-fluorouracil.

Differences in preferential regional uptake of radiolabelled oil droplets have been reported, depending on the emulsifier composition (phospholipids or Pluronic F68). Some studies have been performed in which surface modification of either polystyrene microspheres or emulsified oil droplets gave a significant reduction in uptake by the liver (show in rabbits by sacrifice/tissue analysis and by gamma scintigraphy), indicating that hepatic macrophages interacted less with treated particles than with untreated particles.

The major difficulty in the use of emulsified oil droplets (as well as liposomes and other colloidal or particulate drug carrier systems) as a means of directed drug delivery is that the capillary bed in most organs does not have large enough pores (fenestrae) for the droplets to migrate out of the vascular space. Only where the capillary walls are particularly "leaky" can diffusion occur. This is particularly the case in the RES and lymphatic systems and in the presence of inflammation with concurrent edema. It is possible that the greatest potential of O/W emulsions will be used for systemic delivery of therapeutic substances. Interestingly, the drug in an emulsion in such a way that the oil droplets were destabilized on injection into the blood stream, giving a rapid release of the drug into the blood stream (Prankerd and Stella 1990 : 145).

General features of lipid emulsions for drug delivery are their good *in vivo* tolerability, i.e. low systemic toxicity and low cytotoxicity, their composition of physiological compounds, the relatively low costs of the excipients and the ease of industrial scale production by high pressure homogenization. Incorporation of drugs can reduce distinctly drug side effects, e.g. thrombophlebitis associated with i.v. injected diazepam or etomidate. However, disadvantages of lipid emulsions is stated due to the liquid state of the oil droplets, a prolonged drug release cannot be achieved. After i.v.injection, there will be a partitioning of the drug within milliseconds or seconds between the liquid oil phase and the aqueous phase of the

blood. Even very lipophilic drugs will exhibit a burst release due to the relatively large volume of the water phase compared to the few milliliter of the oil phase of the emulsion. A prolonged drug liberation from emulsions obtained in *in vivo* studies can be attributed to the experimental set up used. When applying a dialysis tube or inverted dialysis tube method one measures rather the distribution between the two aqueous phases inside and outside the tube than the release form the oil droplets. Hence, a major disadvantage of emulsions as drug carrier systems is therefore the burst release due to the lack of a solid matrix. Furthermore, emulsions are relatively fast metabolized limiting additionally prolonged drug release.

1.3 Emulsion Metabolism and Bioavailability

The safety of emulsions as drug delivery system is inherent to the biodegradable excipients commonly used, such as the emulsifiers egg and soy lecithin, and the oleagenous substrate (for example soybean, safflower oil, structured triglycerides or Miglyol oil). It is generally agreed that parenteral lipid emulsion is taken up along similar routes as natural chylomicrons. It was clearly shown that the rate of elimination is similar for fat emulsions and chylomicrons. However, more detailed studies show differences in the molecular mechanisms of elimination from the bloodstream. In chylomicrons, the triglyceride substrate is eliminated by two processes. One, is removal of triglyceride from the particle through lipolysis which is the hydrolysate by the capillary endothelial enzyme lipoprotein lipase to release glycerol, fatty acids and diglycerides into the adjacent tissues, with the remainder of the emulsion particles removed from the plasma by the liver. Lipoprotein lipase is found predominantly in the adipose tissue, heart and skeletal muscles. The other metabolic pathway is the removal of the particle itself. For lipid emulsions, this is the predominant route for elimination. It takes in the removal of greater than 50% of the emulsion particles from the bloodstream into the extrahepatic tissues with little or no preceeding lipolysis.

The clearance rate of an emulsion from the blood is intrinsic to the relationship between the physicochemical properties of the emulsion droplets and the physiological response by the reticuloendothelial system (RES). Small emulsion particles are removed slower than larger droplets, and negatively or positively charged

emulsified particles are removed quickly in comparison to neutral emulsion droplets. Emulsion droplets containing large molecular weight emulsifiers, surfactants or phospholipids containing polyethylene glycol are also found to clear slowly from the bloodstream. The liver absorbs 90% of the recognized emulsion particles within five minutes after injection, with minor fractions found in the spleen, lung and bone marrow. Diversion from the liver allows passive targeting to the lung, kidneys and area of inflammation. Active targeting can be achieved by the used of conjugated antibodies or vectors to the polyoxyethylene side chains of the emulsifiers (Stevens et al. 2003 : 2-3).

1.4 Lipid emulsion compositions

In order to meet the requirements for parenteral emulsion, carefully selection of excipients must be considered. Moreover, the composition in lipid emulsion are affecting the physicochemical properties, stability, bioavailability, and metabolism of formulation. These constituents including oil, emulsifier, co-emulsifier, and drug are described in the detail as below :

1.4.1 Oil

In the early development phase of a project, the solubility of the drug substance usually drives the oil selection. If the formulation is intended to scale-up, purity and cost must also be considered (Floyd 1999 : 136). The oil used for pharmaceutical emulsions are generally of natural origin. Davis et al. (1985) and Boyett and Davis (1989) reported that oil phases of emulsion were based mainly on long chain triglyceride (LCT) from vegetable source such as soybean, safflower, and cottonseed oils (Table 1) (Klang and Benita, in Benita, ed. 1998 : 121). The quality of an oil processed from a potentially variable source must be closely controlled in order to minimize oxidation and remove “unsaponifiable” materials (such as waxes and steroidal components). The oils need to be purified and winterized to allow removal of precipitated wax materials after prolonged storage at 4°C. Known contaminants (hydrogenated oils and saturated fatty materials) should be minimized. Checking for possible presence of aflatoxins, herbicides, pesticides, which may be inadvertent contaminations is necessity. In general, high-quality food grade oils are likely sources for the preparation of injectable emulsions or emulsions for oral administration.

Table 1 Commercially available lipid emulsions.

Trade Name	Oil Phase (%)	Emulsifier (%)	Other Components (%)
Intralipid (kabi-Pharmacia)	Soybean 10 and 20	Egg lecithin 1.2	Glycerol 2.5
Lipofundin S (Braun)	Soybean 10 and 20	Soybean lecithin 0.75 or 1.2	Xylitol 5.0
Lipofundin (Braun)	Cottonseed 10	Soybean lecithin 0.75	Sorbitol 5.0
Lipofundin N (Braun)	Soybean and MCT (1:1) 10 and 20	Egg lecithin 0.75 and 1.2	Glycerol 2.5
Liposyn (Abbott)	Safflower 10 and 20	Egg lecithin 1.2	Glycerol 2.5
Abbolipid (Abbott)	Safflower and Soybean (1:1) 10 and 20	Egg lecithin 1.2	Glycerol 2.5
Lipovenos (Fresenius)	Soybean 10 and 20	Egg lecithin 1.2	Glycerol 2.5
Travemulsion (Travenol)	Soybean 10 and 20	Egg lecithin 1.2	Glycerol 2.5

Source: S. Klang and S. Benita, “Design and Evaluation of submicron emulsions as colloidal drug carriers for intraveneous administration,” In Submicron Emulsions in Drug Targeting and Delivery (Singapore : Harwood academic publishers, 1998), 120.

The use of medium chain triglycerides (MCT) in fat emulsion formulations grew extensively during the 1970s. These MCTs are obtained from hydrolysis of coconut oil and fractionation into free fatty acids (mainly caprylic and capric acids) that contained between 6 and 12 carbon atoms. The MCTs are re-esterification with glycerol. MCTs are reported to be 100 times more soluble in water than LCT and to have an enhanced solubilizing capability. Levy et al (1990) showed that MCT was also used in medicated emulsions owing to its increased ability to dissolve large concentrations of liposoluble drugs (Klang and Benita, in Benita, ed. 1998 : 121).

MCT has mostly been used in fat emulsions (Table 1) in combination with LCT. MCTs are oxidized more quickly and more completely, preventing lipolysis, and thus lower the plasma level of free fatty acids and cholestin systhesis.

The amount of soybean oil (and thus the amount of unsaturated fatty acids) was reduced, as an unfavorable influencing of the liver and lung function, and immune reactions were observed due to the high linoleic acid content (Lucks, Müller, and Klütsch, in Nielloud and Marti-Mestres, eds. 2000 : 237).

The use of structured lipids in lipid emulsions is also possible. In contrast to the physical mixtures of MCT and LCT, these lipids contain a mixture of middle chain and long chain fatty acids within a triglyceride. Distinctions are made between chemically defined and randomized structured lipids. In clinical examinations with structured lipids of this type similar advantages over the pure LCT emulsions could be shown, as could also be observed with the physical mixtures with the reduced LCT amount (Lucks et al., in Nielloud and Marti-Mestres 2000 : 238).

The other oil phases e.g. triacetin, squalane and castor oil were investigated to solubilize Taxol and lipophilic anticancer drugs. These emulsions were monodispersed with a small average particle diameter. Furthermore, they are expected to dissolved rapidly in the body, thus, preventing phagocytosis and accumulation in the RES (Klang and Benita, in Benita, ed. 1998 : 144).

Care must be taken to minimize or eliminate oxidation during the processing and storage of oils. Antioxidants, such as α-tocopherol can be

incorporated to prevent oxidation during processing of the oil and subsequent emulsion. Plastic containers should be avoided, as they are permeable to oxygen. The optimal bulk container should be well-filled, light-resistant, and its integrity tested to ensure maintenance of the protective nitrogen environment. Storage temperatures should not be exceeding the controlled room temperature (Floyd 1999 : 137).

1.4.2 Emulsifier

Since emulsions are thermodynamically unstable systems, a mixture of surfactants should be added for improving stability (Klang and Benita, in Benita, ed. 1998 : 121). The main functions of the surfactants are to form a thin film at the interface, lower the surface tension, hence, preventing flocculation and coalescence of the dispersed oil phase.

Consideration of parenteral toxicity mainly as a result of hemolytic reaction has eliminated many emulsifying agents that might be used in parenteral emulsion. Also, stringent requirements must be met regarding stability. For example, synthetic lecithins have been tried but are readily hydrolysed so the resulting emulsion is not stable (Boyett and Davis, in Liberman, Rigger, and Banker, eds. 1988 : 381). Emulsifiers such as natural phospholipids, block copolymers of polyoxyethylene polyoxypropylene (poloxamer), polyoxyethylene castor oil derivatives (Cremophors®) and polyoxyethylene sorbitans (polysorbate, Tweens®) are now approved by the various pharmacopoeias for parenteral emulsion formulations (Klang and Benita, in Benita, ed. 1998 : 121). However, the most commonly used emulsifier is natural lecithin, which is a mixture of the triglycerides of stearic, palmitic and oleic acids, linked to the choline ester of phosphoric acid named as phosphatides or fat-like compounds that is found in all living organisms and is either of animal (egg yolk) or vegetable (soybean) origin. Other descriptive names include purified egg lecithins, egg yolk phospholipids, phosphatidylcholine and soybean lecithin. Some phosphatide emulsions are very stable toward hydrolysis and oxidation. Another reason that natural lecithin has been widely used is because this type of emulsifier is metabolized in the same way as fat and are not excreted via the kidneys as are many synthetic agents (Boyett and Davis, in Liberman, Rigger, and Banker, eds. 1988 : 381-382).

The toxicological effects of natural phospholipids were studied. Soybean phospholipids were the principle cause of granulomatous lesions in rats, and high concentration of soybean lecithin greater than 1% was increased blood pressure. These lecithin-associated adverse reactions were attributed to impurities in the lecithin (Boyett and Davis, in Liberman, Rigger, and Banker, eds. 1988 : 382). Although it is rare, type I allergic reactions to soybean lecithins emulsified-lipid solutions have been observed. Even after purification, lecithin contains a distribution of related substances (Floyd 1999 : 137).

Due to their natural origin, lecithins are multicomponent mixtures, which may also contain compounds unsuitable for intravenous injection. They must therefore be purified (e.g. chromatographically) before use in injectable emulsions. Until egg lecithin had been chromatographically purified, emulsions made with this emulsifier could not be used clinically because they were too toxic. This was attributed to the hydrolysis of lecithin to lyso-lecithin i.e. lysophosphatidylcholine, lysophosphatidyl-ethanolamine. Levels of these lyso-derivatives must be controlled to reduce their hemolytic potential.

Although purified lecithins reduce the incidence of side effects, some purified lecithins are insufficient as emulsifiers. Auxiliary emulsifying agents have been added to increase stability of an emulsion by forming complexed interfacial films. It is important to note that the physical properties of lecithin and the resulting emulsion stability can vary greatly, depending on the source and degree of purification of the emulsifier. Deliberate use of auxiliary emulsifying agents can be employed. With combinations of surfactants, in which one is a natural lecithin and the other a synthetic surfactant, formation of lecithin hydrolysis products is minimized and the shelf life of the product is extended (Floyd 1999 : 137).

One group of nonionic materials that have been investigated as potential auxiliary emulsifing agents and free from toxic effects; is the pluronics (poloxamers or polyoxyethylenepolyoxypropylene derivatives) (Boyett and Davis 1988 : 382). Other emulsifiers, such as the polyoxyethylene castor oil derivatives (Cremophors®) and polyoxyethylene sorbitans (polysorbate, Tweens®), are already approved by various pharmacopoeias for parenteral administration and can therefore

be considered for emulsion formulation design. However, it should be kept in mind that heat exposure following stream sterilization can alter the emulsifying ability by reducing aqueous solubility resulting in final phase separation (Klang and Benita, in Benita, ed. 1998 : 121).

In order to achieve better stability of lipid emulsions, poloxamer-188 and polysorbate-80 were used in this study as co-surfactant and the properties of these surfactants is reviewed as follows:

1.4.3 Co-emulsifier

1.4.3.1 Poloxamer-188

Poloxamers, primarily used in pharmaceutical formulations as emulsifying or solubilizing agent, are nonionic polyoxyethylene-polyoxypropylene copolymers consist of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a triblock structure: EO_x-PO_y-EO_x The polyoxyethylene segment is hydrophilic whilst the polyoxypropylene segment is hydrophobic, resulting in an amphiphilic copolymer. All poloxamers are chemically similar in composition, differing only in the relative amounts of propylene and ethylene oxides added during manufacture, in which the number of hydrophilic EO (x) and hydrophobic PO (y) units can be altered to vary the size, hydrophilicity and lipophilicity. The copolymers with various x and y values are characterized by distinct hydrophilic-lipophilic balance (HLB).

Poloxamers do not consist of single molecules but rather in a more or less stochastic distribution of molecules over a broad molecular weight range. Their physical and surface-active properties vary over a wide range and a number of different types are commercially available. The structure formula of poloxamer is presented below:

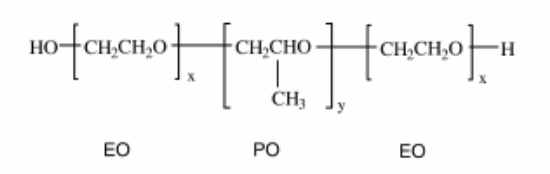


Figure 3 Poloxamers structure consisting of two terminal polyoxyethylene (PO)blocks flanking a central polyoxyethylene (EO) block.

Source: A. V. Kabanov et al., "Pluronic® block copolymers: novel functional molecules for gene therapy," Advanced Drug Delivery Reviews 54 (2002) : 224.

The nomenclature for poloxamers includes two- or three-digit numeric code. The numeric code defines the structural parameter of block copolymer. The first two digits refer to the molecular weight of the PO block, while the last digit indicates the percentage of EO in the polymer when multiply by 100 and 10, respectively. For example, Poloxamer-188, using in this study, the central PO block has an average molecular weight of ca 1800, while the EO chains represent about 80% of total molecule and have molecular weight of approximately 3600 each (Krafft, in Benita, ed. 1998 : 263).

When poloxamers is used to emulsify oil in water, the latter segment, which is essentially hydrophobic, will adhere onto the surface of the oil droplets, while the two hydrophilic EO chains extend as a brush into the aqueous phase. The EO chains are substantially hydrated due to hydrogen bonding between water molecules and the ether oxygen of the EO chains (Krafft, in Benita, ed. 1998 : 262-263).

All addition of poloxamer into aqueous phase provides a good physical stability lipid emulsions. There were partial penetration of poloxamer molecules into the phospholipids monolayers (Benita and Levy 1993 : 1069). These complexes interfacial film among these substances improve the overall stability of an emulsion. Moreover, The branched hydrophilic segments of poloxamer may provide the steric hindrance activity by covering a larger area on the surface of the emulsion and preventing aggregation of emulsions. Poloxamer-188, due to high cloud point

resulting in more resistance against dehydration during autoclaving and subsequently no emulsifier damage (Floyd 1999 : 137-138).

1.4.3.2 Polysorbate-80

Polysorbates are the series of fatty acid esters of Sorbitol and its anhydrides copolymerized with approximately 20 moles of ethylene oxide for each mole of Sorbitol and its anhydrides. Polysorbates are hydrophilic nonionic surfactants used widely as emulsifying agents in the preparation of stable O/W pharmaceutical emulsions. They may also be used as solubilizing agents for a variety of substances including essential oils and oil soluble vitamins, and as wetting agents in the formulation of oral and parenteral suspensions. Polysorbates are also widely used in cosmetics and food products (Wade and Weller 1994 : 376).

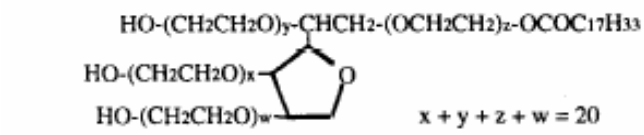


Figure 4 Structure of polysorbate-80 (polyoxyethylenesorbitan monooleate)

Source : T. Hara et al., "Emulsion formulations as a vector for gene delivery *in vitro* and *in vivo*," Advanced Drug Delivery Reviews 24 (1997) : 267.

Polysorbate-80 contains three short polyoxyethylene chains per molecules. As described before, an addition of Polysorbate-80 into aqueous phase provide a good physical stability lipid emulsions from partial penetration of polysorbate molecules into the phospholipids monolayers (Benita and Levy 1993 : 1069). These complex interfacial film among these substances improve the overall stability of an emulsion. The branched hydrophilic segments of polysorbate-80 may provide the steric hindrance activity by covering a larger area on the surface of the emulsion and preventing aggregation of emulsions (Floyd 1999 : 138).

1.4.4 Additives

Since the emulsion of oil in water emulsion has low osmotic pressure, it requires the addition of other materials to adjust the physiological tonicity to allow peripheral administration. Reducing sugars such as dextrose and glucose

have been reported to interact with phosphatides to produce a brown discoloration on sterilization or on storage. Sorbitol used as an additional source of readily available calories, is claimed to affect the pH and produce phase separation on autoclaving (Hansrani, Davis, and Groves 1983 : 147). Ionic agent such as sodium chloride are unsatisfactory, due to strong interaction with the lecithin emulsifying agent. The ion led to a reduction in the zeta-potential and at the same time to a disturbance in the lamella structure of the phospholipids causing severe stability difficulties. For this reason, the tonicity of emulsions is generally adjusted with glycerol, hence, glycerol is usually recommended as an isotonic agent and can be found in almost every parenteral emulsion (Benita and Levy 1993 : 1070). In addition to its contribution to tonicity, glycerol, in combination with propyleneglycol has shown to reduce the globule size and improve the creaming stability of oil in water emulsions (Floyd 1999 : 138).

Small quantities of electrolyte are added to adjust the pH of the system to around pH 8.0 prior to sterilization. The pH falls on autoclaving and as a function of time owing to glyceride and phosphatide hydrolysis, resulting in the production of small quantities of free fatty acids (FFA). The toxicity of emulsions could be correlated to FFA levels in the products. The rate of FFA production was minimal if the pH of the emulsion was between 6 and 7, after sterilization (Hansrani et al. 1983 : 147).

Furthermore, emulsion stabilizers are often needed to prevent peroxidation of unsaturated fatty acids in the oil as well as oxidation of the drug substance and to protect emulsions from phase separation. The first step is easy to accomplish by the addition of various antioxidants such as α-tocopherol, deferoxamine mesylate and ascorbic acid, α-tocopherol is the most commonly selected probably because of its successful incorporation into two commercial lipid emulsions (Lipofundin® and Trivé 1000®).

The second one is much more difficult to accomplish because it is related to interfacial degradation. The properties of the emulgator interfacial film are altered by the incorporation of liposoluble drugs. Thereafter, a stabilizer agent capable for localizing in the interfacial film should be added. Such

molecules are generally amphipathic and are poor surface-active agents but can stabilize the film by enhancing molecular interactions and increasing electrostatic surface charge droplets. A well-known stabilizer is oleic acid or its sodium salt, cholic acid, deoxycholic acid and their respective salts, cationic lipid such as stearylamine, oleylamine, 3β-[N-(N′, N′-dimethylaminoethane)- carbamoyl] cholesterol (DC-Chol) were also shown to markedly improve during-incorporated emulsion stability. (Klang and Benita, in Benita, ed. 1998 : 121).

Antimicrobial agent should be added in small-volume because the aqueous, phase is most vulnerable to in advertent contamination. These agents can be dissolved in the aqueous prior to emulsification. Preservatives include the methyl and butyl derivatives of p-hydroxybenzoic acid e.g. methyl and butyl derivatives, and quaternary ammonium compounds of their high aqueous solubility is suggested. (Floyd 1999 : 138).

1.4.5 Drug

Lipid emulsions intended for parenteral administration are designed for the incorporation of lipophilic and hydrophobic drugs, which exhibits poor aqueous solubility. Inclusion of hydrophobic drugs in the innermost oil phase presents special problems related to the solubilization of the drugs. However these problems generally can be overcome by techniques such as the elevation of the temperatures and the use of additives to increase the oil solubility of hydrophobic drugs. The addition of other drugs to emulsions for i.v. application was also resulted in reduced stability or cracking (Klang and Benita, in Benita, ed. 1998 : 126).

The typical compositions of lipid emulsions used in parenteral nutrition are shown in Table 2:

Table 2 Typical compositions of lipid emulsions used in parenteral nutrition.

Compound	concentration (% w/w)
Oil (e.g. soya, safflower, MCT)	10 – 30 %
Phospholipids (egg or soya lecithin)	0.6 – 1.5 %
Isotonicity agent (glycerol, xylitol)	2.2 – 5.0 %
NaOH to adjust pH	q.s.
Water for injection q.s. to	100 %

Source: B. Siekmann and K. Westesen, "Submicron lipid suspensions (solid lipid nanoparticles) versus lipid nanoEmulsions: similarities and differences," In Submicron Emulsions in Drug Targeting and Delivery (Singapore: Harwood academic publishers, 1998), 206.

1.5 Incorporation method of drugs in lipid emulsions

There are two different approaches to incorporate lipophilic drugs into the lipid emulsions.

1.5.1 De novo emulsification

In principle, the drug molecules should be incorporated by this method, particularly poorly water soluble drugs. Thus, the drug is initially solubilized or dispersed together with an emulsifier in suitable single oil or oil mixtures by means of slight heating in order to overcome solubility limitation. The water phase containing the osmotic agent with or without an additional emulsifier is also heated and mixed with the oil phase by means of high-speed mixers. Further homogenization takes place to obtain the needed small droplet size range of the emulsion. A terminal sterilization by filtration or steam then follows. The formed lipid emulsions contain most of the drug molecules within innermost oil phase. This is a generally accepted and standard method to prepare lipophilic drug-loaded lipid emulsions. This process is normally carried out under aseptic conditions and nitrogen atmosphere to prevent both contamination and potential oxidation of sensitive excipients. Terminal heat sterilization must also be performed and light protection is optional.

1.5.2 Extemporaneous drug addition

A concentrated sterile solution of the drug is added in a solvent such as dimethylacetamide, dimethylsulphoxide or ethanol to lipid emulsion base or a commercial intravenous lipid emulsion such as Intralipid® or Liposyn® by aseptic technique addition. This method is not recommended because the solvent used can disrupt the emulsion integrity, and any particulate matter generated by the drug precipitating out of solution may go unnoticed in an opaque white matrix. Although this approach has been used successfully in preliminary animal and clinical studies with cytotoxic agents, it is not a suitable procedure for routine use because of emulsion stability problems. However, if necessary case of use, the addition procedure must be performed with great care due to the prevention of precipitation of the drug in aqueous phase of the emulsion and also prevent cracking of the emulsion.

1.6 Steps of preparation of lipid emulsions

Lipid emulsion proposing for intravenous used should be prepared with a sufficiently small droplet size and narrow droplet size distribution. If the droplets are larger than a certain size, 1 μm, generally ranges between 100-500 nm, they can get stuck in narrow blood vessel, e.g., in lung, pulmonary thereby causing emboli. A typical production flow diagram is presented in Figure 5.

1.6.1 Mixing

The best method of preparing a fine (sub-micrometer) oil-in-water emulsion is firstly prepared a water-in-oil system and then invert it by adding more water. All manufacturers of the commercial system favor this technique. However, the inversion method may not be used on the large scale. One problem is that phosphatides are waxy and hydroscopic which makes them are water dispersable rather than soluble and require to be adequately dispersed in water before addition of the oil phase. The phospholipids, therefore, are mixed in warm water by using a high speed mixer fitted with a high shear impeller or dissolve in anhydrous ethanol. Nitrogen atmosphere is beneficial for prevent oxidation in case excipients and drug

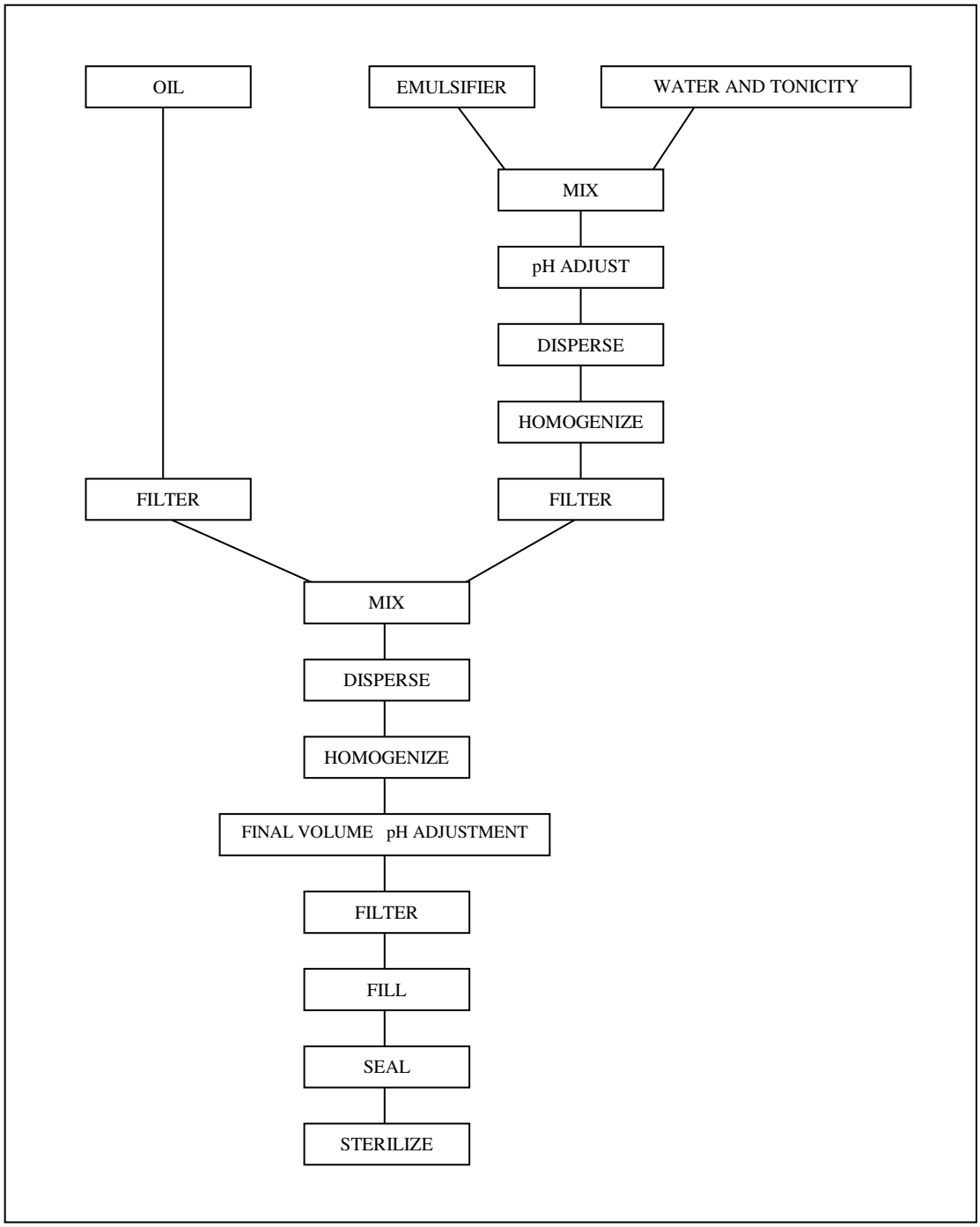


Figure 5 Production flow diagram for hypothetical intravenous emulsion.

Source: J. B. Boyett and C. W. Davis, "Injectable Emulsions and Suspensions," In Pharmaceutical Dosage Forms: Disperse Systems, Volume 2 (New York : Marcel Dekker, 1988), 386.

sensitive to oxidation are used, also the alcohol would be flushed off under this nitrogen atmosphere. The oil and aqueous phases are combined at approximately 70ºC, then, coarse emulsion could be formed by emulsification equipments e.g. magnetic stirrer, simple homogenizer of a stator-rotor type, high shear mixer. Subsequently, these coarse dispersion furthermore homogenized to reduce the large droplets to the required submicron sized level and uniform dispersion.

1.6.2 Homogenization

Once the coarse emulsion is formed, it is necessary to reduce the droplets size even further by homogenization. An essential requirement for the mean droplet size of intravenous emulsions must be smaller than the finest capillaries likely to be encountered in the vascular system; otherwise, an oil embolism can occur. The particle size of the droplets needs to be below 1 μm, generally ranges between 100-500 nm (Benita and Levy 1993 : 1070).

Ultrasonic homogenizers have been successfully utilized to produce injectable emulsions on a laboratory scale. However, no documentation was found to indicate that this process has been scaled up for manufacture of emulsions on a commercial scale (Boyett and Davis, in Lieberman et al., eds. 1988 : 387).

Two principle types of homogenizer are employed. They are low shear and high shear devices. Low shear devices include impellers and are the mixers necessary at the initial stage of an emulsification process. High shear devices are widely used for the emulsification of a variety of materials. The first on of this type is a colloid mills which consist of a high speed rotating disc closely set to a static wall, a crude emulsion is sheared and passed between the gaps. Colloid mills are extremely effective at reducing the average droplet size to approximately 5 μm, which is not small enough for intravenous use as described before. The possible way achieve droplet size is by repeated passing through a two-stage high-pressure homogenizer. In this machine, the crude emulsion is forced under pressure through the annular space between a spring loaded valve and valve seat. The second stage is in tandem which the emulsion is rapidly dispersed. Pressure between 2000 to 4000 lbs/sq.inch (150-280 kg/cm^2) and temperature of 40-80ºC are required and repeated passage through the machine to obtained satisfactory size reduction (Hansrani et al. 1983 : 147). The

whole manufacturing process may be performed under nitrogen protection whenever possible.

Another capable equipment to achieve necessary droplet size is microfluidizers, in which processing is based on a submerged jet principle. Two fluidized streams interact at ultrahigh velocities in precisely defined micro-channels with an interaction within an interaction chamber. The interaction field does not vary with time or location and has no moving parts. Process pressure can be varied from 500 to 20,000 psi and the process stream is accelerated to velocities of up to 1,500 ft/sec. a combination of shear, turbulence, and cavitation forces results in the energy efficient production of consistently fine droplets with a narrow size distribution (Boyett and Davis, in Lieberman et al., eds. 1988 : 387-389).

A major concern regarding the homogenization process is the certain contamination produced from gasket materials, packing and metal parts. These contaminants also originate from pumps and metal surfaces wetted by the emulsion (Boyett and Davis, in Lieberman et al., eds. 1988 : 389).

1.6.3 pH adjustment

Since the physical and chemical stability of emulsions is pH dependent, buffering agents are not typically added because there is the potential for buffer catalysis of the hydrolysis of lipids. Alternatively, the pH is adjusted with a small quantity of sodium hydroxide. The optimum pH of the finished emulsion is in the range of 6-7. The benefits of this pH range are twofold. Initially, this pH range allows the ionization of the phosphate groups at the surface of the lecithin film, leading to an optimum surface charge for the globules. Low pH (values lower than 5) should be avoided as the electrostatic repulsion between emulsified oil globules is decreased, resulting in increased globule size and coalescence. Ultimately, the second benefit of this pH range is minimized lecithin hydrolysis. When terminal heat sterilization is employed, however, the pH should be adjusted to approximately 8.0 prior to sterilization. A slightly alkaline pH is preferred in this case because the pH of the emulsion falls on heating, and also as a function of time during storage, as a result of glyceride and phosphatide hydrolysis, which liberates free fatty acids (Floyd 1999 : 138-139).

1.6.4 Filtration

Filtration of every component of the emulsion prior to further processing would be useful to ensure low levels of foreign particulates and a low bioburden. Hydrophilic membrane is suitable for the aqueous components and hydrophobic filters for the oil components and also used for ethanol solution of phospholipids. However, after homogenization the emulsion is required final filtration to remove trash acquired during the homogenization process. This implied a problem since the filter pore size is limited to remove large particles but cannot remove particles with dimensions close to the oil droplets themselves.

1.6.5 Packing

USP Type I or II glass bottles are generally used to pack injectable emulsions due to plastic containers are generally permeable to oxygen that would limit application to a labile oxidized lipid emulsions. Besides, the impurity in plastic containers containing oil-soluble plasticizers might be extracted by the emulsion, although siliconization of the inner surface of these bottles is a proper choice to provide a hydrophobic surface in contact with the emulsion. Freitas and Muller demonstrated that siliconization of the glass vials almost eliminated particle growth in dispersions of solid lipid nanoparticles.

The stoppers used to package injectable emulsions are also carefully selected. The stopper must not be permeable to oxygen or become softened by contact with oil phase of the emulsion. Teflon-coated stoppers are available and prove to be suitable for the intravenous application. The headspace of the final packing of the finished emulsion must be probable flushed with nitrogen or evacuated prior to sealing to minimize oxidation of the emulsion.

1.6.6 Sterilization

The practical issue of injectable emulsion is sterility. The maintenance of low bioburden during manufacture and sterility assurance in the final packaged product is of extreme importance because the lipid component renders the formulation growth-promoting to in advertent microbiological contamination. Following homogenization, the complete emulsion requires final filtration to remove large particles, but this cannot remove particles with dimensions close to those of the

oil droplets themselves. Consideration should be given to the flow rate through the final filter because the presence of small oil droplets will lower the throughput of the membrane filter. For large-volume (100-1000 ml) injectable fat emulsions, sterilization is achieved by autoclaving. Sterilization conditions must be selected carefully to ensure a sterile product but should minimize degradation of the thermo-labile product. An essential requirement is a low initial or low presterilization bioburden in the emulsion to allow the minimum heat input to the final product. In addition, the product can be agitated during the cycle to facilitate heat transfer and maintain emulsion integrity. Sterilization causes some hydrolysis of lipids and lecithins resulting in the liberation of free fatty acids (FFA), which are known to lower the pH of the emulsion. The recommended autoclaving condition with no effect to emulsion stability was 110ºC for 40 min (Rosoff, in Lieberman et al., eds. 1988 : 247).

Besides, in addition to avoid the terminal sterilization an aseptic process in which is to sterilize individual components and then mix and homogenize by aseptic technique, the final emulsion is aseptically filtered through a sterilizing membrane, 0.22 µm cartridge filter, into the aseptic suite for subsequent aseptic filling.

The final product of emulsions should be kept under nitrogen as described previously and not exposed to direct sunlight oxidative degradation. However, the final products should not be exposed to temperature exceeding 30ºC. The oscillatory movement may cause separation of emulsions, and this has been observed with some phospholipids stabilized emulsions, possibly due to rupture of the stabilizing interfacial film around the oil droplets. So, it is revealed that the transportation should be considered.

1.7 Characterization of physical properties of lipid emulsions

Physical properties of lipid emulsions can be investigated by the observation of droplet size, particle size distribution, surface charge, pH and drug content. All of these properties are important to predict emulsion stability. Lipid emulsions must be sufficiently stable throughout manufacturing, terminal sterilization,

transportation, storage, and also clinical administration. The physical properties of lipid emulsions are described as following:

1.7.1 Size and droplet size distributions

One of the prime physical characteristics of multiphase systems such as lipid emulsions is the size and shape of the particle distribution. The particle size of an emulsion is usually expressed as the diameter of the globules in the internal phase, which strictly required mean diameter ranging from 100-500 nm as an intravenous administration proposing for possible safety liability. Size distributions are influenced by the emulsifier characteristics as well as by the method of manufacture; the well-formed emulsions should display a narrow. The width of the size distribution can be expressed as polydispersity index (PDI), distribution which is zero for monodisperse particles, parenteral fat emulsions are typically in the range of 0.1-0.2 (Jumaa and Muller 2001 : 1117).

The emulsions droplets size affected not only the toxicity but also the system stability. Although increasing the volume of oil phase promoted the amount of the liposoluble drug, which could be incorporated in the emulsion dosage form, high oil concentration often leads to an increase in particle size and viscosity of lipid emulsions. In general, emulsions containing the smallest globules (usually 200-500 nm) also tend to be the most physically stable (Floyd 1999 : 136).

Moreover, the emulsions particle size is directly affected the biofate of fat emulsions. The size of particulate carriers is known to influence both the phagocytic uptake by the mononuclear phagocyte system and the binding of apolipoproteins to emulsions. The particle size is a major determinant of the transfer to extravascular spaces from the blood compartment (Nishikawa, in Benita, ed. 1998 : 105). Lipid emulsion particles as drug carriers is the need to produced small particles, as large colloidal particles administered intravenously are rapidly taken up by the cells of the mononuclear phagocyte system (Trotta, Pattarino, and Ignoni 2002 : 203). On the other hands, there is also an evidence that particle size of emulsion directly affects on the rate in which emulsion is utilized by the body (Boyett and Davis, in Lieberman, eds. 1989 : 390). Emulsions with particle size ranging from 0.5 to 1 μm are utilized more rapidly by the body than that with 3 to 5 μm particle size (Floyd

1999 : 136). Thus, lipid emulsion should be considered in the selection of appropriate particle size.

1.7.1.1 Droplet surface charge

The surface charge of the droplets is assayed by zeta potential measurements typically performed by Doppler electrophoresis apparatus such as Zetasizer (Malvern, England) or determined by the moving boundary electrophoresis technique, in which the electrophoretic mobility is measured and used to calculate the zeta potential. In addition, a new technique for measuring zeta potential, electrokinetic sonic analysis, can be performed using a Matec ESA 8000®. By this technique the zeta potential can be determined for a concentrated (up to 40%) dispersion without the requirement for dilution, which could affect emulsion stability (Floyd 1999 : 136).

The surface potential of lecithin-stabilized emulsions plays an important role in stabilizing drug-containing emulsions, not only by producing electrostatic repulsion, but also by formation of a mechanical barrier. The electrical surface charge of the droplets is produced by the ionization of interfacial film-forming components. The surface potential (zeta potential) of an emulsion droplet will be dependent upon the extent of ionization of the emulsifying agent. The ionization extent of some phospholipids comprised in lecithin is markedly pH-dependent (Klang and Benita, in Benita, ed. 1998 : 125).

Commercial lecithins are a mixture of phospholipids, which vary in composition. They may comprise phosphatidylcholine (PC) as the major component, zwitterionic in form, neutral over a wide pH range, together with negatively charged phospholipids such as phosphatidylethanolamine. For this reason, surface charge could be optimized by selection of lecithins with varying amounts of negatively charged phosphatides such as phosphatidic acid, phosphatidylserine or phosphatidylinositol. In addition, other components such as cholesterol are presented and may affect the interfacial film-charge extent. In lipid emulsion consisting of phosphatidylglycerol (PG), an increase in the droplet charge may occur. Based on this basis, high zeta potential values (above 30 mV) should be achieved in most of the emulsion preparations in order to ensure a high energy barriers, which caused

repulsion of droplets resulting in the formation of stabilized emulsions (Klang and Benita, in Benita, ed. 1998 : 125).

The drug incorporated oil droplets can possibly, affect surface charge. Since the drugs have some surface activity due to polar or ionized groups, they can fully ionized at the formulation pH, which approach the pKa of the drug. Thus, the charge on these drugs will contribute some surface charge, which will influence overall surface charge of the emulsion.

1.7.1.2 pH

It has already been shown that the main degradation pathway of lipid emulsions led to the formation of the fatty acids which gradually reduce the pH of the emulsion (Hansrani et al. 1983 : 147 ; Herman and Groves 1993 : 774). The initial pH of the emulsion might decrease during storage period. However, this pH decrease can be controlled by adjusting the initial pH of the emulsion in order to minimize the hydrolysis rate of the phospholipids and triglycerides. Therefore, the pH of the emulsion should be monitored continuously over the entire shelf life of the emulsion to detect free fatty acid formation.

2. Solid lipid nanoparticles

2.1 Definitions and physical structure of solid lipid nanoparticles

From a number of advantages as described before, lipid emulsions have been used for decades as parenteral nutrition and also as colloidal drug carrier system for delivery poorly water substances. However, disadvantage of this carrier system is prolongation of drug release cannot be achieved regarding to the liquid state of the oil droplets. Another interesting parenteral carrier systems are the liposomes, which have been described for the first time in 1960's by Bangham et al. and were introduced as drug delivery vehicles in the 1970's. This carrier have been developed in order to reduce toxic side effects of the incorporated highly potent drugs and increase the efficacy of the treatment. Major obstacles for the liposomal formulation development were limited by physical stability, drug leakage and difficulties in upscaling.

In the 1990's solid lipid nanoparticles (SLN or liposphere or nanosphere) were developed as an alternative colloidal carrier system for emulsions, and liposomes in controlled drug delivery. SLN are particles prepared by replacing the liquid lipid (oil) of lipid emulsion by a solid lipids, i.e. the lipid particle matrix being solid at room temperature and also body temperature, and stabilized by surfactant(s). The lipid can be highly purified triglycerides, complex glyceride mixtures or even waxes (Wissing et al 2004 : 1259). SLN formulations for various application routes (parenteral, pulmonary, oral, dermal, ocular, pulmonary, rectal) have been developed and thoroughly characterized *in vitro* and *in vivo* (Wissing et al 2004 : 1258).

2.2 Models for incorporation of active compounds into SLN

There are basically three different models for the incorporation of active ingredients into SLN (Figure 6). The structure obtained is a function of the formulation composition (lipid, drug, surfactant) and the production conditions (hot or cold homogenization)

2.2.1 Homogeneous matrix model : A homogeneous matrix with moleculary dispersed drug or drug being presented in amorphous clusters can be mainly obtained when applying the cold homogenization method and when incorporating very lipophilic drugs in SLN with the hot homogenization method. In the cold homogenization method, the bulk lipid contains the dissolved drug in molecularily dispersed form, mechanical breaking by high pressure homogenization leads to nanoparticles having the homogeneous matrix structure (Figure 6a). Similary, this will happen when the oil droplet produced by the hot homogenization method is being cooled, crystallizes and no phase separation between lipid and drug occurs during this cooling process. This model is assumed to be valid for incorporation of e.g. the drug prednisolone, which can slow release from 1 day up to 20 weeks.

2.2.2 Drug-enriched shell model : This model (Figure 6b) could be obtained when phase separation occurs during the cooling process from the liquid oil droplets to the formation of a solid lipid nanoparticles. The lipid can precipitate first forming a practically compound –free lipid core. At the same time, the concentration of active compound in the remaining liquid lipid increases

continuously during the forming process of the lipid core. This model is assumed, for example, coenzyme Q10- the enrichment leads to a very fast release. A fast release can be highly desired when application of SLN to the skin should increase the drug penetration, especially when using the occlusive effect of SLN at the same time.

2.2.3 Drug-enriched core model : A core enriched with active compound can be formed when the opposite occurs, which means the active compound starts precipitating first and the shell will have distinctly less drug (Figure 6c). This leads to a membrane controlled release governed by the Fick law of diffusion (Müller et al. 2002 : S135)

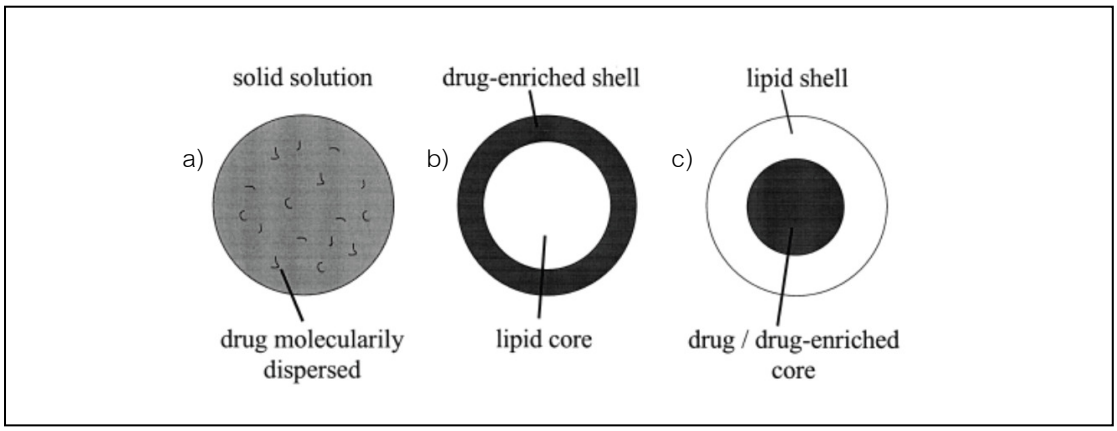


Figure 6 Models of incorporation of active compounds into SLN (a) homogeneous matrix; (b) drug-enriched shell model; (c) drug-enriched core model.

Source: R. H. Müller, K. Mäder and S. Gohla, "Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art," European Journal of Pharmaceutics and Biopharmaceutics 50 (2000) : 167.

2.3 Method of preparation of SLN

Different approaches exist for the production of SLN, and are described briefly as follow (Wissing et al. 2004 : 1260-1262).

2.3.1 High pressure homogenisation (HPH)

HPH is a suitable method for the preparation of SLN can be performed at elevated temperature (hot HPH technique) or at or below room temperature (cold HPH technique) The particle size is decreased by cavitation and turbulences. Briefly, for the hot HPH, the lipid and drug are melted (approximately 5°C above the melting point of the lipid) and combined with an aqueous surfactant solution having the same temperature. A hot pre-emulsion is formed by high speed stirring. The hot pre-emulsion is then processed in a temperature controlled high pressure homogeniser, generally a maximum of three cycles at 500 bar are sufficient. The obtained nanoemulsion recrystallises upon cooling down to room temperature forming SLN. The cold HPH is a suitable technique for processing temperature labile drugs or hydrophilic drugs. Here, lipid and drug are melted together and then rapidly ground under liquid nitrogen forming solid lipid microparticles. A pre-suspension is formed by high speed stirring of the particles in a cold aqueous surfactant solution. This pre-suspension is then homogenized at or below room temperature forming SLN, the homogenising conditions are generally five cycles at 500 bar. The influence of homogeniser type, applied pressure, homogenisation cycles and temperature on particle size distribution has been studied extensively. Both HPH techniques are suitable for processing lipid concentrations of up to 40% and generally yield very narrow particle size distributions (polydispersity index < 0.2)**.**

2.3.2 Microemulsions

The group of Gasco has developed and optimised suitable method for the preparation of SLN via microemulsions which has been adapted and/or modified by different labs. Firstly, a warm microemulsion is prepared by stirring, typically ~10% molten solid lipid, 15% surfactant and up to 10% co-surfactant. This warm microemulsion is then dispersed under stirring in excess cold water (typical ratio ~1:50) using an especially developed thermostated syringe. The excess water is removed either by ultra-filtration or by lyophilisation in order to increase the particle

concentration. Experimental factors such as microemulsion composition, dispersing device, temperature and lyophilisation on size and structure of the obtained SLN have been studied intensively. Also, high concentrations of surfactants and co-surfactants (e.g. butanol) are necessary for formulating purposes, however it is less desirable with respect to regulatory purposes and application.

2.3.3 Preparation by solvent emulsification-evaporation or -diffusion

Different academic groups have attempted to the produce SLN via precipitation technique. In the solvent emulsification-evaporation, the lipid is dissolved in a water-immiscible organic solvent (e.g. toluene, chloroform) which is then emulsified in an aqueous phase before evaporation of the solvent under reduced pressure. Upon evaporation of the solvent, the lipid precipitates forming SLN. An important advantage of this method is the avoidance of heat during the preparation, which makes it suitable for the incorporation of highly thermolabile drugs. Problems might arise due to solvent residues in the final dispersion; in 1995, Sjöström et al. have calculated the amount of toluene residues as 20–100 ppm in final dispersions. Also, these dispersions are generally quite dilute, because of the limited solubility of lipid in the organic material. Typically, lipid concentrations in the final SLN dispersion range around 0.1 g/L, therefore, the particle concentration has to be increased by means of, e.g. ultra-filtration or evaporation. In the solvent-diffusion technique, partially watermiscible solvents (e.g. benzyl alcohol, ethyl formate) are used. Initially, they are mutually saturated with water to ensure initial thermodynamic equilibrium of both liquids. Then, the lipid is dissolved in the water-saturated solvent and subsequently emulsified with solvent-saturated aqueous surfactant solution at elevated temperatures. The SLN precipitate after the addition of excess water (typical ratio: 1:5–1:10) due to diffusion of the organic solvent from the emulsion droplets to the continuous phase. Similar to the production of SLN via microemulsions, the dispersion is fairly dilute and needs to be concentrated by means of ultra-filtration or lyophilisation. Average particle sizes around 100 nm and very narrow particle size distributions can be achieved by both solvent evaporation methods.

2.3.4 Preparation by W/O/W double emulsion method

Recently, a novel method based on solvent emulsification–evaporation for the preparation of SLN loaded with hydrophilic drugs has been introduced. Here, the hydrophilic drug is encapsulated-along with a stabiliser to prevent drug partitioning to the external water phase during solvent evaporation-in the internal water phase of a W/O/W double emulsion. This technique has been used for the preparation of sodium cromoglycate- containing SLN. Their average size was in the micrometer range.

2.3.5 Preparation by high speed stirring and/or ultrasonication

The SLN were developed from lipid microparticles produced by spray congealing followed by lipid nanopellets produced by high speed stirring or sonication. A great advantage of this method is the fact that the equipment is common in every lab and the production can easily be done. The problem of high speed stirring was a broader particle size distribution ranging into the micrometer range. This lead to physical instabilities such as particle growth upon storage. This could be improved by higher surfactant concentrations, which may then result in toxicological problems after parenteral administration. A further disadvantage is potential metal contamination due to ultra sonication. Therefore, studies have been performed by various research groups in order to improve the stability of the obtained SLN dispersions. Generally, high speed stirring and ultra sonication are combined and performed at elevated temperatures for some time. Quite narrow and physically stable distributions can be achieved, however the lipid concentration is low (< 1%) and the surfactant concentration is comparably high.

2.4 Advantages and disadvantages of SLN

The SLN combine advantages of emulsions, composition of physiological compounds, good tolerability, the ease of scaling up. Moreover, with advantages of solid polymeric particles, solid matrix for controlled drug release, protection of incorporated drugs against chemical degradation, slowlier *in vivo* metabolism (Müller and Runge, in Benita, ed. 1998 : 219). SLN have shown the advantages over the liposomes and lipid emulsions in terms of physical stability,

protection of labile drugs from chemical, photochemical or oxidative degradation, and more flexibility in modulating drug release (Joshi and Müller 2009 : 162) .

However, the potential problems associated with SLN are

1. Pay-load for a number of drugs too low**.**
2. Drug expulsion during storage**.**
3. High water content of SLN dispersion**.**

Since the SLN is prepared from solid lipid or blend of solid lipids. after preparation by the hot homogenization technique, the particles crystallize, at least partially, in higher energy modification α and β'. During storage, these modifications can transform to the low energy, more ordered β modification. Due to its high degree of order, the number of imperfections in the crystal lattice is reduced thus leading to drug expulsion (Figure 7)

The creation of a less ordered solid lipid matrix is the per-requisite for a sufficiently high drug-load. In general, the drug can be located in between the lipid layers and also in imperfections (e.g. amorphous drug clusters). In case of spacially very similar lipid molecules, especially when mono acid highly purified glycerides such as tristearin are used, drug load is very limited and drug expulsion occurs, within hours or a few days due to the formation of the perfect β modification.

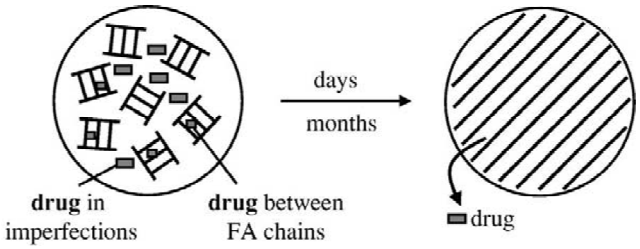


Figure 7 : Mechanism of drug expulsion during storage of SLN dispersions; transition to highly ordered lipid crystal.

Source : R. H. Müller, M. Radtke and S. A. Wissing, "Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations," Advanced Drug Delivery Reviews 54, Suppl.1 (2002) : S144.

According to the SLN patent, the lipid concentration in the dispersion ranges from 0.1 to 30%. The limitation was set to 30% because, according to the patent , bicoherent creams were formed in the homogenization process above 30%. The resulting water content of 99.9 to 70% can potentially create problems when incorporating the SLN dispersion in to a conventional cream. One might not achieve the desired percentage of solid lid particle mass in the cream. In addition, for the preparation of creams and pastes consisting only of lipid particles, water needs to be removed. That means there was also need to improve the production process, i.e. to reduce the water content (Müller et al. 2002 : S144).

Since early 1990's, a number of SLN or SLN-based systems for the delivery of cytotoxic drugs have been successfully formulated and tested. Table 3 provide a list of drugs formulated in these systems, the works are focus on cytotoxic agents and their derivatives. Compared to many other drug delivery systems, the history of SLN is relatively short. As can be seen in Table 3, there is still a lack of clinical studies of the use of SLN for cancer management. Nevertheless, the findings in the preclinical studies using cell culture systems or animal models have so far been very promising (Wong et al. 2007 : 493).

Examples of solid lipid nanoparticles on the market is shown in Table 4. SLN with cationic lipids have also been considered as new transfection agents. For example, SLN prepared with a cationic lipid (DOTAP) had the same transfection efficiency as the liposomes from the same cationic lipid, but with SLN the range of strong non-viral transfection agents that can be produced in large scale is widened. A study of methotrexate-loaded solid lipid nanoparticles (MTx-SLN) for topical treatment of psoriasis, and its formulation and clinical implication was published. The formulation and preparation of MTx-SLN gel were optimized for the cetyl alcohol lipid, Tween 80, as surfactant and sodium tauroglycocholate as co-surfactant.The optimized SLN particle size was 123 nm and an entrapment efficiency of 52% was obtained.The use of MTx-SLN improved the therapeutic response and the MTx-SLN base gel was observed to reduce adverse effects of therapy, promoting better patient compliance.It is therefore possible to consider it as a supplementary to oral therapy, particularly in the final stage of psoriasis treatment.

Table 3 A summary of SLN formulations used for delivery of drugs with anticancer properties and the significant works based on these formulations.

Drug	Research group	Focus of studies
Anticancer drugs		
Camptothecin	Yang 1 Yang 2	SLN prep[a] char[b] SLN charb, PKc/BDd studies in mice
Cholesteryl butyrate	Serpe/Gasco	*in vitro* evaluation in colon cancer cell line
Doxorubicin	Gasco1 Gasco2 Gasco3 Serpe/Gasco Wu1 Wu2 Wu3 Wu4	SLN prep and char PK/BD studies on rats Non-stealth/stealth, PK/BD studies in rats *in vitro* evaluation in colon cancer cell line Dox and Dox/verapamil-SLN prep and char *in vitro* evaluation (cytotoxicity, drug uptake) In drug-resistant breast cancer cell lines *in vitro* evaluation of Dox/GG918-SLN
Etoposide	Murthy	SLN prep and char, BD studies in tumor-bearing Mice (sc, ip, iv)[e]
FudR[f]	Wang	SLN prep and char, PK/BD studies in mice
Idarubicin	Gasco1 Gasco2	SLN prep and char PK/BD studies on rabbit (Duodenal)
Paclitaxel	Gasco Lee Müller Serpe/Gasco Zhang	SLN prep and char SLN prep, *in vitro* and *in vivo* cytotoxicity Evaluation of stealth SLN for delivery of Paclitaxel prodrug SLN prep and char *in vitro* evaluation in colon cancer cell line SLN prep and char (2 types, Brij78 or Pluronic F68 as surfactants), PK studies in mice
Retinoic acid	Kim	SLN prep and char, drug stability studies, *in vitro* cytotoxicity on various human cell lines
SN-38 (irinotecan analog)	Unger	SLN prep, drug stability studies, PK and *in vivo* Cytotoxicity in tumor-bearing mice
Chemosensitizers		
Verapamil	Wu	Dox/verapamil-SLN prep and char
Cyclosporin-A	Gasco	SLN prep and char
GF120918(or GG918)	Wu	SLN prep and char *in vitro* evaluation of Dox/GG918-SLN

The last names of the corresponding authors are used in the "research group" Category.

[a] prep – preparation

[b] char – characterization

[c] PK – pharmacokinetics

[d] BD – biodistribution

[e] sc – subcutaneous, ip – intraperitoneal, iv - intravenous.

[f] FuDR – 3',5'-dioctanoyl-5-fluoro-2'-deoxyuridine, a derivative of 5-fluorouracil (5-FU).

Source : Ho Lun Wong et al., "Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles," Advanced Drug Delivery Reviews 59 (2007) : 494.

Table 4 Example of solid lipid nanoparticles approved by FDA in the market.

Pharmaceutics or therapeutic agent	Market	Indication	Application
Nanobase®	Market	Hepatitis C	Injection
Nanopearl®	Market	Hydration mask	Topical

Source : Priscyla D. Marcato and Nelson Durán, "New aspects of nanopharmaceutical delivery systems," Journal of Nanoscience and nanotechnology 8 (2008) : 5.

2.5 Characterization of physical properties of SLN

An adequate characterization of the resulting dispersion is a prerequisite for the control of the quality of the product. Characterization of SLN is a challenge due to small size of the particles and the complexity of the system. Several parameters have to be considered which have direct impact on the stability and release kinetics (Müller et al. 2000 : 167) :

2.5.1 Particle size and droplet size distributions

The definition of colloidal particles is based on the size characteristic of the structure, i.e. a structure with a size below 1μm as liposomes, nanospheres, nanocapsules. Well-formulated systems should display a narrow particle size distribution in the submicron range. Furthermore, as described above, particles greater than 1 μm and the increase of their number in time can be an indicator of physical instability. Intravenous injection of particles with average diameter above 5 μm might cause death due to embolism. Thus, size control and avoidance of nanoparticles growth are important considerations in preparing dispersions and particular attention is paid to size. Moreover, particle size can modulate the capture mechanism by macrophages and influence their biological stability. It appeared that the quantitative contribution of pinocytosis declined, and that of phagocytosis increased, with increasing particle size. These results may influence the biodistribution behavior of the particles (Heurtault et al. 2003 : 4290).

2.5.2 Zeta potential

The measurement of the zeta potential (ZP) allows predictions about the storage stability of colloidal dispersion. In general, particle

aggregation is less likely to occur for charged particles (high ZP) due to electric repulsion. However, this rule cannot not be strictly applied for systems which contain steric stabilizers. This is due to a presence of steric stabilizer will decrease the ZP according to the shift in the shear plane of the particle (Müller et al. 2000 : 168).

2.5.3 Degree of crystallinity and lipid modification

Since particle size analysis is necessary, but not a sufficient step to characterize SLN quality. Special attention must be paid to characterization of the degree of lipid crystallinity and the modification of the lipid, because these parameters are strongly correlated with drug incorporation and release rates. Thermodynamic stability and lipid packing density increase, and drug incorporation rates decrease in the following order:

supercooled melt < α-modification < β'-modification < β-modification.

Due to the small size of the particles and the presence of emulsifiers, lipid crystallization and modification changes might be highly retarded.

Differential scanning calorimetry (DSC) and X-ray scattering (XRD) are widely used to investigate the status of the lipid. DSC uses the fact that different lipid modifications posses different melting points and melting enthalpies. By means of X-ray scattering it is possible to assess the length of the long and short spacings between lipid lattice. It is highly recommended to measure the SLN dispersion themselves because solvent removal will lead to modification changes. However, the use of conventional X-ray sources has sensitivity problem and take a long time for investigation, the use of synchrotron irradiation might overcome the problem and it permits the detection of intermediate states of colloidal systems which conventional X-ray methods cannot performed. Infrared and Raman spectroscopy are useful tools to investigate structural properties of lipids. However, their potential to characterize SLN dispersions remains to be investigated (Müller et al. 2000 : 168).

3. Nanostructure lipid carriers

3.1 Definitions and Physical structure of nanostructured lipid carriers

At the end of 1990's, modifications of SLN, the so-called nanostructured lipid carriers (NLC) have been introduced. NLC have been proposed as the SLN of a new generation, it is particles with solid lipid matrix with an average diameter in the nanometer range. This carrier system can be used to overcome observed limitations of conventional SLN, to increasing the payload and prevent drug expulsion (Joshi and Müller 2009 : 162). Three different types of NLC were proposed including the presence of oil droplets in a solid lipid matrix (which should combine high drug loading caused by the liquid lipid and controlled release caused by the solid lipid) (Figure 8) :

3.1.1 Imperfect type NLC (Figure 8b) : the spatially different lipids. e.g. glycerides composed of different fatty acids are mixed. Using spatially different lipids leads to larger distances between the fatty acid chains of the glycerides and general imperfections in the crystal and thus to more room for the accommodation of guest molecules. The highest drug load could be achieved by mixing solid lipids with small amounts of liquid lipids (oils).

3.1.2 Multiple type NLC (Figure 8c) : the higher amount of oil are mixed with the solid degradation by the surrounding solid lipids. this type of NLC can be regarded as an analogue of W/O/W emulsions since it is an oil-in-solid lipid-in-water dispersion.

3.1.3 Amorphous type NLC (Figure 8d) : since drug expulsion is caused by ongoing crystallization or transformation of the solid lipid, this can be prevented by the formation of this NLC type. The particles are solid but crystallization upon cooling can be avoided by mixing special lipids (e.g. hydroxyoctacosanylhydroxystearate and isopropylmyristate) (Wissing et al. 2004 : 1259).

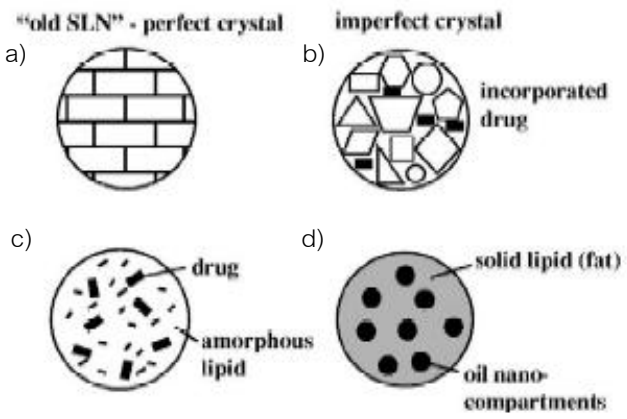


Figure 8 : The three type compared to the relatively ordered matrix of (a) SLN, NLC types: (b) imperfect type, (c) amorphous type, (d) multiple type.

Source : R.H. Müller, M. Radtke and S. A. Wissing, "Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations," Advanced Drug Delivery Reviews 54,1 (2002) : S145.

3.2 Advantages and disadvantages of NLC

For the production of NLC, especially very different lipid molecules are mixed, i.e. blending solid lipids with liquid lipids (oils). The resulting matrix of the lipid particles shows a melting point depression compared to the original solid lipid (Müller et al. 2002 : S144). Hence, an increase in drug loading capacity can avoid/minimize potential expulsion of the active compounds during storage and a lower water content of the particle suspension (Pardeike et al. 2009 : 171). However, some data reported that NLC posses no advantages over nanoemulsions (Mäder and Mehnert, in Nastruzzi, ed. 2005 : 19).

By now many different drugs have been incorporated in NLC but are mainly investigated for dermal application with seldom investigations focused on the parenteral route (Joshi and Müller 2009 : 162). The NLC containing cosmetic products are listed in Table 5.

Table 5 Examples of cosmetic products currently on the market containing lipid nanoparticles.

Product name	Producer/distibuter	Market introduction	Main active ingredients
Cutanova Cream Nano Repair Q10	Dr. Rimpler	10/2005	Q 10, polypeptide, hibiscus extract, ginger extract, ketosugar
Intensive Serum NanoRepair Q10		10/2005	Q 10, polypeptide, mafane extract
Cutanova Cream NanoVital Q10		06/2006	Q 10, TiO_2, polypeptide, ursolic acid, oleanolic acid, sunflower seed extract
SURMER Crème Legère Nano-Protection	Isabelle Lancray	11/2006	Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, milk extract from coconut, wild indigo, noni extract
SURMER Crème Riche Nano-Restructurante			Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, milk extract from coconut, wild indigo, noni extract
SURMER Elixir du Beauté Nano-Vitalisant			Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, milk extract from coconut, wild indigo, noni extract
SURMER Masque Crème Nano-Hydratant			Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, milk extract from coconut, wild indigo, noni extract
NanoLipid Restore CLR	Chemisches Laboratorium	04/2006	Black currant seed oil containing ω-3 and ω-6 unsaturated fatty acids
Nanolipid Q10 CLR	Dr. Kurt Richter, (CLR)	07/2006	Coenzyme Q10 and black currant seed oil
Nanolipid Basic CLR		07/2006	Caprylic/capric triglycerides
NanoLipid Repair CLR		02/2007	Black currant seed oil and manuka oil
IOPE SuperVital	Amore Pacific	09/2006	Coenzyme Q10, ω-3 und ω-6 unsaturated fatty acids
Cream			
Serum			
Eye cream			
Extra moist softener			
Extra moist emulsion			
NLC Deep Effect Eye Serum	Beate Johnen	12/2006	Coenzyme Q10, highly active oligo saccharides
NLC Deep Effect Repair Cream			Q10, TiO_2, highly active oligo saccharides
NLC Deep Effect Reconstruction Cream			Q10, acetyl hexapeptide-3, micronized plant collagen, high active oligosaccharides in polysaccharide matrix
NLC Deep Effect Reconstruction Serum			
Regenerationscreme Intensiv	Scholl	6/2007	Macadamia ternifolia seed oil, avocado oil, urea, black currant seed oil
Swiss Cellular White Illuminating Eye Essence	La prairie	1/2007	Glycoprotiens, panax ginseng root extract, equisetum arvense extract, Camellia sinensis leaf extract, viola tricolor extract
Swiss Cellular White Intensive Ampoules		1/2007	Glycoprotiens, panax ginseng root extract, equisetum arvense extract, Camellia sinensis leaf extract, viola tricolor extract
SURMER Creme Contour Des Yeux Nano-Remodelante	Isabelle Lancray	03/2008	Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, hydrolized wheet protein
Olivenöl Anti Falten Pflegekonzentrat	Dr. Theiss	02/2008	Olea europaea oil, panthenol, acacia senegal, tocopheryl acetate
Olivenöl Augenpflegebalsam			Olea Europaea oil, prunus amygdalus dulcis oil, hydrolized milk protein, tocopheryl acetate, rhodiola rosea root extract, caffeine

Source : J. Pardeike, A. Hommoss and R. H. Müller, "Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical products," International Journal of Pharmaceutics 366, (2009) : 175.

Although, NLC have not been extensively studied as delivery systems for anticancer agents. There are few studies are mentioned :

1. The study by Li et al., encapsulating 9-nitrocamptothecin in NLC showed that stealth 9-nitrocamptothecin had sustained release characteristics and could resist the adsorption of plasma proteins to a certain extent. In the tissue distribution studies 9-nitrocamptothecin was mainly found in the lung, liver, pancreas, ovaries and uterus, and the AUC of 9-nitrocamptothecin-loaded NLC was higher than

that of the solution. Also these NLC were shown to effectively target liver and lung (Joshi et al 2009 : 165).

2. Bondì et al. have described the development of NLC as carrier for two antitumor compounds that possess a remarkable antineoplastic activity. But their limited stability and low solubility in water could give a very low parenteral bioavailability. The results revealed an enhancement of the cytotoxicity effect of drug-loaded NLC on human prostate cancer (PC-3) and human hepatocellular carcinoma (HuH-6, HuH-7) cell lines with respect to that of both free drugs. Results of characterization studies strongly support the potential application of these drugs-loaded NLC as prolonged delivery systems for lipophilic drugs by several administration routes, in particular for i.v. administration (Bondì et al. 2007 : 61).

Body distribution after intravenous injection of colloidal carriers

Drug substances administered parenterally will normally distribute throughout the body as a function of the intrinsic properties of the drug molecule, thereby interacting not only at the target site, but also other sites where they are not required. This may lead to adverse reactions and unwanted side effects as in the case of cancer chemotherapy where agents that show activity against cancer cells also have a toxic effect on normal tissue. In order for a drug selectivity to reach a special target area, where it normally has limited access because of low affinity to the tissue, it is necessary to deliver the drug selectivity to the target site. A possible way of achieving site specific drug delivery is by means of colloidal drug carrier systems. Before considerating the design and evaluation of an efficient carrier system it is essential to understand the nature of the target and interaction of the colloidal carrier particles with the biological environment (Illum and Davis, in Buri and Gumma, eds. 1985 : 65).

When colloidal drug carriers is administered intravenously to the bloodstream, they are taken up rapidly from circulation by the reticuloendothelial system (RES), also called the mononuclear phagocytic system (MPS). The RES functions in the phagocytosis of foreign material and microorganisms in the bloodstream. It comprises, together with the neotrophils, the major antimicrobial

system in the body (Hyl Tander, Sandström, and Lundholm, in Benita, ed. 1998 : 12). The intravenously injected colloids mainly distribute into the RES-related tissues such as liver (60-90% of the injected dose), spleen (2-10%), lungs (3-20 and more percent), and low amount (>1%) into the bone marrow (Kreuter, in Kreuter, ed. 1994 : 262). The drug accumulation in RES-related tissues may be favorable in certain instances, e.g., when the site of action of the drug is in one or several of these tissues. In general case however, the RES uptake poses a problem for i.v. administration of colloidal drug carriers due to the efficiency of RES, the uptake of the colloidal drug carriers is generally rapid, and therefore the bloodstream circulation time of the latter short. This risks resulting in a low bioavailability in tissues other than RES-related ones. Furthermore, through the RES uptake, most of the drug is accumulated in RES-related tissues. Therefore, the local concentration of the drug in these tissues may be quite high, resulting in a dose-limiting local toxicity. Therefore, in the general case, a long circulation time and an even tissue distribution is advantageous from a therapeutic point of view (Malmsten 2002 : 104).

The uptake of injected colloidal carriers by RES is governed by macrophages through a process called phagocytosis. It is initiates by adsoption of certain serum protein, so-called opsonins, at the carrier surface, including albumin, globulins such as immunoglobulins, and glycoproteins, such as fibronectin After the colloidal carriers is attached to the macrophage surface, there are series of processes leading to the engulfment of the drug carrier. Since the process of coating with opsonins serves to render particles more hydrophobic, i.e. more liable to capture by the reticuloendothelial system (RES) of the body. This system, that consists of macrophages fixed at tissue sites and free circulating macrophages is able to effectively remove effectively particles that are recognized as being foreign. The first organ site in which significant numbers of particles can accumulate following intravenous administration is the lungs. Large particles (> 7 μm) are normally trapped mechanically in the smallest pulmonary vessels whereas particles less than 7 μm can pass through the capillary beds of the lungs and reach the liver and the spleen. These organs are well supplied with fixed macrophages (e.g. Kupffer cells in the liver) that can engulf foreign particles by a process of endocytosis. This process consists of an

adhesion of the particles to the cell surface followed by internalization and is controlled by the nature of the surface coating (the opsonin) on the particle. The Kupffer cells of the liver and the macrophages of the spleen represent about 80% of the available phagocytic macrophage cells in the body. Consequently, these organs are the primary sites for deposition of small colloidal particles. In addition to the Kupffer cells, the hepatocytes and the endothelial cells of the liver are also considered to be able to taken up foreign particles (Illum and Davis, in Buri and Gumma, eds. 1985 : 65).

It has been found that the uptake of colloidal drug carriers by RES depend on several factors, e.g. the size, surface properties of carrier, and the total amount of serum proteins adsorbed at the drug carrier surface. The opsonisation is highly dependent on the surface characteristics of the particles (e.g. surface charge, relative hydrophilicity/hydrophobicity, and chemical functionality). There is an inverse correlation between the serum protein adsorption and the bloodstream circulation time. Thus, the higher the total serum protein adsorption, the faster the clearance from bloodstream circulation. Thus hydrophobic particles will be removed from the circulation rapidly while more hydrophilic particles will remain in the circulation for longer periods of time (Malmsten 2002 : 104).

It follows that if the drug carrier can be made to adsorb little or no serum protein, its circulation time in the blood stream is prolonged. Different approaches are being tested to change the distribution pattern of colloidal carriers in the body to yield high concentration of the drug in the plasma and tissues other than RES-rich organs. This can be achieved in many ways. For example, phospholipids are usually used for preparation of liposomes for i.v. administration. Through choice of the structure of the polar headgroup of the phospholipid, a desire protein adsorption may be achieved. In particular, phospholipids which carry a zero net surface charge, (e.g. phosphatidyl choline (PC), sphingomyelin (SM)), or where the charge is shielded by an outer group (e.g.. ganglioside GM1 or phosphatidylinositol (PI)), the serum protein adsorption is low and the circulation time is long. For phospholipids carrying a bare charge (e.g., phosphatidic acid (PA) or diphosphatidylglycerol (DPG)), on the other hand, the serum protein adsorption is substantial, and the bloodstream circulation time short.

Therefore, through choice of the phospholipid or phospholid mixture composition, the clearance rate may be tune (Malmsten 2002 : 107). A particularly efficient way of reducing serum protein adsorption at colloidal drug carriers is to use hydrophilic polymer. Polyethylene glycol (PEG) or poly(ethylene oxide) is a water-soluble material widely employed in pharmaceutical applications, as its terminal hydroxyl groups can be easily converted into reactive functional groups by a number of routine reactions of organic chemistry. The technique of attaching PEG to any drug, peptide, polymer or other compounds has been denominated as "PEGylation" and its biological applications have been well documented. Pegylation improved ability to deliver drugs to the intended tissues. For example, pegylated liposomal doxorubucin has shown efficacy in breast cancer treatment. The next generation of liposomes for delivery systems will include molecular targeting, as in the case of immunoliposomes that represent an integration of biological components capable of tumor recognition with delivery technologies. Furthermore, PEG is non-toxic and resistant to recognition by the immune system, and may be used to enhance biological activity of conjugate drugs. PEG can also be used in block copolymers. With the hydrophobic polylactide (PLA), the resulting copolymer led to microcapsules that were more soluble in water than PLA (Marcato and Durán 2008 : 6).

In general, the electrostatic, hydrophobic, and van der Walls interactions dominate and drive the adsorption. Since PEG-based surface coatings are uncharged, hydrophilic, and also contain a lot of water (the latter reducing van der Walls attractive interactions between the surface and the protein, this reduces the adsorption driving force. Furthermore, the PEG chains give rise to a repulsive steric interaction which opposes protein adsorption to a PEO-modified surface. Together, these two contributions result in a very low serum protein adsorption. However, for the attractive interactions between the serum proteins and the underlying carrier to be fully screened the PEG layer needs to be sufficiently thick, and hence a minimum PEG molecular weight of the order of 1000-2000 is generally required. Furthermore, if the PEG chain density is not sufficiently high, small serum proteins may "slip through" the PEG layer, and also the repulsive osmotic interaction may be insufficient to withstand a strongly attractive protein-carrier interaction. Thus, the

PEG-based coating of the drug carrier which sufficient thick and dense, the adsorption of essentially all serum proteins is reduced dramatically. This resulting in a drastically prolonged bloodstream circulation time, a decreased accumulation in RES-related tissues, and an increased accumulation of the drug also in tissues and cells not related to the RES (Malmsten 2002 : 107).

Passive targeting by colloidal nanocarriers

Nanoparticles encounter numerous barriers en route to their target, such as mucosal barriers and non-specific uptake. Since chemotherapeutic drugs often kill healthy cells and cause toxicity to the patient. Owing to better understanding of tumor biology, it would therefore be desirable to develop chemotherapeutics that can passively target cancerous cells (Peer et al. 2007 : 751).

Since, Fast-growing of cancer cells demand the recruitment of new vessels (neovascularization) or rerouting of existing vessels near the tumor mass to supply them with oxygen and nutrients. The resulting imbalance of angiogenic regulators such as growth factors and matrix metaloproteinases makes tumor vessels highly disorganized and dilated with numerous pores showing enlarged gap junctions between endothelial cells and compromised lymphatic drainage (Cho et al.2008 : 1313). Furthermore, the dysfunctional lymphatic drainage in tumors retains the accumulated nanoparticles and allows them to release drugs into the vicinity of the tumor cells (Peer et al. 2007 : 751). These unique pathophysiologic characteristics of tumor vessels (leaky blood vessels and poor lymphatic drainage) enable macromolecules, including nanoparticles, can extravasate (escape) to selectively accumulate in tumor tissues. These features are called the Enhanced permeability and retention (EPR) effect (Figure 9) (Cho et al.2008 : 1313). Experiments using liposomes of different mean size suggest that the threshold vesicle size for extravasation into tumors is about 400 nm, but other studies have shown that particles with diameters < 200 nm are more effective (Peer et al. 2007 : 751). Maeda (2001) reported nanoparticles with a molecular weight above 50 kDa, can selectively accumulate in the tumor interstitium (Cho et al.2008 : 1313).

Hence, nanoparticles that satisfy the size and surface characteristics requirements described above for escaping RES capture have ability to circulate for longer times in the bloodstream and greater chance of reaching the targeted tumor tissues.

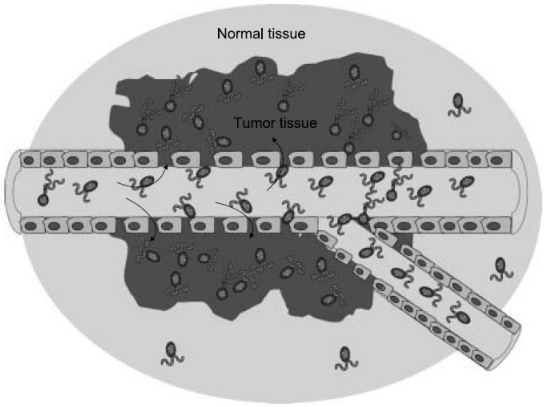


Figure 9 Tumor targeting of nanoparticles passively by enhanced permeability and retention. Long-circulating therapeutic nanoparticles accumulate passively in solid tumor tissue by the enhanced permeability and retention effect. Angiogenic tumor vessels are disorganized and leaky. Hyperpermeable angiogenic tumor vasculature allows preferential extravasation of circulating nanoparticles.

Source: K. Cho et al., Therapeutic nanoparticles for drug delivery in cancer, Advances in Cancer Research 14,5 (2008) : 1314.

Several anticancer agents have been encapsulated in lipid nanoparticles, and their *in vitro* and *in vivo* efficacy has been evaluated by suitable studies. Some studies are lists for example as follow :

1. Rossi et al. were developed oil-in-water emulsions (100–120 nm in diameter) and to correlate the surface properties of the emulsions with blood residence time and accumulation into neoplastic tissues by passive targeting. We investigated the effect of phospholipid and sphingolipid emulsifiers, hydrogenated soybean phosphatidylcholine (HSPC) and egg sphingomyelin (ESM), in combination with polysorbate 80 (PS-80) and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine

(DSPE)-PEG lipids of various PEG chain lengths and structures in prolonging circulation time and enhancing accumulation into B16 melanoma or C26 colon adenocarcinoma. The relationship between amphiphile molecular packing at the air/water interface on emulsion stability upon dilution in albumin and circulation longevity *in vivo* was also explored for non-PEGylated emulsions. PEGylation of the droplet surface with 10–15 mol% of DSPE-PEG 2000 or 5000 enhanced the circulation time of the emulsions, however, accumulation was only observed in the C26 tumor model. The tighter molecular packing observed with ESM/PS-80 monolayers at the air/water interface compared to HSPC/PS-80 correlated with improved emulsion stability *in vitro*, however, enhanced circulation time *in vivo* was not observed. A better understanding of the relationships between composition and performance will result in improved emulsion-based drug delivery vehicles for cancer therapy (Rossi et al. 2007 : 329).

2. Submicron-sized lipid emulsions was coated with polyethylene glycol modified phosphatidylethanolamine (PEG-PE). Physical stability was obtained by using a mixture of phosphatidylcholine and polysorbate 80 as emulsifying agent. Sonicated preparations with the standard composition; triolein (TO): dipalmitoyl phosphatidylcholine (DPPC): polysorbate 80: PEG-PE at mass ratios of 2: 1: 0.4:0.1 exhibited a mean particle size of 44 nm (by quasi-elastic light scattering) and an excellent physical stability. *In vivo* plasma clearance data were obtained by intravenous injection of emulsions into mice. Coating of the emulsion droplets with PEG-PE gave a considerable increase in circulation lifetime. A further notable effect was obtained when the cosurfactant polysorbate 80 was introduced into the system, apparently as a result of decreased particle size. Lipid emulsions with the standard composition showed first order kinetics during 6 h with a circulation half-life of about 3 h. Phospholipid transition temperature and emulsion particle size were found to be important factors while the clearance rate was essentially independent of lipid dose (Lundberg, Mortimer and Redgrave 1996 : 119).

3. Sterically stabilized SLN comprising of trymyristin and egg phosphotidylcholine and pegylated phospholipids as stabilizers and having average particle size around 200 nm were prepared using high pressure homogenization. The

important finding of the study was that in the *in vitro* release studies SLN showed a slow but time-dependent release, and their *in vitro* cytotoxicities against human ovarian and breast cancer cell lines as determined by MTT assay were comparable to those of a commercially available cremophor EL-based paclitaxel formulation (Joshi et al. 2009 : 164).

4. An *in vitro* cellular uptake studies on vinorelbine bitartrate encapsulated in PEG-modified SLN revealed that there was no phagocytosis of these SLN by RAW264.7 cells, but there was a significant improvement in the uptake by cancer cells (MCF-7 and A549) due to this PEG modification. Also the *in vitro* studies anticancer activity of vinorelbine bitartrate was found to be enhanced significantly after its incorporation in SLN and pegylated SLN (Joshi et al. 2009 : 165).

CHAPTER 3
MATERIALS AND METHODS

1. Materials

1.1 1,2-distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000](Ammonium Salt) Mw.2805.54 (DSPE-PEG_{2000}) (Lot No.180PEG2PE-58, Avanti Polar Lipids; USA)

1.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich; Germany)

1.3 Acetronitrile (Labscan Asia; Thailand)

1.4 All-trans retinoic acid (Lot No.048K1516, Sigma-Aldrich.; Switzerland)

1.5 Cetylpalmitate (Sabowax CP ,Sabo S.P.A.; Italy)

1.6 Dimethylsulfoxide (DMSO) (Riedel-de Haën; Germany)

1.7 Disodium hydrogen phosphate (Na_2HPO_4) (Merck; Germany)

1.8 Ethyl alcohol (Labscan Asia; Thailand)

1.9 Glacial acetic acid (Labscan Asia; Thailand)

1.10 Human acute promyelocytic leukemia (HL-60 cell line) (American Type Culture Collection (ATCC); USA)

1.11 Human hepatocellular carcinoma (HepG2 cell line) (American Type Culture Collection (ATCC); USA)

1.12 Isopropyl alcohol (Labscan Asia; Thailand)

1.13 Medium chain triglyceride (Estasan® 3580, Lot No.70923J, Uniqema Asia Pacific; Malaysia)

1.14 Glacial acetic acid (Labscan Asia; Thailand)

1.15 Human acute promyelocytic leukemia (HL-60 cell line) (American Type Culture Collection (ATCC); USA)

1.16 Human hepatocellular carcinoma (HepG2 cell line) (American Type Culture Collection (ATCC); USA)

1.17 Isopropyl alcohol (Labscan Asia; Thailand)

1.18 Medium chain triglyceride (Estasan® 3580, Lot No.70923J, Uniqema Asia Pacific; Malaysia)

1.19 N-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (mPEG)(PLC-g-mPEG) (kindly provided from Assoc. Prof. Suwabun Chirachanchai)

1.20 Oleic acid (Lot & Filling Code 1333645 51107P25, Fluka Chemie AG; Germany)

1.21 Phospholipids (Epikuron® 200, Lot No.149059, Degussa BioActives GmbH; Germany)

1.22 Polysorbate 80 (Tween® 80, Lot No.709705, NOF Corporation; Japan)

1.23 Potassium chloride (Ajax Fine Chem; Australia)

1.24 Potassium dihydrogen phosphate (KH_2PO_4) (Merck; Germany)

1.25 Sodium chloride (Labscan Asia; Thailand)

1.26 Sodium hydroxide (Labscan Asia; Thailand)

1.27 Soybean oil (Lot No.1133367 14904174, Fluka Chemie AG; Germany)

1.28 Tissue culture reagents (Gibco®, Invitrogen; USA)

a. Foetal bovine serum EU Approved origin
b. Glutamax-I
c. Iscove's modified dulbecco's medium (IMDM medium)
d. Minimum essential medium (MEM medium)
e. Non-essential amino acids 100x w/o L-Glutamine
f. Trypsin-EDTA (1x) 0.5 g Trypsin (1:250) and 0.2 g EDTA.4Na/L in HBSS

1.29 Trypan blue stain 0.4% (Gibco®; USA).

2. Equipments

2.1 1.5 ml microtubes

2.2 25 cm^2, 75 cm^2 tissue culture flasks

2.3 25, 50 ml Sterile plastic centrifuge tubes

2.4 Analytical balances (AX-205 DR, PL3002, Mettler Toledo;Switzerland)

2.5 Autoclave (Tattnauer; Germany)

2.6 Bath sonicator (Transsonic® T460/H, Elma Hans Schmidbaür, Germany)

2.7 Biohazard carbinet (Nuaire; USA)

2.8 Centrifuge (Tomy; Japan)

2.9 Column HPLC (Symmetry® C18 5 µm, 3.9x150 mm column, Serial No. 021 4363421 40 84, Waters; USA)

2.10 Dialysis bag (CelluSep® MWCO 6,000-8,000 Membrane Filtration Product; USA)

2.11 Double jackets beakers (SP Glass; Thailand)

2.12 Filter set and sterilization filter 0.22 µm

2.13 Freezer/Refrigerator 4°C, -20°C, -80°C (Thermo Sciencetific; USA)

2.14 Hemocytometer 0.1 mm deep chamber (HBG; Germany)

2.15 High performance liquid chromatography (HPLC) instrument consisted with the following

a. Autosampler (AS-3000, Thermo Separation Products; USA)Liquid chromatography pumps (P-1500, Thermo Separation Products; USA)

b. Degasser (Thermo Separation Products; USA)

c. Liquid chromatography pumps (P-1500, Thermo Separation Products; USA)

d. Software ChromQuest (Thermo Separation Products; USA)

e. UV-VIS detector (UV-1000, Thermo Separation Products; USA)

2.16 Humid CO_2 Incubator (37°C, 95% RH, 5% CO_2) (Sanyo, Japan)

2.17 Inverted microscope (Eclipse TE2000-S, Nikon; Japan and Olympus; USA)

2.18 Micropipette 2-20 µL, 20- 200 µL, 100-1000 µL

2.19 Microplate centrifuge (Haraeus® Multifuge X1, Thermo Sciencetific; USA)

2.20 Microplate reader (SpectraMax® M2, Molecular Devices; USA)

2.21 Multipoint pipette with 8 channel aspiration manifold 20- 200 µL

2.22 Multi-position hotplate stirrer (RT10P, IKA; Germany)

2.23 Multi-well tissue culture plates (96 well plates)

2.24 Peristatic pump on the suction line (Schuett; Germany)

2.25 pH meter (MP220, Mettler Toledo; Switzerland)

2.26 Photon correlation spectroscopy (Zetasizer Nano ZS, Malvern; England)

2.27 Pipette aid, Measuring pipette (2, 5, 10, 25 ml)

2.28 Probe sonicator with stepped microtip 1/8" (VCX500, Sonics; USA)

2.29 Rotating Mixer (Intelli-Mixer RM-2®, Elmi; Latvia)

2.30 Sterilized plastic tips

2.31 Syringe filter (25 mm diameter, 0.45 µm pore size, Chrom Tech; USA)

2.32 Vortex mixer (Vortex Genie-2, Scientific Industries; USA)

2.33 Water Bath thermostat circulator (Tomy, Japan)

3. Methods

3.1 ATRA solubility determination

3.1.1 Solubility of ATRA in water and liquid lipids

The solubility of ATRA in water, liquid lipids such as soybean oil (S), MCT (M), oleic acid (O), and mixture thereof by various ratios were performed at 25°C. An excess quantity of ATRA was added to each solvent and mixed throughly at 25°C for 24 h. Each experiment was performed in triplicate. The samples were filtered with 0.45 μm membrane filters. The concentrations of ATRA were determined by HPLC.

3.1.2 Solubility of ATRA in the mixture of solid lipid and liquid lipids

The solubility of ATRA in the mixture of solid lipid (cetylpalmitate, CP) and liquid lipids (S, M, mixture of S and O, and mixture of M and O) in various ratios were investigated. ATRA in amount of 1, 3, 5, 7,and 9 mg/g was added to the mixtures of oil and CP in the ratio of 1:5, 3:3, and 5:1 then placed in bath-sonicator. The time consuming for complete dissolving of ATRA in each sample was recorded. The temperature was controlled at 70°C throughout the experiment. Each experiment was performed in triplicate.

3.2 Preparation of ATRA loaded lipid nanoparticles

3.2.1 Lipid Emulsion (LE)

ATRA-loaded lipid emulsions (LE) were prepared by De-novo emulsification method. The composition of the oil phase were ATRA, liquid lipid (S, SO, M, MO), lecithin, and butylated hydroxytoluene (BHT), whereas the aqueous phase were composed of polysorbate-80, and distilled water. After separately preheating the oil phase and aqueous phase to 70°C, the aqueous was transferred to the oil phase and stirred with magnetic stirrer at 1,400 rpm for 1 min. To reduce the droplet size to the nanometer range, these mixtures were placed in an ambient temperature water bath and sonicated five times for 5 min with an interval of 10 sec at 40% amplitude by probe type ultrasonicator (Sonics & Materials, CT, USA). The pH of the samples were subsequently adjusted to 8 ± 0.1 with 1 N NaOH, and then filtered through 0.45 μm membrane filter to remove precipitated ATRA. The

formulations were stored in light-protected and sealed containers at 4°C in the refrigerator.

3.2.2 Nanostructured lipid carriers (NLCs)

ATRA-loaded nanostructured lipid carriers (NLCs) were also prepared by De-novo emulsification method and followed the same procedures as LEs in all respects, except liquid lipid (S, M, SO, MO), which was reinstated by mixture of liquid lipid and solid lipid (S:CP, M:CP, SO:CP, MO:CP). The formulations were kept in light-protected and sealed containers at 4°C in the refrigerator.

3.2.3 polymer coated NLC

Coating of colloid surface with a hydrophilic and flexible polymer such as poly(ethylene glycol) (PEG) is widely used to prolong residence time in blood circulation. The presence of hydrophilic surface coating offers steric hindrance to opsonin adsorption on bilayer which further reduces the uptake by the cells of reticuloendothelial systems (RES) results in a considerable extension of the circulation lifetime (Sharma and Sharma 1997 : 129). Previously, Opanasopit and coworkers have been synthesized N-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (PLC-g-mPEG) (PPEG) and successfully incorporated ATRA into the hydrophobic inner core of polymeric micelles which could remarkably improve the stability of ATRA (Opanasopit et al. 2006 : 430).

In this study 1,2-distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethyleneglycol)-2000] (Ammonium Salt) (DSPE-PEG_{2000}) (DPEG) and PLC-g-mPEG with 95% degree of deacetylation (PPEG) were selected for the hydrophilic coating of the lipid droplets. The coating of NLC by selected PEGs was prepared according to the De-novo emulsification method as previously described. DPEG or PPEG was dissolved in aqueous phase composed of polysorbate-80 and distilled water. The mixture was stirred at room temperature until completely dissolved. The composition of the oil phase were ATRA, mixture of liquid lipid and solid lipid (S:CP, M:CP, SO:CP, MO:CP), lecithin, and BHT. Each phase was heated to 70°C, the further process was carried out as indicated in LE preparation in all respects. The formulations were kept in light-protected and sealed containers at 4°C in the refrigerator.

3.3 Lipid nanoparticles characterization

3.3.1 Percentage yield

The concentrations of ATRA incorporated in lipid nanoparticles were directly determined by HPLC after appropriate dilution with isopropanol. All samples were measured in triplicate. The percentage yields of ATRA in the lipid emulsion were calculated using the following equation:

$$\text{Percentage yield} = (CL / CI) \times 100$$

Where CL is the concentration of ATRA measured in the lipid emulsion; CI is the initial concentration of ATRA added in lipid emulsion.

3.3.2 Physicochemical characterization

Nanoparticles droplet size, polydispersity index (PDI), and zeta potential of ATRA-loaded lipid nanoparticles were determined at 25°C by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS. A medium viscosity of 1.054 cP and a refractive index of 1.33 were used for the aqueous phase, with a sample refractive index 1.460 and absorption 0.00 (similarly to the particles named Intralipid® in the Malvern software). Prior to the measurement, 1 µl of lipid nanoparticles was diluted with 1 ml distilled water which was filtered through a 0.22 µm membrane filter. All samples are measured in triplicate.

3.3.3 Photostability study

In order to investigate the effects of lipid nanoparticles on photodegradation protection over the time, samples of the lipid nanoparticles were taken into a 1 ml of tightly closed glass bottle and are irradiated up to 6 h using a UVA lamp, which placed at an 50 cm high from the samples. Eighty microliters of aliquots were taken from each sample at time intervals (0.5, 1, 2, 4, 6 h), followed by dilution with isopropyl alcohol. The amounts of intact ATRA were analyzed by HPLC. For comparison, an isopropyl alcohol solution of ATRA in the same concentration of lipid nanoparticles was investigated by the same procedures as the lipid nanoparticles.

3.3.4 *In vitro* drug release study

The *in vitro* release studies of lipid nanoparticles were operated using dialysis bag (MWCO 6000-8000, CelluSep®, Membrane Filtration Products,

USA). Fifty ml of 10: 15: 75 % (v/v) mixture of ethanol: polysorbate-80: phosphate buffer pH 7.4, were used as the receptor medium. One ml of the lipid nanoparticles was placed in the dialysis bag, and then put the dialysis bag in the double jacket beaker with an external constant temperature circulation water bath under constant stirring at 37±0.5°C. At certain time interval of 1, 2, 4, 8, 12, 24, and 48 h, 3 ml aliquots of the medium were withdrawn and the same volume of fresh medium added. The content of ATRA in the sampling solutions was assayed by HPLC. All experiments were performed in triplicate.

3.3.5 Stability study

Two conditions affecting the physical and chemical stability of lipid nanoparticles were evaluated. The first condition, accelerated condition, was performed by autoclaving the formulations at 121°C for 15 min. The latter, normal condition was done by keeping the formulations in refrigerator at 4°C for 2 months. The particle size, size distribution, zeta potential, pH, and percentage yield were determined before and after stability testing.

3.3.6 Cytotoxicity assay

The anticancer activity of ATRA-loaded lipid nanoparticles were investigated from the inhibition of the proliferation in two different types of cancer cells, Human acute promyelocytic leukemia cells (HL-60 cells) and Human hepatocellular carcinoma (HepG2 cells), by MTT-based cytotoxicity assay.

3.3.6.1 HL-60 cells were routinely cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% foetal bovine serum, 1% non-essential amino acid, 1% glutamax®, in humidified atmosphere (5% CO_2, 95% air, 37°C). The cells were seeded in 96-well plates at $2x10^4$ cells/well densities. After 4 h incubation, ATRA solution, ATRA-loaded lipid nanoparticles and bare-lipid nanoparticles (as a control) with various concentrations were added to the cells. ATRA solution was prepared by dissolving ATRA in DMSO and then diluted with culture medium to obtain various concentrations. The ATRA-loaded lipid nanoparticles were also diluted with culture medium. After 4 days of incubation under 5% CO_2 at 37°C, the medium was removed and 20 µl of MTT (3-(4,5-dimethy-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, 5 mg/ml) in serum free-medium was

added to each well and the cells were incubated for 4 h to allow formation of formazan, then the medium was removed and 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance of formazan was measured at 550 nm using a microplate reader (SpectraMax®M2, Molecular Devices; USA). The relative cell viability was calculated by using the follow equation:

$$\text{Cell viability (\%)} = \left(\frac{OD_{550}\text{ (sample)}}{OD_{550}\text{ (control)}} \right) \times 100$$

Where the OD_{550} (sample) represents the absorbance from the wells treated with samples and the OD_{550} (control) means the absorbance from the wells treated with medium alone.

3.3.6.2 HepG2 cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acid, 1% glutamax®, in humidified atmosphere (5% CO_2, 95% air, 37°C). The cells were seeded in 96-well plates at $5x10^4$ cells/well densities. After 24 h incubation, free ATRA, ATRA-loaded lipid nanoparticles with various concentrations were added to the cells. After 24 h of incubation under 5% CO_2 at 37°C, cell viabilities were determined by MTT assay as described above. Each experiment was performed in triplicate.

3.4 HPLC analysis of ATRA

3.4.1 Preparation of stock solution

ATRA (2.5 mg) was accurately weighed and diluted to volume with isopropyl alcohol (IPA) in a 25 ml-volumetric flask. A series of standard solution was prepared giving the final concentrations ranging as follow: 1.00, 10.00, 25.00, 50.00, 75.00, 100.00 µg/ml.

3.4.2 HPLC condition

The HPLC system consisted of mobile phase delivery pump (P-1500, Thermo Separation Products, USA), UV detector (UV-1000, Thermo Separation Products, USA), and integrator (I-1000, Thermo Separation Products, USA). The C18 reverse phase column (Water, USA), 5 µm, 250 x 0.5 mm was used.

The mobile phase was 84.5:15.0:0.5 (v/v) mixture of acetonitrile:water:glacial acetic acid, and detected at 342 nm. The injection volume was 20 µl and the flow rate was 1.5 ml/min. Under these conditions, the linear calibration curve of ATRA was obtained in the range of 1-100 µg/ml ($R^2 > 0.999$).

3.4.3 Validation characteristics

Validation for ATRA analysis i.e. system suitability, accuracy, precision and linearity was determined. The method for validation analysis was described in the appendix.

3.5 Statistical analysis

All values were express as mean value ± standard deviation (SD). Statistical significance of differences was examined using one-way-analysis of variance (ANOVA) followed by least significant difference post hoc test. The significance level was set at $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

1. ATRA solubility determination

1. Solubility of ATRA in water and liquid lipid
2. Solubility of ATRA in mixture of liquid lipid and solid lipid

2. Preparation of ATRA lipid nanoparticles

1. lipid emulsions
2. NLC
3. polymer coated NLC

3. Characterization of ATRA-loaded lipid nanoparticles

1. Physicochemical characterization of ATRA-loaded lipid nanoparticles
   - Particle size, Polydispersity index (PDI), zeta potential
2. *In vitro* release of ATRA-loaded lipid nanoparticles
3. Photostability study of ATRA-loaded lipid nanoparticles
4. Stability studies of ATRA-loaded lipid nanoparticles
   - Percentage yield, Physicochemical characterization, pH
   4.1. After autoclaved
   4.2. After kept in 4°C for 8 weeks

4. Cytotoxicity assay

1. HL-60 cells
2. HepG2 cells

1. ATRA solubility determination

1.1 Solubility of ATRA in water and liquid lipid

The solubility of ATRA in soybean oil (S), medium chain triglyceride (M), oleic acid (O), mixture of S:O (1:1, 1:3, 3:1), M:O (1:1, 1:3, 3:1), S:M:O (1.5:1.5:1, 1:1:2, 0.5:0.5:3) and water (W) at 25°C is shown in Figure 10.

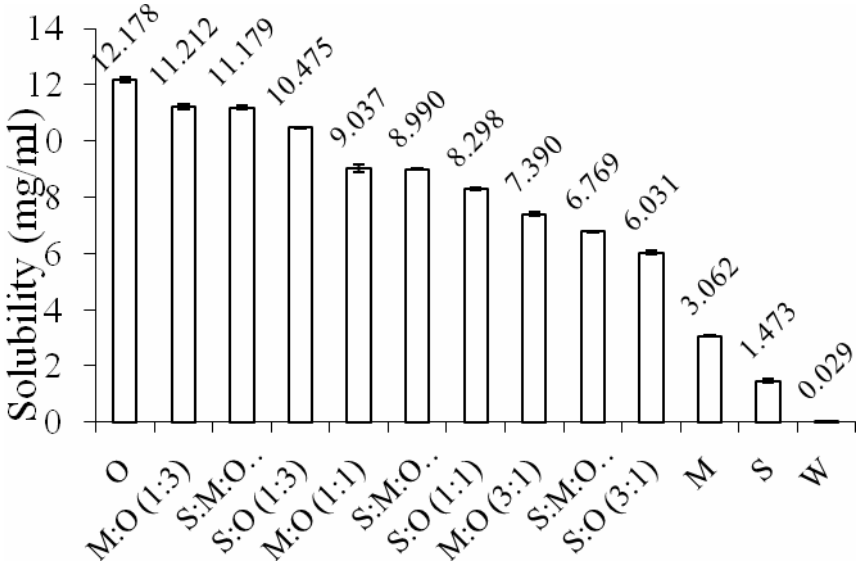


Figure 10 The solubility of ATRA in various solvents at 25ºC

The ATRA-solubility in various solvents was in order of O > M:O(1:3) > S:M:O(0.5:0.5:3) > S:O(1:3) > M:O(1:1) > S:M:O(1:1:2) > S:O(1:1) > M:O(3:1) > S:M:O(1.5:1.5:1) > S:O(3:1) > M > S > W. ATRA is hydrophobic substance; in order to overcome its solubility limitation, lipid emulsions (LEs) systems were applied. Both soybean oil and medium chain triglyceride are commonly used as an oil phase in LE due to the low toxicity and high biocompatibility. The solubility of ATRA in water was found to be extremely low (0.029 mg/ml), while its solubility in soybean oil and in medium chain triglyceride was 1.473 mg/ml and 3.062 mg/ml, respectively. The solubility of ATRA in oleic acid showed the highest value of 12.178 mg/ml. In comparison with water, the solubility of ATRA in soybean oil, medium chain triglyceride and oleic acid was approximately 50, 105 and 420 folds, respectively.

From the results, oleic acid gave the highest solubility of ATRA, while a mixture of oleic acid and soybean oil or medium chain triglyceride showed higher solubility than soybean oil or medium chain triglyceride alone. An increase in the amount of oleic acid in the oil mixtures resulted in increasing the solubility of ATRA. In the preliminary study LE formulations of these oil mixtures, only the lowest ratio of oleic acid (S:O and M:O = 3:1) could form LE. Therefore soybean oil, medium chain triglyceride and the mixture with the lowest ratio of oleic acid (S:O and M:O = 3:1) were chosen to be an oil phase for future LEs formulation and characterization.

1.2 Solubility of ATRA in mixture of liquid lipid and solid lipid

The solubility of ATRA in the mixture of liquid lipid i.e. soybean oil (S), medium chain triglyceride (M), oleic acid (O), mixture of S:O (3:1), M:O (3:1) and solid lipid (cetyl palmitate, CP), by ratio 5:1, 3:3, 1:5, with initial ATRA loaded at 1, 3, 5, 7 mg/g, at 70°C is shown in Table 6. In general, liquid lipids could be solubilized drugs much higher than solid lipids. Non-surprisingly, the time consuming for complete dissolving of ATRA in the mixture composed of the chosen liquid lipid to CP (S:CP, M:CP, SO:CP, MO:CP) in a ratio of 5 to 1 required short dissolved time (≤5 min) in all initial ATRA loaded (1, 3, 5, 7 mg/g). In contrast, the invert ratio of S:CP, M:CP, SO:CP at 1:5 took longer time (≤10 min) for ATRA completely dissolved in such lipid mixtures. Interesting, there was no difference in dissolved time for all ratios of MO:CP mixtures (≤5 min) for entire initial ATRA concentration loaded of ATRA. These results concordantly agreed with the previous solubility studied that MO gave the highest solubility of ATRA among the chosen liquid lipids.

Table 6 The dissolved time of ATRA in various mixtures of liquid lipids and solid lipid at 70ºC

Liquid lipid (g)				**Solid lipid (g)**	**ATRA (mg/g)**	**Dissolved time (min)**
S	**M**	**S:O (3:1)**	**M:O (3:1)**	**CP**		
5				1	1	5
3				3	1	5
1				5	1	10
5				1	3	5
3				3	3	5
1				5	3	10
5				1	5	5
3				3	5	5
1				5	5	10
5				1	7	5
3				3	7	10
1				5	7	10
	5			1	1	5
	3			3	1	5
	1			5	1	10
	5			1	3	5
	3			3	3	5
	1			5	3	10
	5			1	5	5
	3			3	5	5
	1			5	5	10
	5			1	7	5
	3			3	7	10
	1			5	7	10

Table 6 (Continue)

Liquid lipid (g)				Solid lipid (g)	ATRA (mg/g)	Dissolved time (min)
S	**M**	**S:O (3:1)**	**M:O (3:1)**	**CP**		
		5		1	1	5
		3		3	1	5
		1		5	1	10
		5		1	3	5
		3		3	3	10
		1		5	3	10
		5		1	5	5
		3		3	5	10
		1		5	5	10
		5		1	7	5
		3		3	7	10
		1		5	7	10
			5	1	1	5
			3	3	1	5
			1	5	1	5
			5	1	3	5
			3	3	3	5
			1	5	3	5
			5	1	5	5
			3	3	5	5
			1	5	5	5
			5	1	7	5
			3	3	7	5
			1	5	7	5

2. Preparation of lipid nanoparticles

2.1 Preparation of ATRA-loaded lipid emulsions (LEs)

Previous study revealed that ATRA-loaded lipid emulsions (ATRA-LEs) consisting of 30% MCT as an oil phase, 1.2% lecithin as an emulsifier with combination of 8% polysorbate-80 as an co-emulsifier gave good stability (Chinsriwongkul et al. 2007 : 469). Therefore, such compositions of LEs were selected in the present study with the addition of the antioxidant (BHT 0.002%). The compositions of ATRA-loaded lipid emulsions are shown in Table 7. Oil phases were S, M, SO, and MO. Probe sonication was used to reduce emulsion droplets to achieve size in nanometer-range. The obtained ATRA-loaded LE formulations exhibited milky emulsion with an off-white to pale-yellowish color (Figure 11). Since ATRA is yellow, it could be observed that ATRA-loaded amount was the major factor for the color of emulsion.

Figure 11 Appearance of ATRA-loaded lipid emulsions

Table 7 Composition of ATRA-loaded nanoparticles.

Composition	**Lipid emulsions (LEs)**				**Nanostructured lipid carriers (NLCs) liquid lipid:Solid lipid = 1:1**			
	S	**M**	**S:O (3:1)**	**M:O (3:1)**	**S**	**M**	**S:O (3:1)**	**M:O (3:1)**
ATRA (mg/g)	1,3,5,7,9	1,3,5,7,9	1,3,5,7,9	1,3,5,7,9	1,3,5,7,9,11	1,3,5,7,9,11	1,3,5,7,9,11	1,3,5,7,9,11
Liquid lipid (%)								
- soybean oil (S)	30	-	22.5	-	15	-	10	-
- medium chain triglyceride (M)	-	30	-	7.5	-	15	-	5
- oleic acid (O)	-	-	7.5	22.5	-	-	5	10
Solid lipid (%)								
- cetyl palmitate (CP)	-	-	-	-	15	15	15	15
Lecithin (%)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
BHT (%)	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
Polysorbate-80 (%)	8	8	8	8	8	8	8	8
Water qs.	100	100	100	100	100	100	100	100

In order to study the effect of ATRA amount loaded in LE formulations on physical properties, the initial addition of ATRA was varied (1, 3, 5, 7 and 9 mg/g of LE). The physical properties, ATRA content and incorporation efficiency of ATRA-LEs with different types of oil phase were examined. The type of the oils and initial ATRA loading affected the loading capacity of ATRA or ATRA content in LE (Figure 12). In all formulations, as the initial ATRA concentration increased from 1 to 9 mg/g, the incorporation efficiency or percentage yield of ATRA decreased. The incorporation efficiency of ATRA loaded in the formula S, M, S:O (3:1) and M:O (3:1) was higher than 80 % with 1, ≤3, ≤7 and ≤5 mg/g of the initial ATRA, respectively. The results indicated that only formula S:O (3:1) showed the highest loaded ATRA at the initial concentration of ATRA of 7 mg/g. The loading capacity revealed that an oil mixtures (S:O and M:O) LE formulations showed higher loading capacity than that of an oil (S or M) without oleic acid. The highest loading capacity of S:O and M:O LE formulations were 5.13±0.10 mg/g and 4.75±0.04 mg/g when the initial concentrations of ATRA were 7 and 5 mg/g, respectively. These might be due to higher solubility of ATRA in oleic acid, which was approximately 4-fold and 8.3-fold higher than in M and S at 25°C, respectively. These results indicated that oleic acid enhanced loading capacity of ATRA in such lipid emulsions.

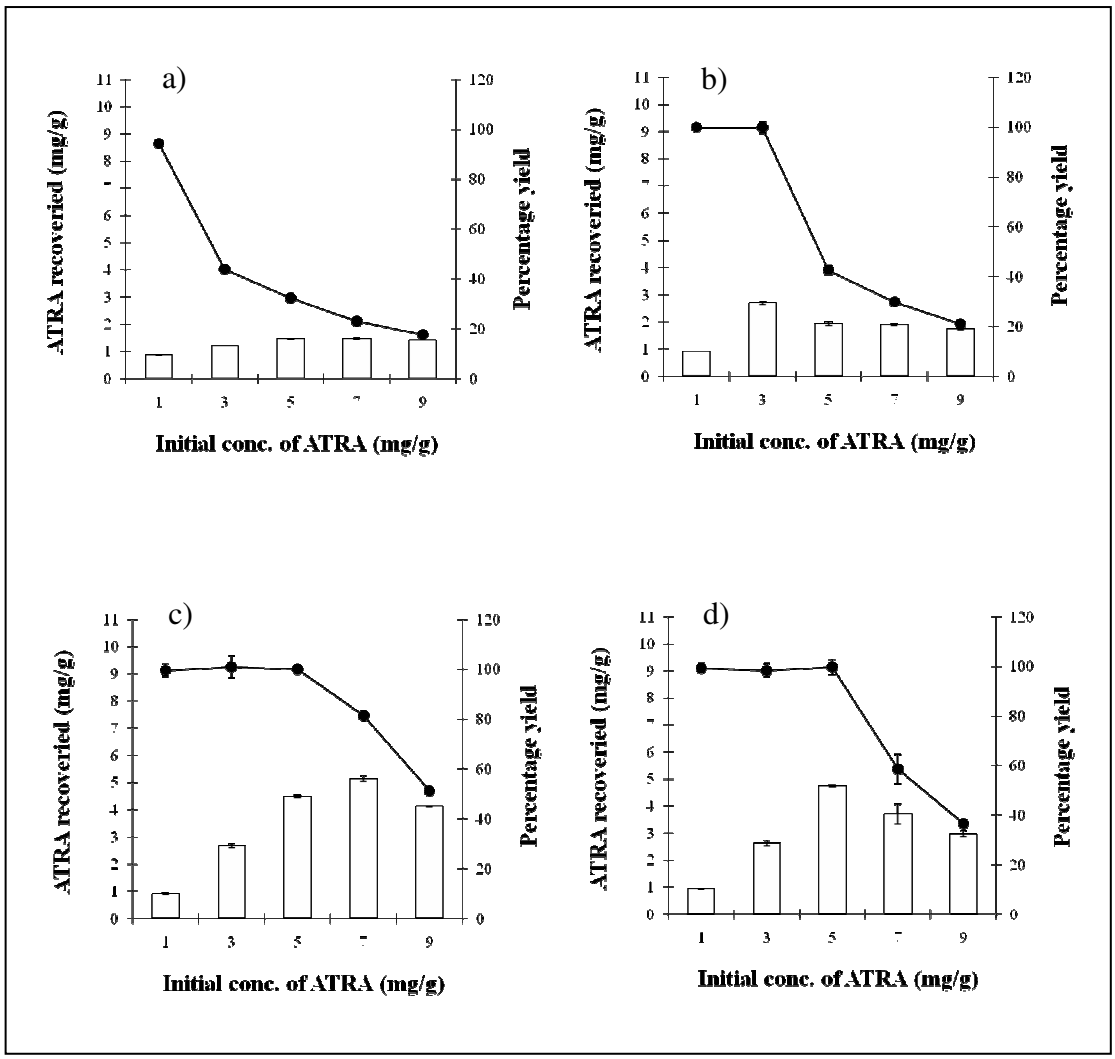


Figure 12 Effect of initial ATRA concentration on the ATRA content and the percentage yield of ATRA incorporated in the lipid emulsion formulations composed with different oil phases. (a) soybean oil; S, (b) medium chain triglyceride; M, (c) soybean oil:oleic acid (3:1); SO, (d) medium chain triglyceride:oleic acid (3:1); MO.

Keys : □ ATRA content

- Percentage yield of ATRA incorporated in the lipid emulsion formulations.

2.2 Preparation of ATRA-loaded nanostructured lipid carriers (NLCs)

In case of nanostructured lipid carriers (NLCs), the same manner as LEs performed by replaced liquid lipid (S, M, SO, MO) with mixture of liquid lipid and solid lipid (S:CP, M:CP, SO:CP, MO:CP). The compositions of ATRA-loaded NLC are shown in Table 7. The obtained ATRA-loaded NLCs provided milky emulsion with off-white to pale-yellowish color similar to the ATRA-loaded LEs as previously described.

To investigate the influence of the ratios of liquid lipid (S, M, SO, MO) : solid lipid (CP) in oil phase on the physical properties of the ATRA-loaded NLC, the mixture of liquid lipid and CP in various proportion i.e. 5:1, 3:3, 1:5 was used. Figure 13 shows the effect of liquid lipid (M) and solid lipid (CP) ratios on ATRA amount loaded in NLC. At 1, and ≤3 mg/g ATRA concentration incorporated into NLC composed of M:CP (5:1) gave percentage yield over 80%. While NLC from oil matrix comprised M:CP (3:3) gave percentage yield higher than 80% with 1, ≤3, and ≤5 mg/g of the initial ATRA. The percentage yield of NLC from M:CP (1:5) higher than 80% with 1 mg/g of initial concentration of ATRA.Since high drug loading in NLC was the major concern for this study, the ratio M:CP (3:3) giving much more loading capacity and possessing good physical appearance by visual observation should be used. NLC which oil phase matrix composed of M and CP in equal amount showed the suitable loading capacity of ATRA among all the investigated proportions. Therefore, liquid lipids with the equal ratio of solid lipid (S:CP, M:CP, SO:CP and MO:CP = 1:1) were chosen to be oil phase for future formulation and characterization.

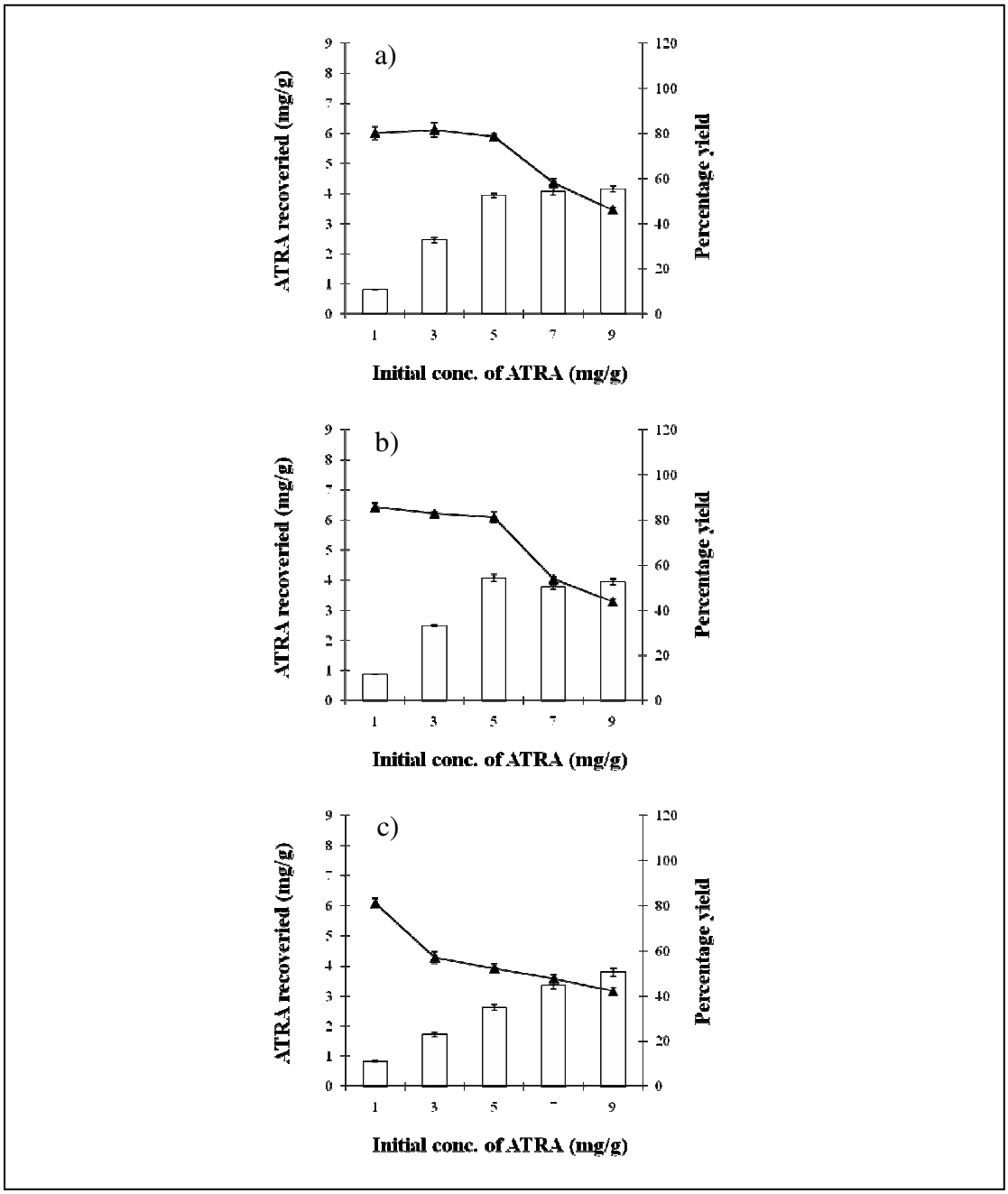


Figure 13 Effect of liquid lipid (M) and solid lipid (CP) ratios in oil phase on the ATRA content and the percentage yield of ATRA incorporated in the NLC formulations. (a) M:CP=5:1, (b) M:CP=3:3, (c) M:CP=1:5

Keys : □ ATRA content

▲ Percentage yield of ATRA incorporated in the NLC formulations.

As the solid lipid (CP) concentration in oil matrix increased, the incorporation efficiency or percentage yield of ATRA decreased. It was found that the similar observations were made on incorporation of ATRA into LEs. The type of the oils and initial ATRA added affected the loading capacity of ATRA or ATRA content in NLCs (Figure 14). The results showed that the incorporation efficiency or percentage yield of ATRA increased when the initial ATRA concentration were increased from 1 to 11 mg/g. The incorporation efficiencies of ATRA-loaded NLC formula composed of S:CP, M:CP, SO(3:1):CP and MO(3:1):CP in an equal ratio (1:1) were higher than 80 % with ≤3, ≤5, ≤9 and ≤9 mg/g of the initial ATRA, respectively. The highest loading capacity of S-, M-, SO-, MO-NLC formulations was 3.74, 5.44, 7.55, and 7.63±0.16 mg/g, respectively. These results demonstrated that NLC formulations consisting of SO(3:1):CP and MO(3:1):CP gave the highest loaded ATRA at initial concentration of ATRA of 9 mg/g. These results indicated the influence of oleic acid on ATRA loading capacity in the same way as those of lipid emulsions. However, regarded on the same type of oil, the loading capacity of ATRA into NLC formulations was higher than that into lipid emulsions. Although it is not clear that how CP increased the ATRA-loading in NLC, it might be possible that NLC composed of solidified lipid which could gave higher viscosity within the lipid droplet of NLC, thus slowed down the mobility of incorporated ATRA or moving out from the lipid particles leading to higher entrapment and better stability. These results demonstrated that NLCs formulation posses advantages on ATRA loading capacity over lipid emulsions.

Interestingly, the NLC formulations in this study especially in the combination of oleic acid have shown drastically higher loading capacity over other colloidal carriers in comparison to many studies, for example, Hwang and coworkers reported loading capacity of ATRA in 15% oil-containing phospholipid-based microemulsion system prepared with soybean oil, phospholipid, and DSPE-PEG_{2000} was only 1.586±0.22 mg/ml (Hwang et al. 2004 : 180). Lim et al. investigated the ATRA incorporation efficiency in SLN powders, with 4.5 mg/g of initial concentration of ATRA, the incorporated concentration of ATRA was 2.9±0.3 mg/g (Lim et al. 2004 : 57). Manconi et al. reported incorporation efficiency of ATRA in

MLV-, LUV-, SUV-liposomal formulations obtained from enriched soya phosphatidylcholine (Phopholipon®90), choloesterol, and dicetyl phosphate was 97.32±1.78% 90.65±1.78, 75.98±2.45% of 4mg/ml initial loading respectively(Manconi et al. 2002 : 245).

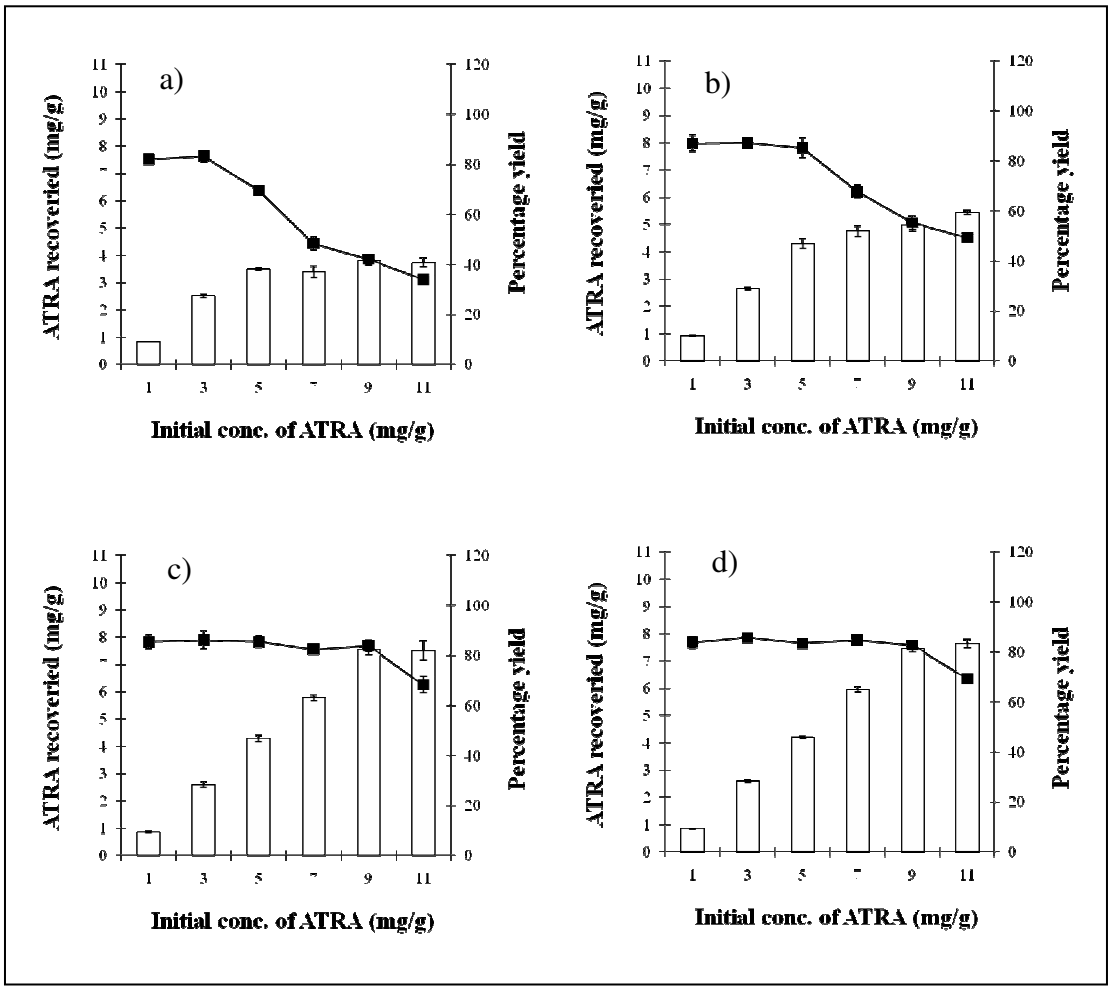


Figure 14 Effect of initial ATRA concentration on the ATRA content and the percentage yield of ATRA incorporated in the NLC formulations composed with different oil phases. (a) soybean oil; S, (b) medium chain triglyceride; M, (c) soybean oil:oleic acid (3:1); SO, (d) medium chain triglyceride:oleic acid (3:1); MO.

Keys : □ ATRA content

- Percentage yield of ATRA incorporated in the NLC formulations.

2.3 Preparation of polymer coated ATRA-loaded NLCs

Since higher loading capacity of ATRA could be obtained by NLCs preparations, furthermore, pure MCT could provide better loading capacity and smaller particles size as well, when compared with pure soybean oil. It was found that the presence of DPEG and PPEG did not significantly affect ATRA loading capacity of NLCs (Figure 15). In this regard, NLCs formulated from two types of oil matrix, M:CP (1:1) and MO:CP (1:1) at 3 mg/g concentration were selected for further study.

3. Characterization of ATRA-loaded lipid nanoparticles

3.1. Physicochemical characterization of ATRA-loaded lipid nanoparticles

The physicochemical characteristics, mean particle size and surface charge are important parameters for predicting the physical stability of colloidal dispersion systems (Lee et al. 2007 : 2144). Particle size, polydispersity index (PDI) and droplet surface charge of ATRA-loaded LE, ATRA-loaded NLC and ATRA-loaded polymer coated NLC are shown in Figure 16, 17 and 18, respectively.

In order to maintain lipid nanoparticles physical integrity by minimizing fatty acid ester hydrolysis of triglyceride and phospholipids (Klang and Benita, in Benita, ed. 1998 : 120), the final pH of the formulations was adjusted to pH 8±0.1 with 1 N NaOH solution. In order to maintain lipid emulsion physical integrity by minimizing fatty acid ester hydrolysis of MCT and phospholipids (Klang and Benita in Benita, ed. 1998 : 122). All lipid nanoparticles, except pure soybean oil-LE, had average sizes less than 200 nm (138.11 to 172.69 nm) with narrow PDI and negative zeta potential (-15.39 to -61.82 mV). Pure soybean oil-LE formulation showed slightly larger mean particle size (249 nm).

The presence of DPEG and PPEG polymer affected the particle sizes of NLC. The particles sizes of DPEG- or PPEG-coated–NLCs were increased (160.39-172.69 nm) compared with uncoated NLCs formulation (NLC-M, -MO). These results indicated the formation of a coating layer on the surface of such lipid nanoparticles.

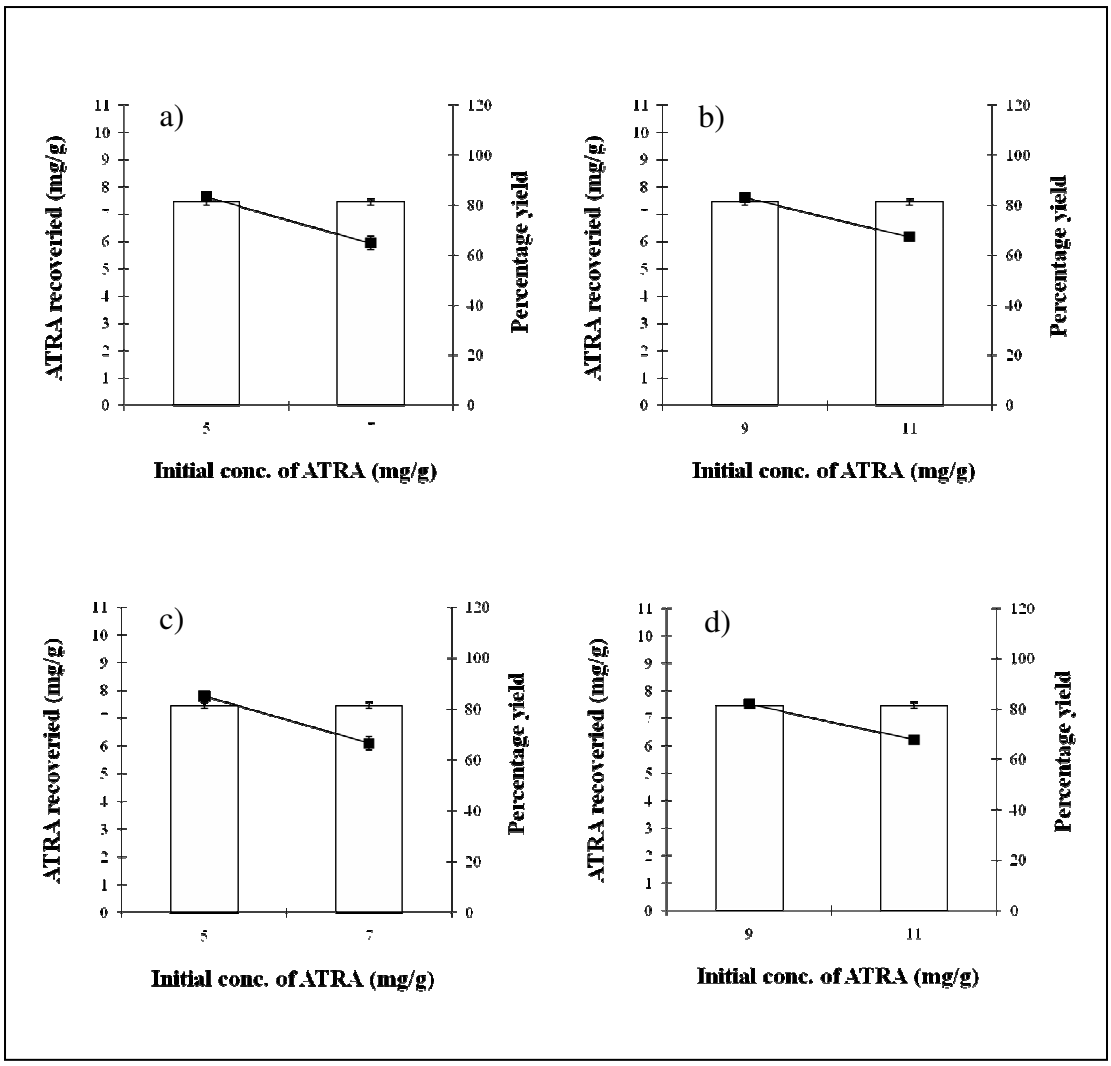


Figure 15 Effect of initial ATRA concentration on the ATRA content and the percentage yield of ATRA incorporated in the DPEG or PPEG coated NLC formulations composed with different oil phases. (a) DPEG-coated NLC-M, (b) DPEG-coated NLC-MO, (c) PPEG-coated NLC-M, (d) PPEG-coated NLC-MO.

Keys : □ ATRA content

- Percentage yield of ATRA incorporated in the NLC formulations.

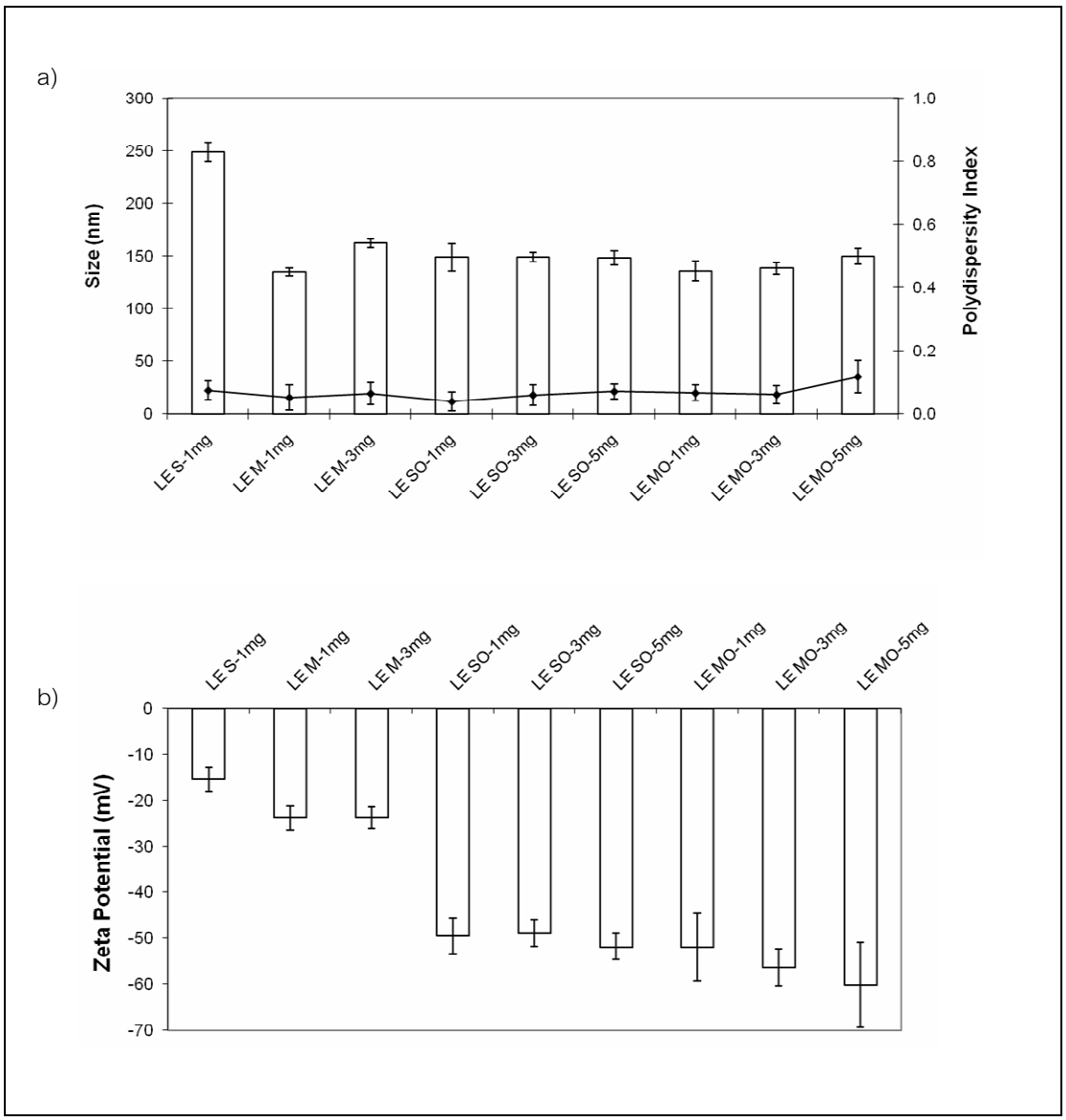


Figure 16 Physical properties of ATRA-loaded LE composed of different oil phase; (a) Mean particles size and particles size distribution (PDI) of LEs (b) surface charge of LEs.

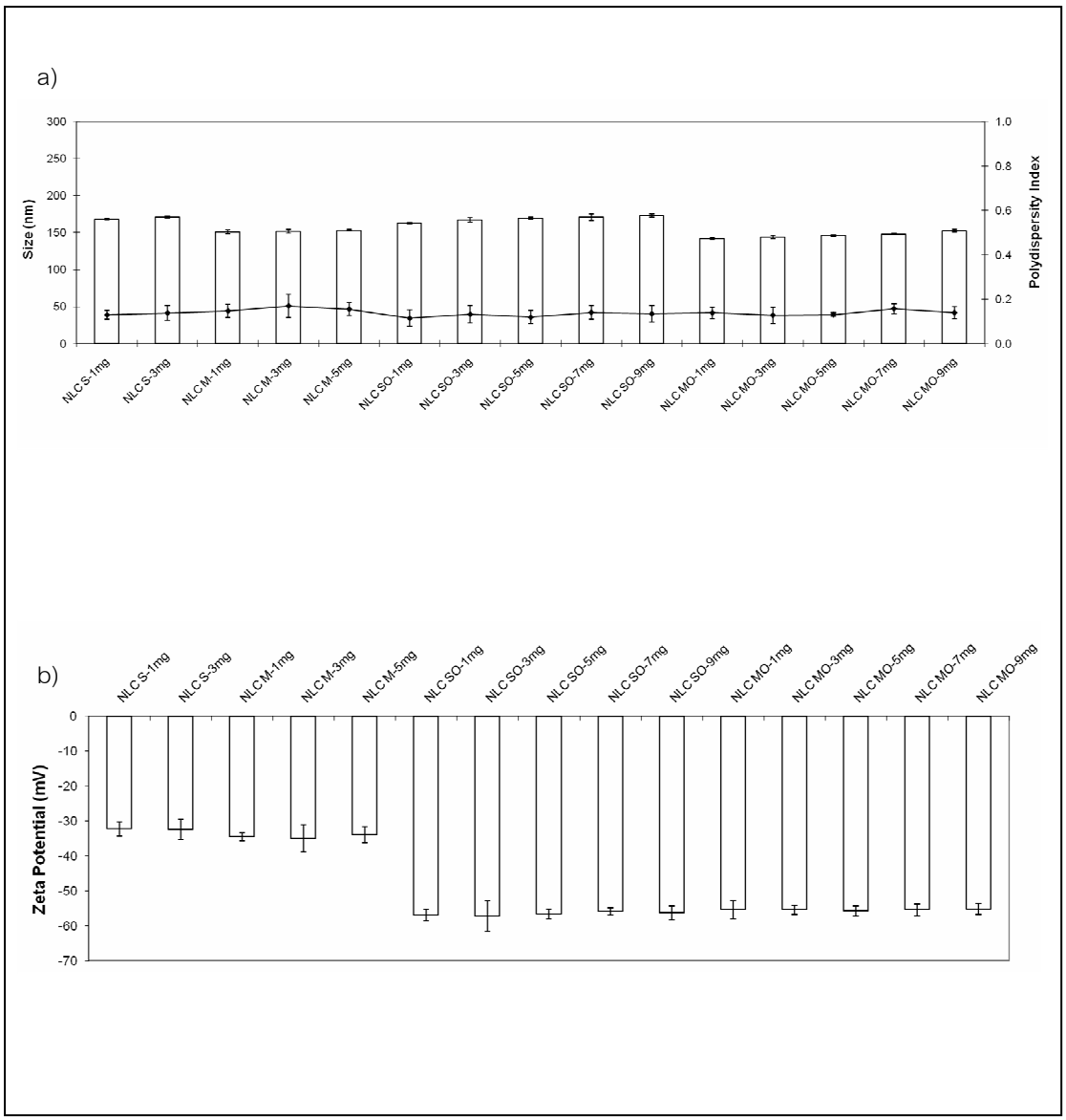
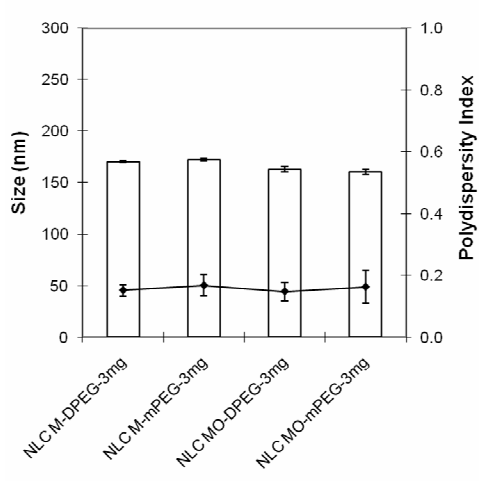


Figure 17 Physical properties of ATRA-loaded NLC composed of different oil phase; (a) Mean particles size and particles size distribution (PDI) of NLCs (b) surface charge of NLCs.

a)



b)

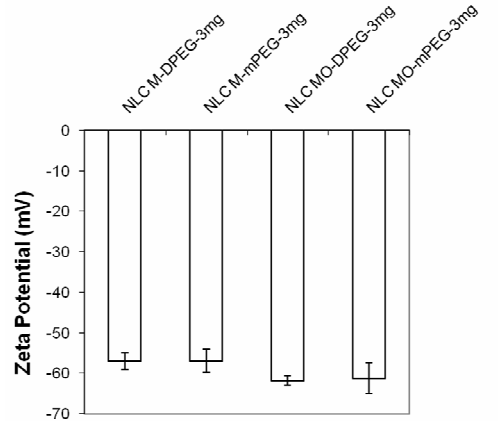


Figure 18 Physical properties of ATRA loaded in polymer coated-NLC composed of different oil phase; (a) Mean particles size and particles size distribution (PDI) of polymer loaded-NLCs (b) surface charge of polymer loaded-NLCs.

For intravenous emulsions dosage form, the particle sizes of oil droplets are generally in the range of 100-500 nm to avoid potential embolism (Klang and Benita, in Benita, ed. 1998 : 119). Hence, the droplet sizes in investigated formula were in an acceptable range, less than 500 nm. The PDI of lipid nanoparticles was also low (0.06-0.17), except for pure soybean oil-LE (0.77). PDI is the measurement of the distribution of nanoparticles population. It is closed to zero for monodispersed particles, whereas lipid nanoparticles are typically in the range of 0.1 to 0.2. These results suggested that the narrow size distribution was obtained in all prepared formulations except for pure soybean oil-LE. These results may be due to chemical differences in the oil phase which significantly affect the energy barriers required for the stability of the dispersion. The shorter chain hydrocarbon of MCT (C8-C10), versus the longer chain hydrocarbons of long-chain triglycerides (LCT) from pure soybean oil (average C-18), would have lower free energy when water is the dispersion medium (Driscoll et al. 2001 : 153). Therefore, the greater the miscibility of the dispersed phase with the continuous phase, the less physicochemical stress upon the same emulsifier to maintain stability.

The zeta potential (ZP) is a measure of the electric charge at the surface of the particles indicating the physical stability of colloidal systems. The ZP value higher than |30 mV| indicates electrostatic long term stability of aqueous dispersions (Teeranachaideekul et al. 2008 : 135). In this study, the ZP values of ATRA loaded lipid nanoparticles were negative due to negative charge of fatty acid, hydrolytic products of phospholipids, which are likely to accumulate at the surface of oil droplets (Benita and Levy 1993 : 1070). High zeta potential values should be achieved in prepared emulsions to ensure a high energy barrier, which causes the repulsion of adjacent droplets and results in the formation of a stable system (Lin et al. 2000 : 267).

The lowest zeta potential was found in lipid emulsion formulated with pure soybean oil, while MCT gave -23.74 ± 2.32 mV. These results were slightly lower than the appropriated value of parenteral emulsions. Lipid nanoparticles formulated with oleic acid exhibited higher negative charge when compared with those without oleic acid. These results indicated that oleic acid affects the

incorporation efficiency and stability of ATRA-loaded lipid nanoparticles from improving negative surface charge of lipid droplets. It is also interesting to consider the role of the fatty acids presence, particularly with oleic acid, in the stability of lipid nanoparticles. The previous study showed that the addition of oleic acid to parenteral fat emulsions has improved the stability of emulsion (Washington and Davis 1987 : 34).

It could also be observed that the presence of DPEG and PPEG polymer dramatically affected the zeta potential of NLC formulated with MCT from -34.92 mV to -57.02 and -56.92 respectively. Since PEG is expected to provide additional steric stabilization of colloidal particles (Lee et al. 2007 : 2139), thus the NLCs prepared with DPEG and PPEG should theoretically provided better NLCs physical stability.

3.2 *In vitro* release of ATRA-loaded lipid nanoparticles

The effect of lipid nanoparticles on the release of ATRA was investigated *in vitro* by determining the drug release across a dialysis bag. The release profiles of ATRA from ATRA-loaded lipid emulsions are shown in Figure 19. The *in vitro* ATRA release data were fitted to proposed models including zero order, first order, and higuchi model as following :

Zero order equation: $C_t = C_0 + k_0 t$

First order equation: $L_n C_t = \ln C_0 + k_1 t$

Higuchi model: $Ct = C_0 + k_H t^{1/2}$

C_t is cumulative amount of ATRA released at any specified time point and C_0 is the dose of drug incorporated in the delivery system. k_0, k_1 and k_H are rate constants for zero order, first order, and Higuchi model respectively.

The regression analysis was performed after fitting such three kinetic models to the experimental data. The correlation (R^2) was used as an indicator of goodness-of-fit of the equation to the *in vitro* ATRA release data. The degradation rate constants (k) of ATRA from the lipid nanoparticles were estimated from the slope of the plots. The predicted half life ($t_{1/2}$), namely the time required when ATRA yield in the lipid nanoparticles remains at 50%, was estimated by substituting k into those

equation kinetics. From the data obtained (Table 8), it was found that the release kinetic data from lipid nanoparticles agree with zero order and higuchi model (square-root-of-time equations) as show in Table 8.

ATRA could be gradually released from emulsions during 48 h. The release of ATRA from lipid emulsion in all formulations was about 1.60-11.38% at 48 h. This indicated that lipid emulsions can be used as carriers for controlled delivery of ATRA. ATRA being hydrophobic prefers to dissolve in lipid emulsion instead of receptor medium. However, the significant differences in the percentages of ATRA released in the receptor medium at 48 h consideration on oil type were observed. Since the release kinetic was not followed first-order kinetic meaning ATRA released was not depended on the starting ATRA concentration, but depended greatly on the type of oil phase. In all ATRA-loaded lipid emulsions with different initial ATRA 1 mg/g (Figure 19a), 3 mg/g (Figure 19b) and 5 mg/g (Figure 19c), the release of ATRA from lipid emulsions with different oil phases showed a same trend increase in the order of MO > SO > M > S. The highest release rate was observed in an oleic acid-contained formula (MO and SO). These results were consistent with the previous report that the progesterone-releasing rate of lipid nanoparticles was enhanced by increasing oleic acid content (Yuan et al. 2007 : 175). In the same type of oil phase but different in initial drug concentration of 1 mg/g, 3 mg/g and 5 mg/g, there was no significant difference in ATRA release from all formulations (Figure 20). These results indicated that the type of oil phase but not initial drug concentration affected the release of ATRA from the lipid emulsion formulations.

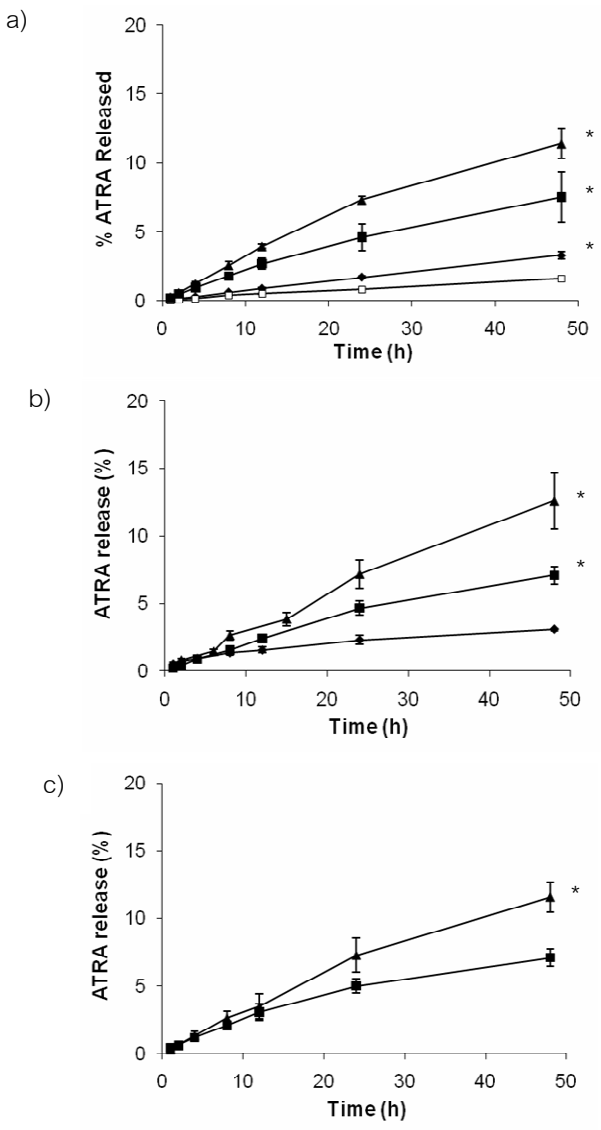


Figure 19 Release profile of ATRA loaded-lipid emulsions composed of different oil phase; soybean oil (S), MCT (M), MCT:oleic acid (MO), soybean oil:oleic acid (SO) (a) ATRA 1 mg/g. (b) ATRA 3 mg/g (c) ATRA 5 mg/g.

Keys : □ Soybean oil (S)

♦ MCT (M)

▲ MCT:oleic acid (MO)

■ Soybean oil:oleic acid (SO)

* Significantly different (p=0.05).

Table 8 The kinetic parameters of ATRA release from the ATRA-loaded lipid nanoparticles.

Formula	Zero order equation		First order equation		Higuchi model	
	k_0	R^2	k_1	R^2	k_H	R^2
LEs						
LE-S (1mg/g)	0.0326	0.9924	0.0283	0.7443	0.2588	0.9700
LE-M (1mg/g)	0.0686	0.9992	0.0315	0.7407	0.5413	0.9622
LE-M (3mg/g)	0.0525	0.9477	0.0144	0.7990	0.4327	0.9980
LE-SO (1mg/g)	0.1550	0.9834	0.0273	0.6968	1.2497	0.9896
LE-SO (3mg/g)	0.1486	0.9763	0.0276	0.7265	1.1990	0.9849
LE-SO (5mg/g)	0.1433	0.9525	0.0234	0.7186	1.1765	0.9945
LE-MO (1mg/g)	0.2398	0.9774	0.0291	0.7048	1.9374	0.9880
LE-MO (3mg/g)	0.2591	0.9951	0.0272	0.7605	2.0619	0.9756
NLCs						
NLC-S (1mg/g)	0.0855	0.9861	0.0241	0.7905	0.6860	0.9826
NLC-S (3mg/g)	0.0887	0.9920	0.0252	0.7797	0.7081	0.9793
NLC-M (1mg/g)	0.0830	0.9873	0.0246	0.7524	0.6666	0.9864
NLC-M (3mg/g)	0.0879	0.9860	0.0234	0.7812	0.7061	0.9857
NLC-M (5mg/g)	0.0843	0.9743	0.0243	0.7726	0.6825	0.9878
NLC-SO (1mg/g)	0.0852	0.9747	0.0254	0.7324	0.6900	0.9899
NLC-SO (3mg/g)	0.0875	0.9883	0.0258	0.7329	0.7023	0.9857
NLC-SO (5mg/g)	0.0822	0.9735	0.0238	0.7388	0.6656	0.9886
NLC-SO (7mg/g)	0.0841	0.9739	0.0254	0.7401	0.6811	0.9899
NLC-SO (9mg/g)	0.0841	0.9821	0.0238	0.7582	0.6781	0.9893
NLC-MO (1mg/g)	0.0831	0.9801	0.0250	0.7393	0.6696	0.9861
NLC-MO (3mg/g)	0.0873	0.9864	0.0253	0.7460	0.7015	0.9868
NLC-MO (5mg/g)	0.0852	0.9735	0.0261	0.7116	0.6908	0.9901
NLC-MO (7mg/g)	0.0850	0.9786	0.0247	0.7315	0.6878	0.9921
NLC-MO (9mg/g)	0.0846	0.9759	0.0253	0.7191	0.6858	0.9920
Polymer coated-NLCs						
NLC-M-DPEG (3mg/g)	0.0847	0.9715	0.0252	0.7215	0.6879	0.9912
NLC-M-PPEG (3mg/g)	0.0857	0.9882	0.0253	0.7528	0.6875	0.9853
NLC-MO-DPEG (3mg/g)	0.0853	0.9827	0.0242	0.7756	0.6872	0.9866
NLC-MO-PPEG (3mg/g)	0.0856	0.9817	0.0265	0.7876	0.6877	0.9809

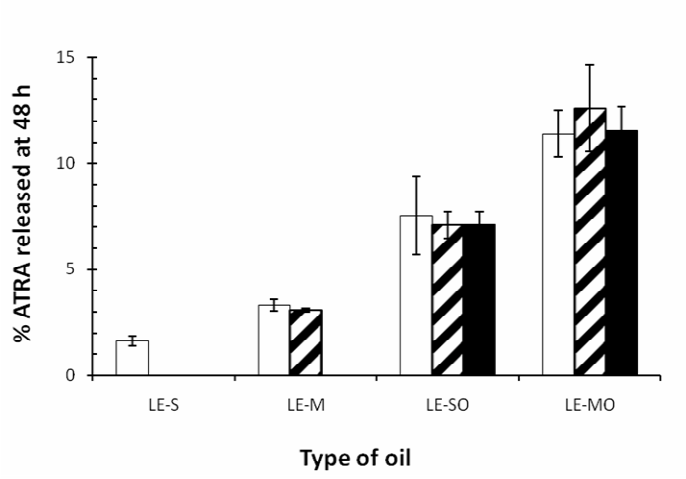


Figure 20 Percentage of ATRA released at 48 h from ATRA loaded-LE composed of different oil phase in different amount of initial ATRA concentration.

Keys : □ ATRA 1 mg/g

▨ ATRA 3 mg/g

■ ATRA 5 mg/g.

The release profiles of ATRA from ATRA-loaded NLC formulations were also investigated. The *in vitro* release of ATRA from ATRA-loaded NLCs system across dialysis tube under the same condition as LE was only 4.06-4.34% at 48 h. (Figure 21). In contrast to LE, there was no significant difference in ATRA released rate from all NLCs in accordance with composition of oil phase (Figure 22). The cumulative amount of ATRA released from NLCs was less than that from the corresponding LEs. It could be due to the presence of solid lipid, cetyl palmitate (CP), the melted blend of solid lipid and liquid lipid increased viscosity of particles leading to slower release rate according to the law by Strokes-Einstein (Teeranachaideekul et al. 2007 : 147). All these results supported that ATRA incorporated into both LEs and NLCs was preferable to remain in these lipid nanoparticles delivery systems. Moreover, no burst release of drug was observed indicating that prolonged release of ATRA from the lipid nanoparticles delivery systems was affordable.

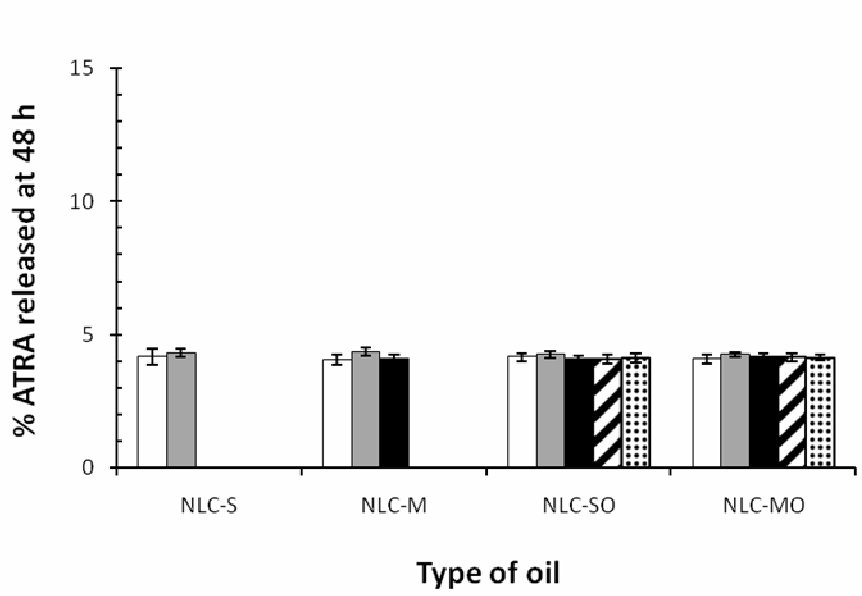


Figure 21 Percentage of ATRA released at 48 h from ATRA loaded-NLC composed of different oil phase in different amount of initial ATRA concentration.

Keys : □ ATRA 1 mg/g

▒ ATRA 3 mg/g

■ ATRA 5 mg/g

▨ ATRA 7 mg/g

⁞⁞ ATRA 9 mg/g

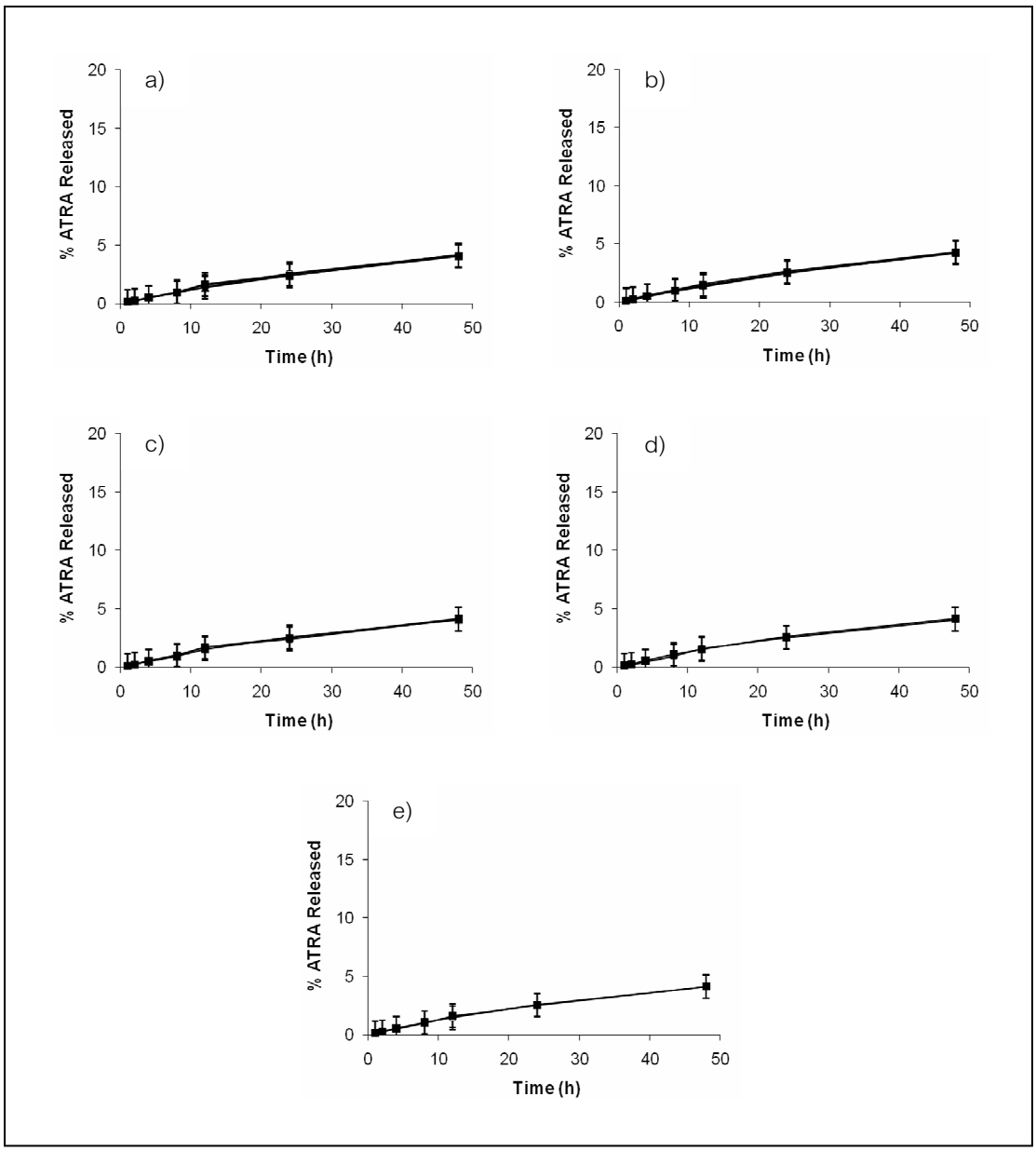


Figure 22 Release profile of ATRA loaded-NLCs composed of different oil phase. (a) ATRA 1 mg/g. (b) ATRA 3 mg/g (c) ATRA 5 mg/g. (d) ATRA 7 mg/g. (e) ATRA 9 mg/g.

Keys : ♦ Soybean oil (S)

▲ MCT (M)

■ MCT:oleic acid (MO)

□ Soybean oil:oleic acid (SO)

The release profiles of ATRA from polymer coated-NLC formulations were evaluated as described in LE and NLC. The results showed that ATRA released from polymer-coated NLC was sustained over 48 h, but it was only 4.10-4.18% ATRA released (Figure 23). There was no significant difference in ATRA released rate from DPEG- and PPEG coated NLCs when compared with uncoated M- and MO-NLC (Figure 24). The cumulative amount of ATRA released from polymer coated-NLCs was in agreement with NLCs which was less than that from the corresponding LEs as stated by type of oil used. These results implied that the accompanying of PEG polymer to NLCs did not affect the ATRA release rate from NLC which could conserve the sustained release properties of lipid nanoparticles system.

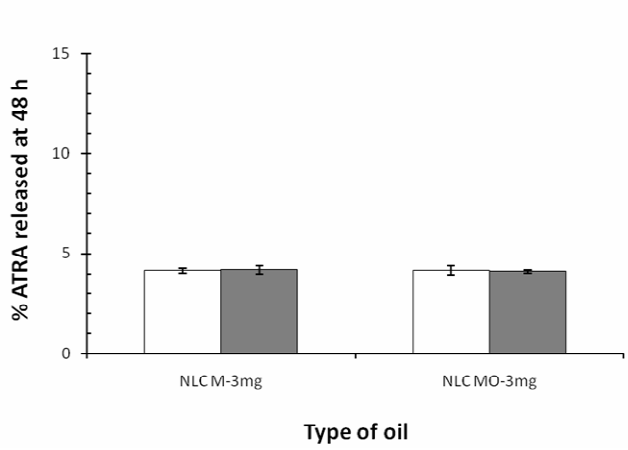


Figure 23 Percentage of ATRA released at 48 h from polymer coated-NLC composed of different oil phase, ATRA loaded at 3 mg/g concentration.

Keys : □ DPEG

■ PPEG

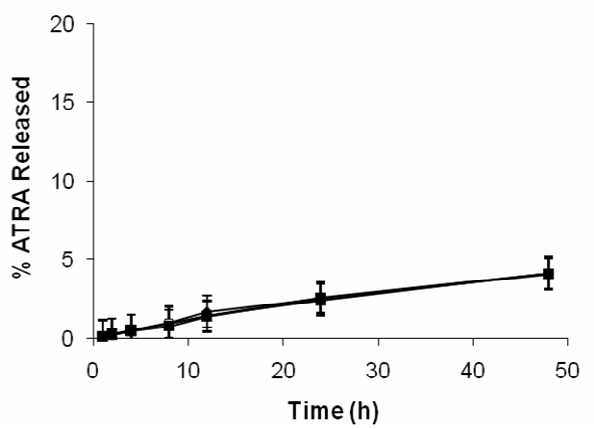


Figure 24 Release profile of ATRA-loaded DPEG or PPEG coated NLCs composed of different oil phase, at 3 mg/g of initial ATRA concentration.

Keys : ♦ NLC-M with DPEG

▲ NLC-M with PPEG

□ NLC-MO with DPEG

■ NLC-MO with PPEG

3.3 Photostability study of ATRA-loaded lipid nanoparticles

With a feature common to all compounds in retinoids class, ATRA undergoes degradation when exposed to light (Brisaert, Everaerts and Plazier-Vercammen 1995 : 161, Carlotti et al. 2002 : 88). Therefore, the development of formulations characterized by high photo-protection towards the ATRA is important. The inclusion of ATRA with β-cyclodextrin (Caddeo et al. 2007 : 293, Lin et al. 2000 : 265), liposomes (Ioele et al. 2005 : 251), niosomes (Manconi et al. 2003 : 261), solid lipid nanoparticles (Yuan et al. 2007 : 174), and polymeric micelles (Opanasopit et al. 2007 : 424) has been reported to protect the drug against photodegradation. Figure 25 showed the photodegradation of ATRA (1, 3, 5, 7 and 9 mg/g of isopropyl alcohol (IPA)) at 25±0.5°C in the presence of UVA light for 6 h. At this temperature, thermal instability problems could not occur since it was found that the samples stored at 25°C, was chemically stable for months. The results revealed that the intact ATRA content significantly decreased with the increase of ATRA concentration.

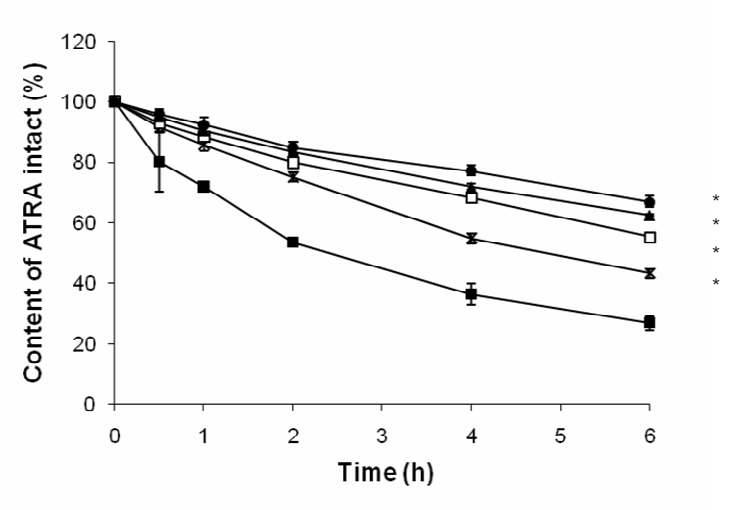


Figure 25 Effect of light on the chemical stability of ATRA in isopropyl alcohol solution (IPA) in different amount of initial ATRA concentration.
Keys : ■ 1 mg/g
X 3 mg/g
□ 5 mg/g
▲ 7 mg/g
● 9 mg/g
* Significantly different (p=0.05) when compared with ATRA 1 mg/g.

The photodegradation of ATRA-loaded LE, ATRA-loaded NLCs, and ATRA-loaded polymer coated NLCs with different oil phases and initial ATRA concentration at 25±0.5°C in the presence of UVA light for 6 h is shown in Figure 26, Figure 27 and Figure 28, respectively. The photostability results revealed that ATRA content in all formulations of lipid emulsion was significantly higher than that in IPA solutions.

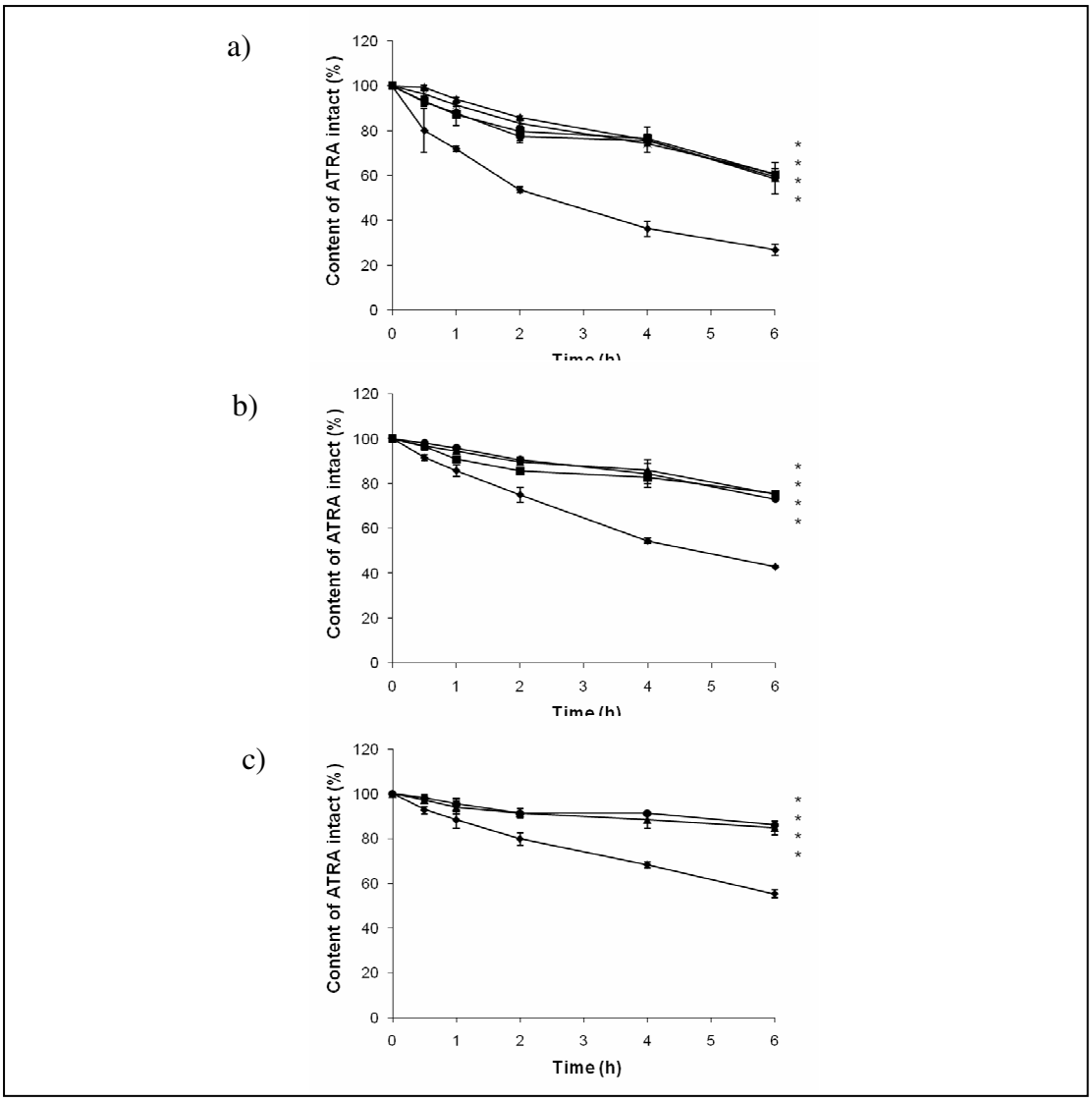


Figure 26 Effect of light on the chemical stability of ATRA–loaded lipid emulsions composed of different oil phase compared with ATRA in isopropyl alcohol solution (IPA) in different amount of initial ATRA concentration; (a) 1 mg/g, (b) 3 mg/g and (c) 5 mg/g.

Keys : x Soybean oil (S)

▲ Soybean oil:oleic acid (SO)

■ MCT (M)

● MCT:oleic acid (MO)

♦ Isopropyl alcohol solution (IPA)

* Significantly different (p=0.05) when compared with ATRA 1 mg/g.

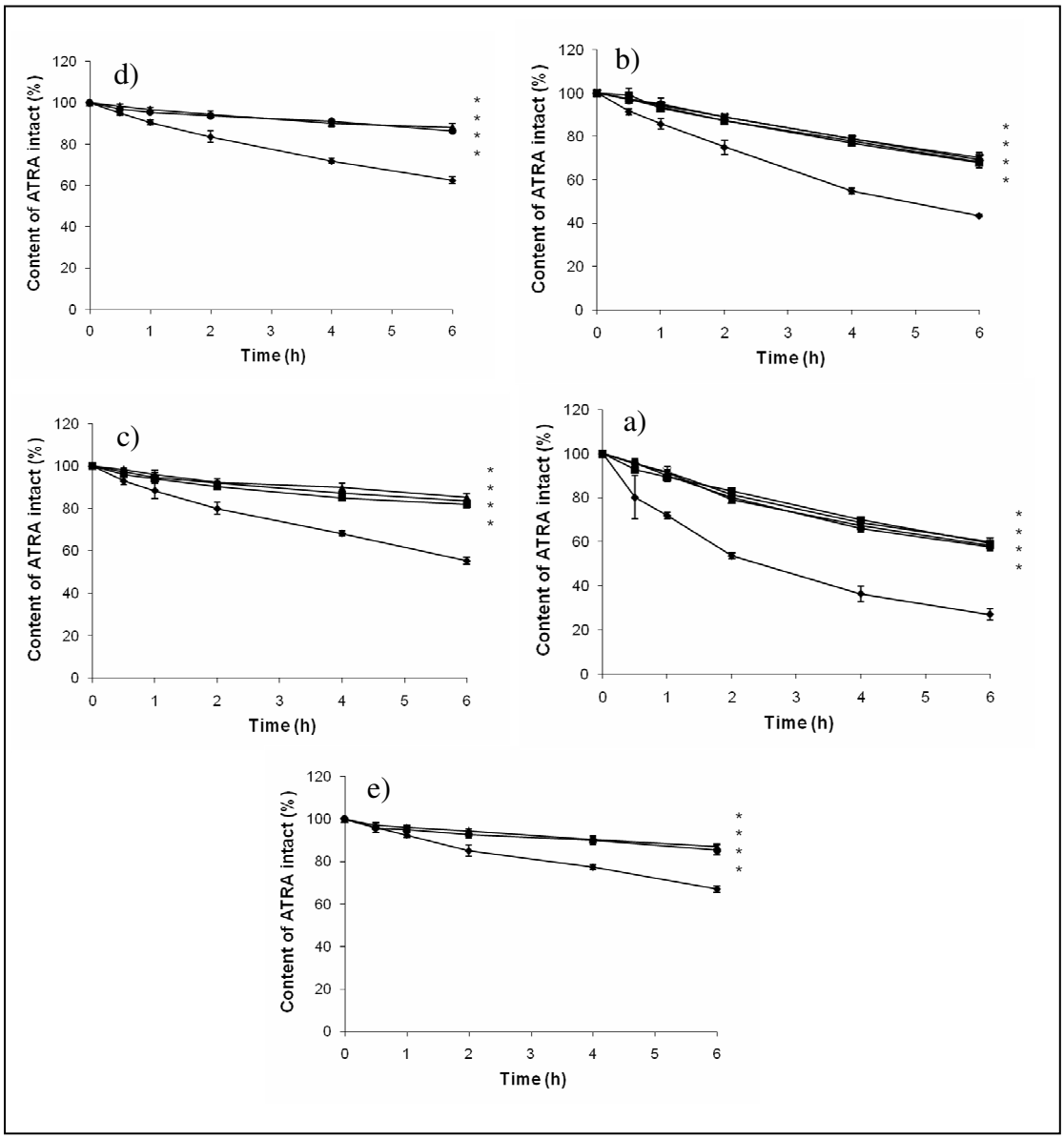


Figure 27 Effect of light on the chemical stability of ATRA–loaded NLCs composed of different oil phase, compared with ATRA in (♦) isopropyl alcohol solution (IPA) in different amount of initial ATRA concentration; (a) 1 mg/g, (b) 3 mg/g and (c) 5 mg/g. (d) 7 mg/g. (e) 9 mg/g.

Keys : **x** Soybean oil (S)

▲ Soybean oil:oleic acid (SO)

■ MCT (M)

● MCT:oleic acid (MO)

♦ Isopropyl alcohol solution (IPA)

* Significantly different (p=0.05) when compared with ATRA 1 mg/g.

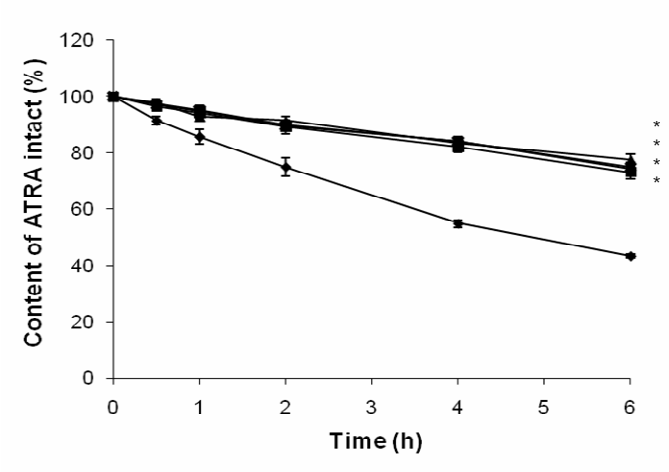


Figure 28 Effect of light on the chemical stability of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase, compared with ATRA in isopropyl alcohol solution (IPA) at 3 mg/g of initial ATRA concentration.

Keys : **x** NLC-M with DPEG ▲ NLC-MO with DPEG

■ NLC-M with PPEG ● NLC-MO with PPEG

♦ Isopropyl alcohol solution (IPA)

* Significantly different (p=0.05) when compared with IPA

The determination of the kinetics of photodegradation of ATRA in isopropyl alcohol and in lipid nanoparticles were performed by plotting concentration of the drug remaining versus time (zero-order kinetic) and log of concentration of the drug versus time (first-order kinetic). The regression coefficients (R^2) were obtained, and the best fit observed indicates the reaction order, the rate constant of photodegradation (k_0 or k_1) of ATRA was carried out from the slopes of the straight lines (Table 9). It was found that most of ATRA photodegradation followed the first order kinetics except for LE-SO 1 mg/ml, LE-SO 3 mg/ml LE-MO 3 mg/ml, and NLC-MO-PPEG 3 mg/g were followed the zero order kinetics. Other studies has also have reported ATRA degradation to follow first order kinetics (Brisaert and Plaizier-Vercammen 2000 : 52 ; Ioele et al. 2005 : 257) while studies reporting degradation to follow zero-order kinetics have appeared (Manconi et al. 2003 : 267).

Table 9 The kinetic parameters of ATRA degradation in the presence of UV light for 6 h, when loaded in lipid nanoparticles and alcoholic solution.

Formula	Zero order equation		First order equation	
	k_0	R^2	k_1	R^2
ATRA in IPA solution				
ATRA 1 mg/g	-0.0928	0.9775	-11.3094	0.8976
ATRA 3 mg/g	-0.0615	0.9975	-9.4629	0.9805
ATRA 5 mg/g	-0.0412	0.9955	-7.1083	0.9848
ATRA 7 mg/g	-0.0337	0.9966	-6.1502	0.9857
ATRA 9 mg/g	-0.0283	0.9930	-5.3570	0.9858
ATRA-loaded LE				
LE-S (1mg/g)	-0.0351	0.9924	-6.3658	0.9894
LE-M (1mg/g)	-0.0322	0.9445	-5.7973	0.9367
LE-M (3mg/g)	-0.0188	0.9323	-3.7500	0.9146
LE-SO (1mg/g)	-0.0384	0.9796	-6.8876	0.9903
LE-SO (3mg/g)	-0.0189	0.9720	-3.7921	0.9744
LE-SO (5mg/g)	-0.0112	0.9396	-2.3481	0.9276
LE-MO (1mg/g)	-0.0335	0.9391	-5.9833	0.9266
LE-MO (3mg/g)	-0.0220	0.9875	-4.3554	0.9923
ATRA-loaded NLC				
NLC-S (1mg/g)	-0.0376	0.9929	-6.7563	0.9794
NLC-S (3mg/g)	-0.0256	0.9999	-4.9653	0.9976
NLC-M (1mg/g)	-0.0367	0.9966	-6.5220	0.9869
NLC-M (3mg/g)	-0.0284	0.9960	-5.4448	0.9896
NLC-M (5mg/g)	-0.0139	0.9582	-2.8831	0.9468
NLC-SO (1mg/g)	-0.0398	0.9873	-7.0591	0.9697
NLC-SO (3mg/g)	-0.0277	0.9993	-5.2944	0.9958
NLC-SO (5mg/g)	-0.0113	0.9666	-2.3890	0.9601
NLC-SO (7mg/g)	-0.0092	0.9731	-1.9774	0.9682
NLC-SO (9mg/g)	-0.0094	0.9777	-2.0020	0.9723
NLC-MO (1mg/g)	-0.0409	0.9911	-7.1939	0.9747
NLC-MO (3mg/g)	-0.0268	0.9990	-5.1622	0.9987
NLC-MO (5mg/g)	-0.0126	0.9708	-2.6325	0.9618
NLC-MO (7mg/g)	-0.0094	0.9620	-2.0018	0.9566
NLC-MO (9mg/g)	-0.0100	0.9464	-2.1094	0.9376
ATRA-loaded Polymer-coated NLC				
NLC-M-DPEG (3mg/g)	-0.0200	0.9914	-4.0000	0.9904
NLC-M-PPEG (3mg/g)	-0.0219	0.9961	-4.3144	0.9944
NLC-MO-DPEG (3mg/g)	-0.0181	0.9848	-3.6720	0.9777
NLC-MO-PPEG (3mg/g)	-0.0207	0.9911	-4.1199	0.9929

The Photodegradation half life (t½) of ATRA where 50% of initial concentration of ATRA in the formulations were left were calculated from

$t½ = 0.693 / k_1$ (First order kinetic)

$t½ = [C_0] / 2k_0$ (Zero order kinetic)

where C_0 is the initial concentration of ATRA in formulation at t=0, and k0, k1 are the photodegradation rate constant of zero order- and first order kinetic respectively.

The photodegradation rate constant (k) and the corresponding half life of ATRA and ATRA-loaded lipid nanoparticles at 25±0.5°C under the UV light for 6 h are summarized in Table 10. The lipid nanoparticles formulation was effective in protecting light-induced destabilization of ATRA. The decrease in the intact ATRA content in IPA solution was greatly faster than in lipid emulsion. An initial ATRA concentration of 9 mg/g and 5 mg/g showed the highest intact ATRA content in NLC and LE, respectively, both in IPA and in lipid nanoparticles. Results obtained from this photostability study led us to suppose that the better photoprotection of ATRA-loaded lipid nanoparticles is the consequence of the higher inclusion value of this drug in lipid nanoparticles. The relationships between the photodegradation rate versus the drug concentration has been well documented (Caddeo et al. 2007 : 293, Ioele et al. 2005 : 258, Manconi et al. 2003 : 268, Tan, Meltzer and Lindenbaum 1993 : 817). At the same initial ATRA concentration, ATRA-loaded lipid nanoparticles in different oil phase were not significantly different in the decrease intact ATRA content (Figure 17, 18). These results were in agreement with the previous study which showed that ATRA-loaded nanocapsules improved ATRA photostability, independent of the type of oil phase used in their study (Ourique 2008 : 3). There was no significant difference between type of oil used in polymer coated-NLCs and photo-protective efficacy as well as found in plain-NLCs. These results indicated that the photo-protective property of NLC was not affected by coating of PEG on NLC surfaces (Figure 28).

Table 10 Photodegradation rate and half life (t½) of ATRA in isopropyl alcohol (IPA) solution and ATRA-loaded lipid nanoparticles at 25 ± 0.5°C in the presence of UV light for 6 h (n=3).

Formulation	ATRA remained (mg/g)	Photodegradation rate (x 10^{-3} h^{-1})	Half-life (t½) (h)
ATRA in IPA solution			
ATRA 1 mg/g	0.239	92.82	7.47
ATRA 3 mg/g	1.237	61.49	11.27
ATRA 5 mg/g	2.697	41.19	16.82
ATRA 7 mg/g	4.251	33.69	20.57
ATRA 9 mg/g	5.845	28.31	24.48
ATRA-loaded LE			
LE S 1mg/g	0.534	35.06	19.77
LE M 1mg/g	0.524	32.25	21.49
LE M 1mg/g	2.159	18.80	36.86
LE SO 1mg/g	0.509	6887.55	7.26
LE SO 3mg/g	2.168	3792.14	13.19
LE SO 5mg/g	4.095	11.17	62.04
LE MO 1mg/g	0.541	33.52	20.67
LE MO 3mg/g	2.169	4355.40	11.48
LE MO 5mg/g	4.174	9.89	70.10
ATRA-loaded NLC			
NLC S 1mg/g	0.537	37.63	18.42
NLC S 3mg/g	2.018	25.57	27.10
NLC M 1mg/g	0.510	36.71	18.88
NLC M 3mg/g	1.849	28.42	24.38
NLC M 5mg/g	3.867	13.93	49.74
NLC SO 1mg/g	0.511	39.82	17.40
NLC SO 3mg/g	1.962	27.67	25.04
NLC SO 5mg/g	4.147	11.27	61.48
NLC SO 7mg/g	6.079	9.18	75.47
NLC SO 9mg/g	7.626	9.36	74.01
NLC MO 1mg/g	0.506	40.90	16.94
NLC MO 3mg/g	1.988	26.83	25.83
NLC MO 5mg/g	4.067	12.56	55.16
NLC MO 7mg/g	5.896	9.36	73.84
NLC MO 9mg/g	7.581	9.98	69.43
ATRA-loaded Polymer-coated NLC			
NLC M-DPEG 3mg/g	2.138	20.00	34.65
NLC M-PPEG 3mg/g	2.095	21.88	31.67
NLC MO-DPEG	2.222	18.14	38.21
NLC MO-PPEG	2.136	4119.86	12.14

4. Stability studies

4.1 Physical stability studies

Stability assessment was studied by two methods. First, accelerated test by steam sterilization was used to evaluate the ATRA-loaded lipid nanoparticles. The samples were subjected to autoclave at 121°C, 15 psi for 15 min. Second, ATRA-loaded lipid nanoparticles were kept at 4°C for 56 days. The pH, percentage yield, particle size, size distribution and droplet surface charge (zeta potential, ZP) were also investigated.

The physical properties by visual observation i.e ATRA recrystlliztion and phase seperation are presented in Table 10. No ATRA crystals were found in all lipid nanoparticles under polarized light microscopy after being freshly prepared and after autoclaved so far. After being kept at 4°C for 56 days, the crystallization of ATRA was found only in LE-SO-5 mg/g, LE-MO-5 mg/g, NLC-SO-7 mg/g, NLC-SO-9 mg/g and NLC-MO-9 mg/g, and there was no phase separation in all formulations.

The pH of lipid nanoparticles were markedly decreased after being autoclaved, since the main degradation pathway of the lipid nanoparticles led the formation of free fatty acid which gradually reduced the pH of the lipid nanoparticles. These results indicated that the degradation of lipid nanoparticles was stimulated by temperature. There were no pH change after storage at 4°C for 56 days as comparing with those of the initial was in the range of 7.95-7.98 (Figure 29-31), suggested the good stability of those all lipid nanoparticles under 4°C storage condition.

Table 11 The physical (visual observation) of ATRA- loaded lipid emulsions following autoclaved and storage at 4°C for 56 days.

Formulation \ Condition	Autoclave		4°C for 56 days	
	ATRA recrystallization	Phase separation	ATRA recrystallization	Phase separation
LE				
LE S-1mg/g	-	No	-	No
LE M-1mg/g	-	No	-	No
LE M-3mg/g	-	No	-	No
LE SO-1mg/g	-	No	-	No
LE SO-3mg/g	-	No	-	No
LE SO-5mg/g	-	No	++	No
LE MO-1mg/g	-	No	-	No
LE MO-3mg/g	-	No	-	No
LE MO-5mg/g	-	No	+	No
NLC				
NLC S-1mg/g	-	No	-	No
NLC S-3mg/g	-	No	-	No
NLC M-1mg/g	-	No	-	No
NLC M-3mg/g	-	No	-	No
NLC M-5mg/g	-	No	-	No
NLC SO-1mg/g	-	No	-	No
NLC SO-3mg/g	-	No	-	No
NLC SO-5mg/g	-	No	-	No
NLC SO-7mg/g	-	No	+	No
NLC SO-9mg/g	-	No	+	No
NLC MO-1mg/g	-	No	-	No
NLC MO-3mg/g	-	No	-	No
NLC MO-5mg/g	-	No	-	No
NLC MO-7mg/g	-	No	-	No
NLC MO-9mg/g	-	No	+	No
Polymer coated-NLC				
NLC M-DPEG-3mg/g	-	No	-	No
NLC M-PPEG-3mg/g	-	No	-	No
NLC MO-DPEG-3mg/g	-	No	-	No
NLC MO-PPEG-3mg/g	-	No	-	No

Key : - unchanged

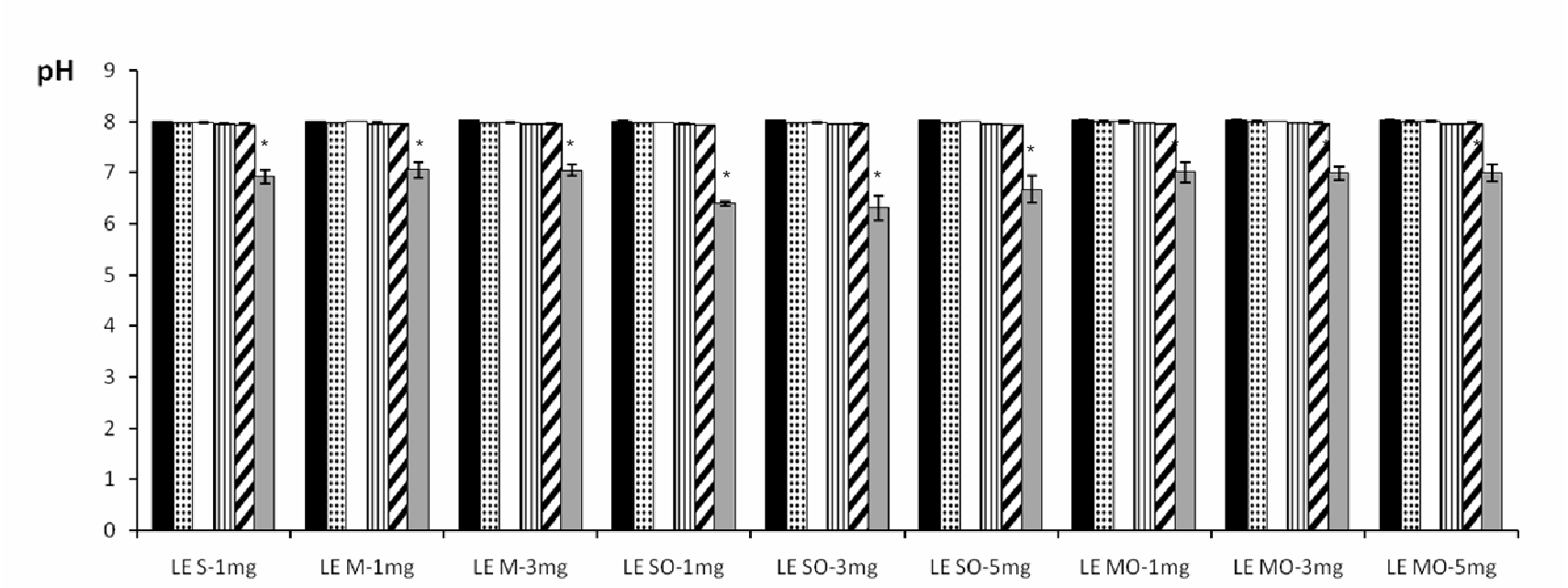


Figure 29 The change in pH of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration.

Keys : ■ pH at the initial ⁞⁞ After 14 days at 4°C □ After 28 days at 4°C

▥ After 42 days at 4°C ▨ After 56 days at 4°C ■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

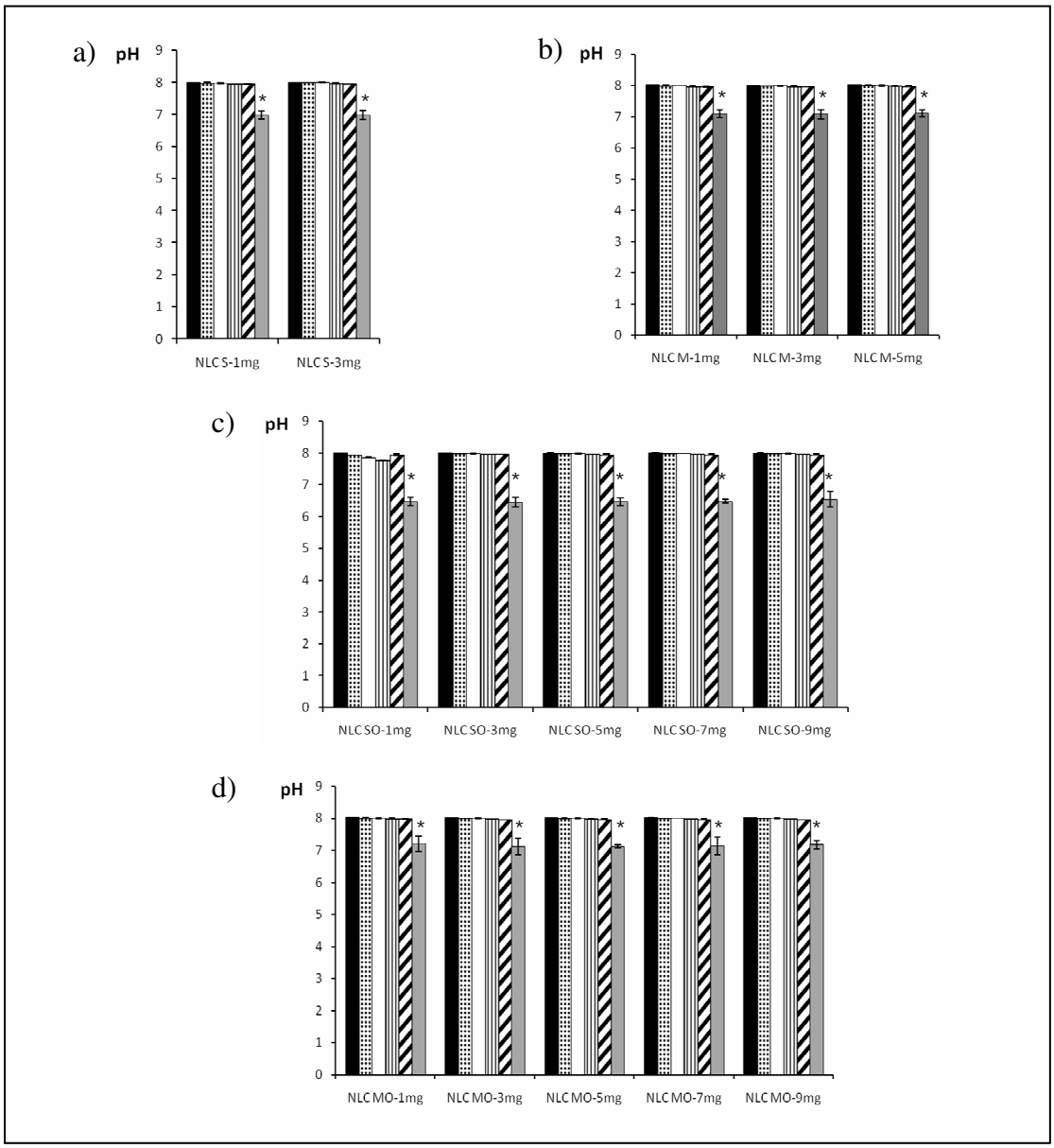


Figure 30 The change in pH of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration; (a)soybean oil (S) ,(b) MCT (M), (c) soybean oil:oleic acid (SO), (d) MCT:oleic acid (MO).

Keys : ■ pH at the initial ⁞⁞ After 14 days at 4°C

□ After 28 days at 4°C ||| After 42 days at 4°C

▨ After 56 days at 4°C ■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

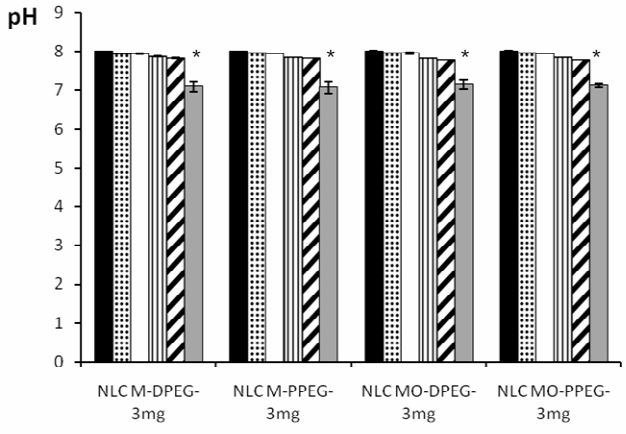


Figure 31 The change in pH of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Keys : ■ pH at the initial

▦ After 14 days at 4°C

□ After 28 days at 4°C

▥ After 42 days at 4°C

▨ After 56 days at 4°C

■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

The chemical stability, as shown in terms of percentage yield of intact ATRA in lipid nanoparticles formulations are presented in Figure 32-34. Since ATRA was readily degraded upon exposure to light and heat, the percentage yield of ATRA was greatly decreased after being autoclaved at 121°C. It was retained about 65.51 – 73.77 %. Whereas the percentage yield of ATRA was slightly decreased during the storage at 4°C. The percentage retained of all preparations was more than 90%. The degradation rate of ATRA kept at 4°C was not significantly different in all formulations. This reveals that the degradation of ATRA is independent of oil phase type.

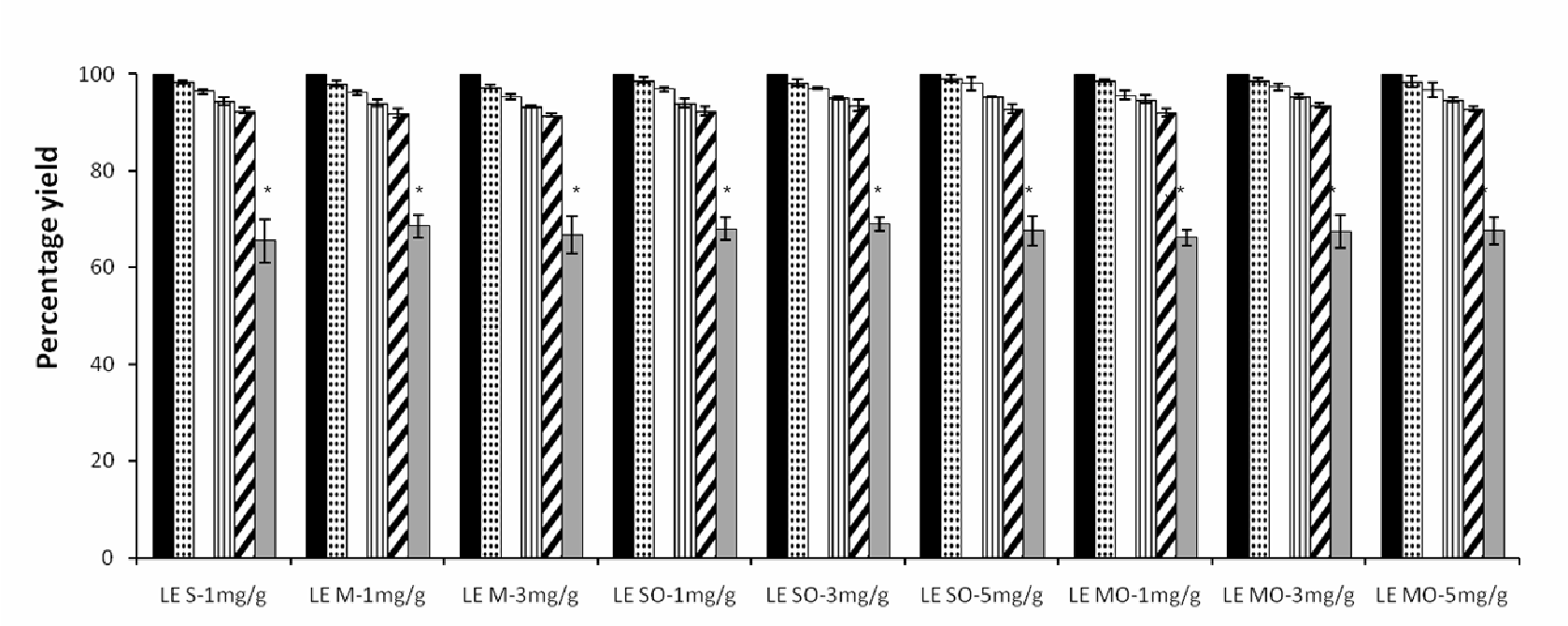


Figure 32 The change in percentage yield of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration.

Keys : ■ Percentage yield at the initial ⁞⁞ After 14 days at 4°C □ After 28 days at 4°C

▥ After 42 days at 4°C ▨ After 56 days at 4°C ■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition

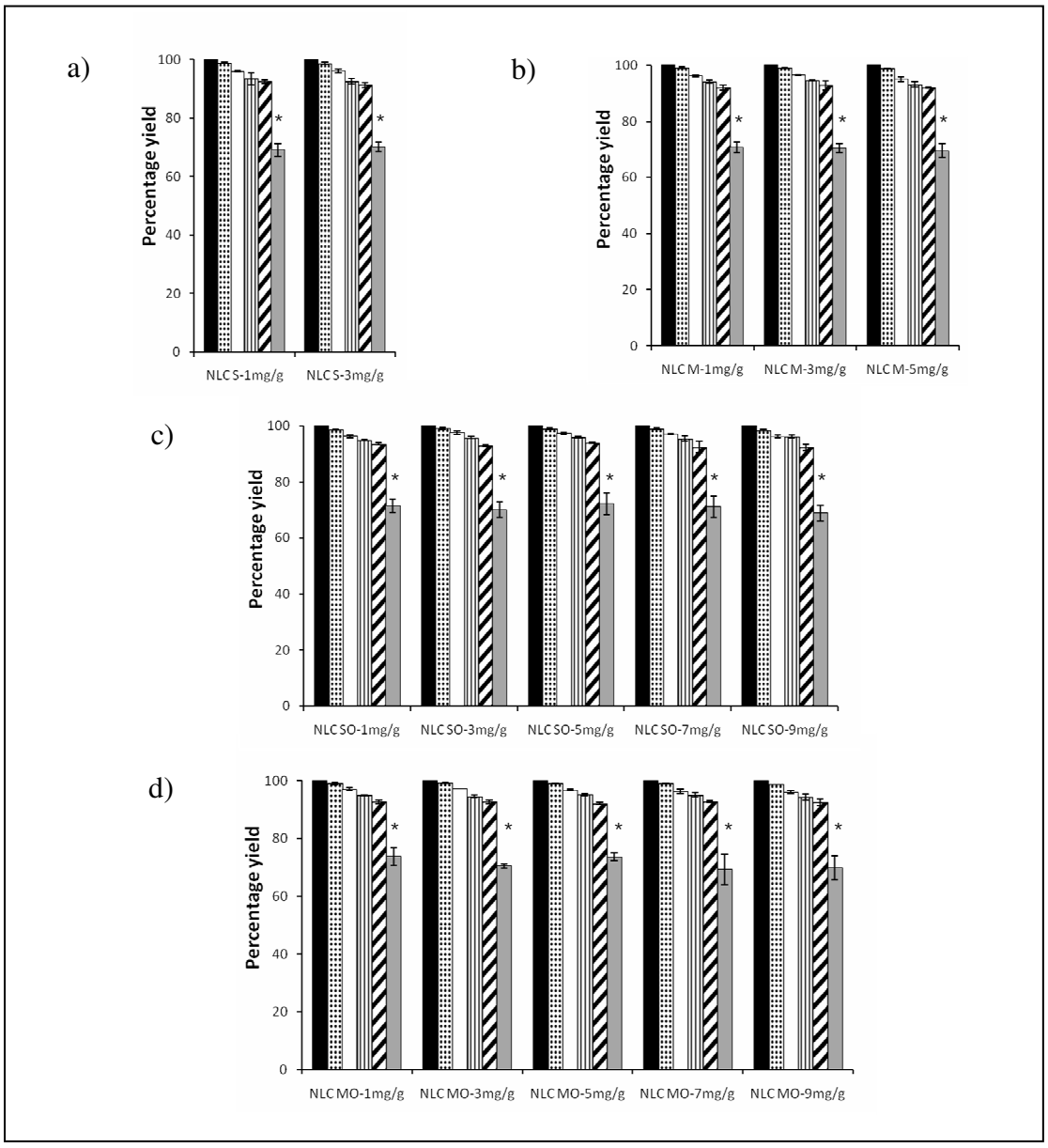


Figure 33 The change in percentage yield of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration; (a)soybean oil (S) ,(b) MCT (M), (c) soybean oil:oleic acid (SO), (d) MCT:oleic acid (MO).

Keys : ■ Percentage yield at the initial ⁞⁞ After 14 days at 4°C

□ After 28 days at 4°C ||| After 42 days at 4°C

▨ After 56 days at 4°C ■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

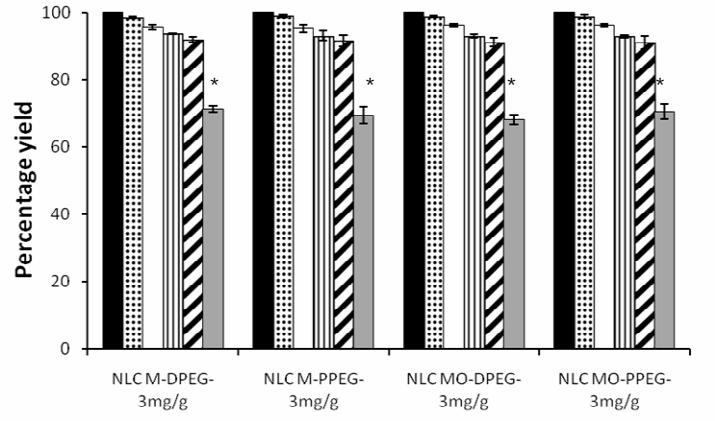


Figure 34 The change in percentage yield of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Keys : ■ Percentage yield at the initial

⁞⁞ After 14 days at 4°C

□ After 28 days at 4°C

▥ After 42 days at 4°C

▨ After 56 days at 4°C

■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

The mean particle size of ATRA-loaded lipid emulsion before and after stability studied by being autoclaved and storage at 4°C is shown in Figure 35-37. From the data obtained, the batches stored at 4°C showed only a slight change in the mean particles size. The size was lower than 200 nm with narrow PDI for 2 months after production (except for LE-S increased to 251.32 nm, which initial particles size was 249 nm) implying that all lipid nanoparticles are physically stable with no flocculation or coalescence of the droplets under this condition. It could be observed that the rate of change in mean particles size tended to be faster when increase the oleic acid content in LE formulation. However, droplet sizes of such lipid nanoparticles containing ATRA were in the acceptable range of parenteral lipid

emulsion. It is generally recognized that applying heat could break down the emulsions. The results demonstrated that 15 minutes of steam sterilization at 121°C greatly affected the mean lipid nanoparticles droplet size. The mean particles size of LEs and NLCs was drastically increased to about 406.95 – 670.53 nm and 341.22 – 497.45 nm, respectively, indicating that the mixed film at the oil-water interface was not altered by exposure to pressure and temperature during sterilization process. However, Levy and Benita have reported that no change in mean droplets size distribution on injectable diazepam submicron-emulsion after 15 min of autoclaving at 121°C (Levy and Benita 1991 : 106). It might be noted that the effect of autoclaving impact on droplets size must be considered case by case. Since the parenteral formulations must be sterilized, thus non-heat sterilization of these ATRA-loaded lipid nanoparticles should be employed.

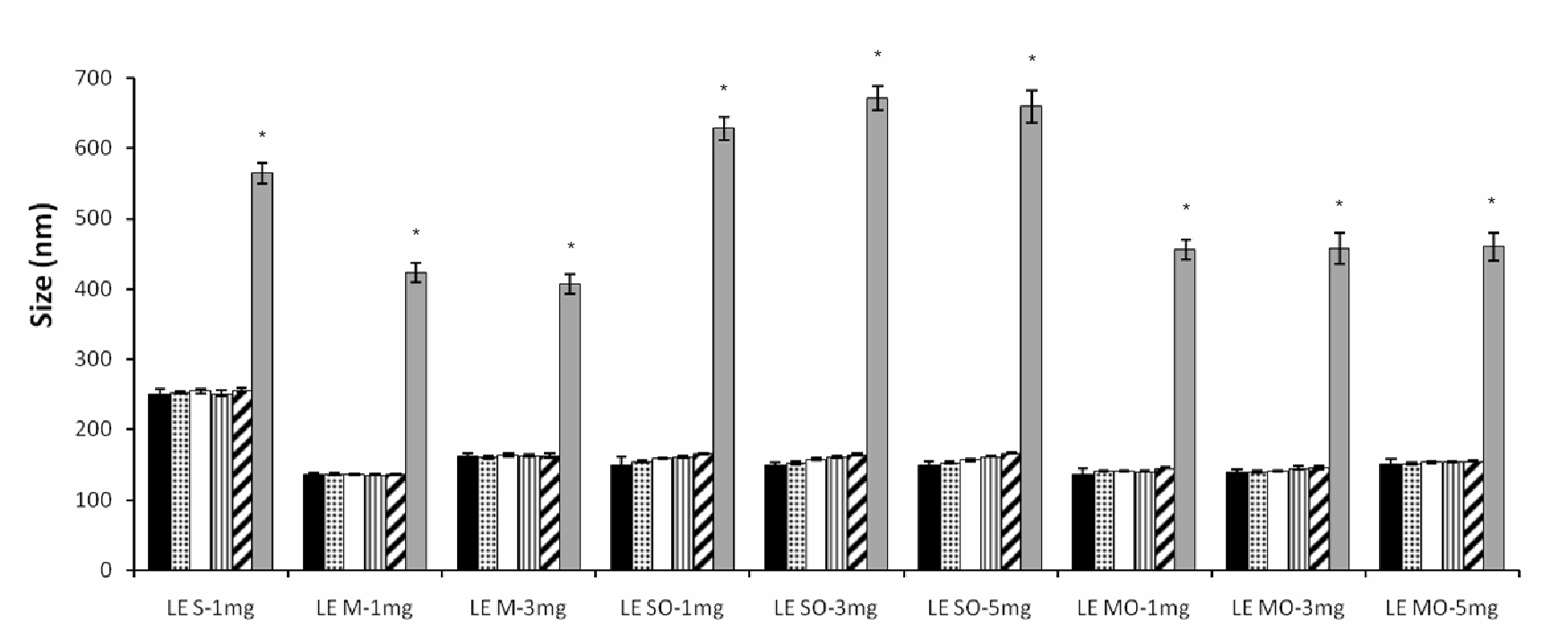


Figure 35 The change in particles size of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration.

Keys : ■ Particles size at the initial ⁞⁞ After 14 days at 4°C □ After 28 days at 4°C

▥ After 42 days at 4°C ▨ After 56 days at 4°C ■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

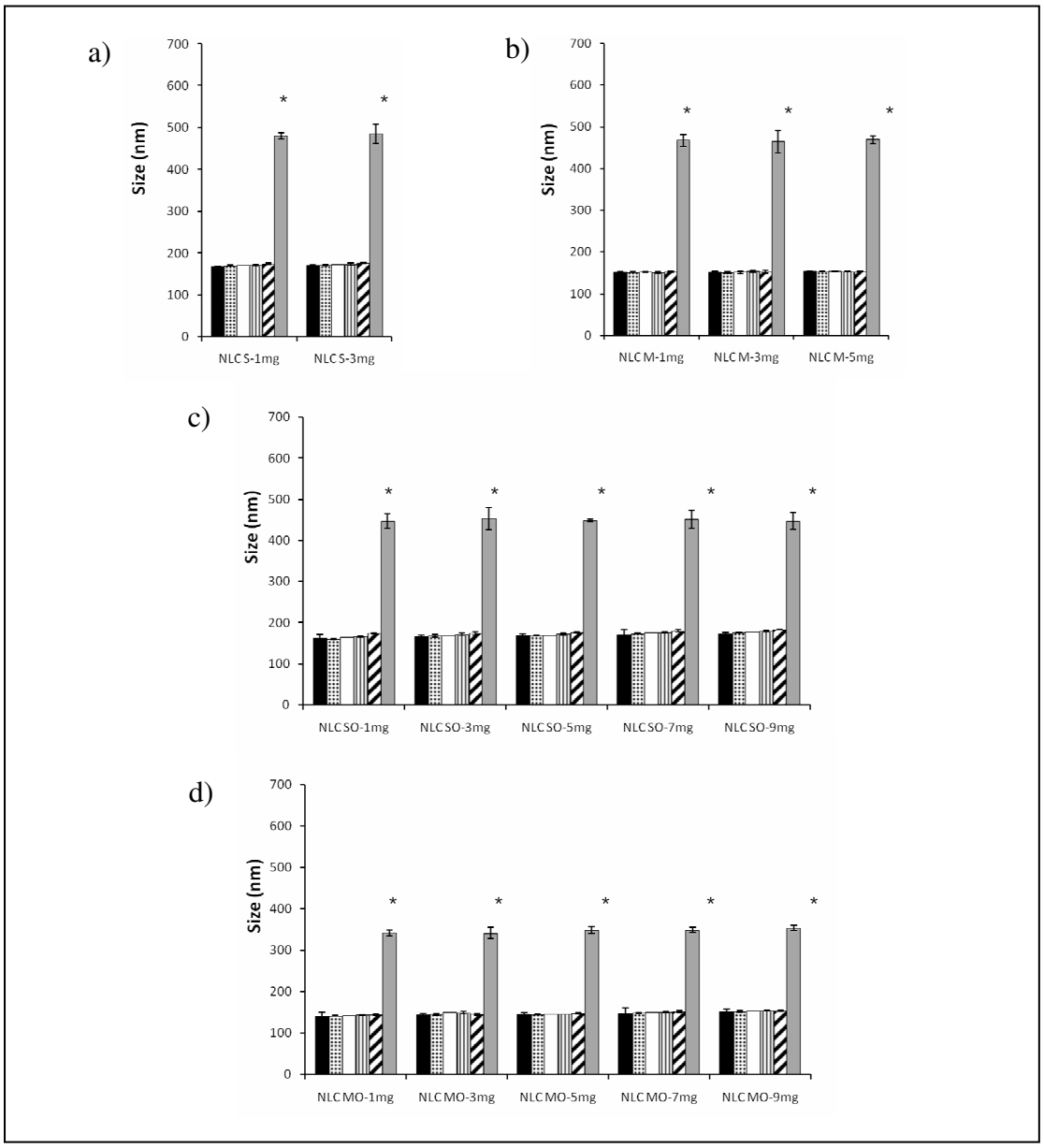


Figure 36 The change in particles size of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration; (a)soybean oil (S) ,(b) MCT (M), (c) soybean oil:oleic acid (SO), (d) MCT:oleic acid (MO).

Keys : ■ Particles size at the initial ▦ After 14 days at 4°C
□ After 28 days at 4°C ▥ After 42 days at 4°C
▨ After 56 days at 4°C ■ After autoclaved
* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

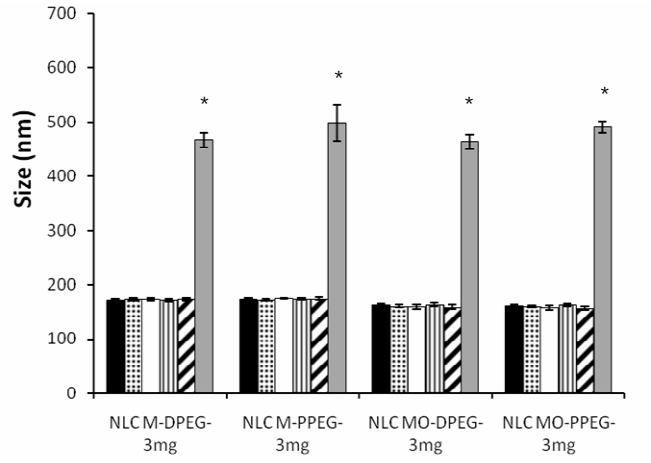


Figure 37 The change in particles size of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Keys : ■ Particles size at the initial

▦ After 14 days at 4°C

□ After 28 days at 4°C

▥ After 42 days at 4°C

▨ After 56 days at 4°C

■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

The zeta potential of lipid nanoparticles before and after stability studied by being autoclaved and storage at 4°C is shown in Figure 38-40, no significant changes in zeta potential value for all lipid nanoparticles formulations was found in both after being autoclaved and 56 days of storage at 4°C.

Taken the physical and chemical stability results together, it is clear that good physical stability of lipid nanoparticles could be obtained especially in NLC composed of MCT. Furthermore, the use of hydrophilic polymers (DPEG, PPEG) purposed for extension of the blood circulation lifetime did not affect the stability of NLC formulation.

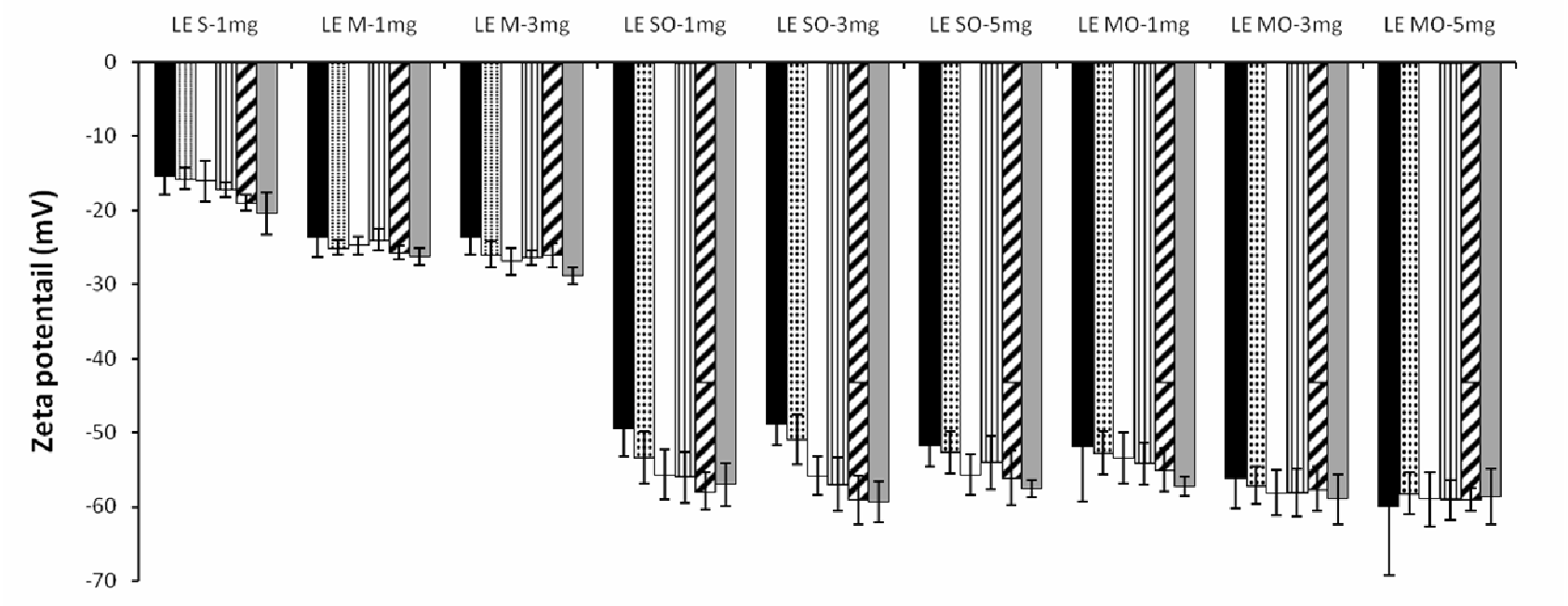


Figure 38 The change in zeta potential of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration.

Keys : ■ Zeta potential at the initial ▦ After 14 days at 4°C □ After 28 days at 4°C
▥ After 42 days at 4°C ▨ After 56 days at 4°C ■ After autoclaved

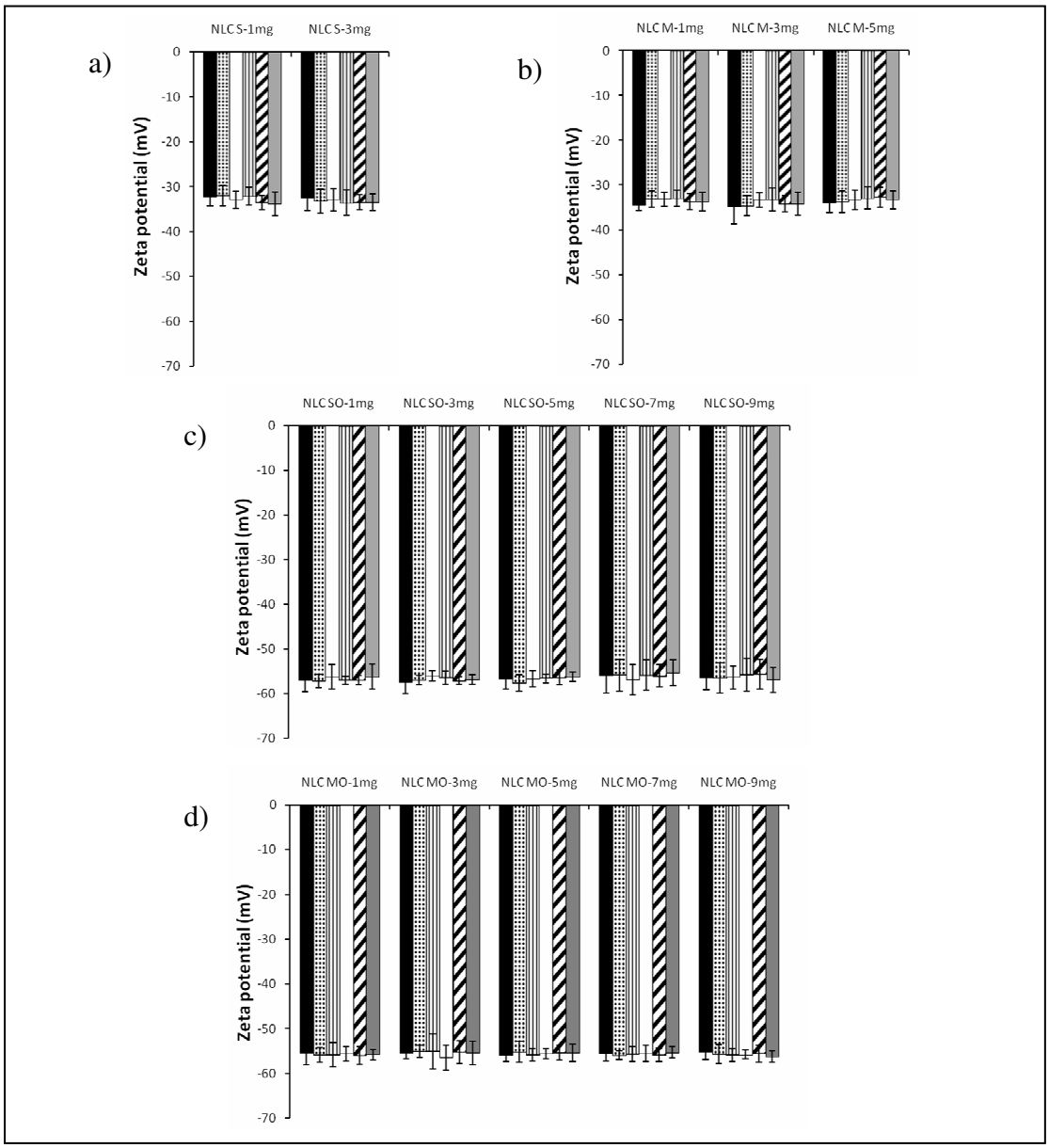


Figure 39 The change in zeta potential of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration; (a)soybean oil (S) ,(b) MCT (M), (c) soybean oil:oleic acid (SO), (d) MCT:oleic acid (MO).

Keys : ■ Zeta potential at the initial ⁞⁞ After 14 days at 4°C

□ After 28 days at 4°C ||| After 42 days at 4°C

▨ After 56 days at 4°C ■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

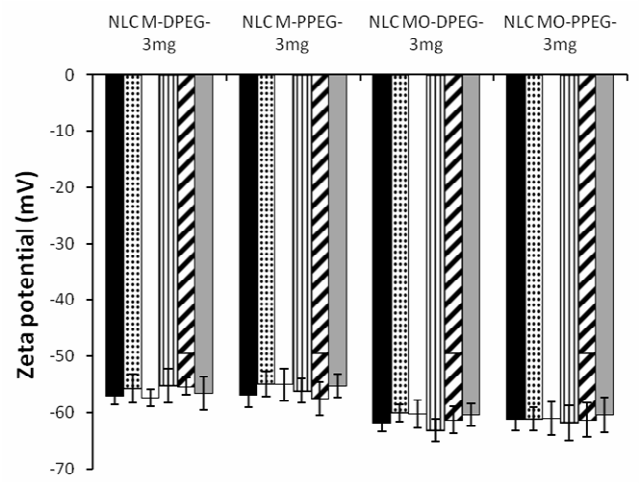


Figure 40 The change in zeta potential of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Keys : ■ Zeta potential at the initial

▦ After 14 days at 4°C

□ After 28 days at 4°C

▥ After 42 days at 4°C

▨ After 56 days at 4°C

■ After autoclaved

* Significantly different (p=0.05) when compared with after 56 days at 4°C storage condition.

5. Cytotoxicity assay

During the preparation of lipid nanoparticles, ATRA was exposed to the heating process. To study whether the anticancer efficacy of ATRA could be still remained after processing, the antiproliferative effects of ATRA on human carcinoma cell lines, acute promyelocytic leukemia cells (HL-60), human hepatoma cells (HepG2) were investigated by MTT assay. Twelve ATRA-lipid nanoparticles formulations i.e. ATRA-loaded LEs (S, M, SO, MO), ATRA-loaded NLCs (S, M, SO, MO), and ATRA-loaded NLCs with DPEG or PPEG (M, MO) were examined.

The survival curves of HL-60 cells (Figure 41) and HepG2 cells (Figure 42) after exposure to ATRA, either in solution or in lipid nanoparticles showed that the growth inhibitory effects of ATRA were significantly different between free ATRA and ATRA-loaded lipid nanoparticles. The activity of ATRA-loaded lipid nanoparticles on HL-60 and HepG2 cells was greater than that of free ATRA. Between two cell lines, HL-60 was more sensitive to ATRA free drug than HepG2. ATRA could inhibit the proliferation of HL-60 and HepG2 cell lines in a dose dependent manner in the range of 1-2,500 ng/ml and 0.1-50 µg/ml, respectively. The results revealed that the HL-60 cells was more sensitive to ATRA than the HepG2 cells. Table 11 reports the growth inhibitory concentrations of ATRA-loaded LE, -NLC, and –solution on the two cell lines. The amount of ATRA required to achieve 50% of growth inhibition (IC_{50}) was much lower in ATRA-loaded lipid nanoparticles than in solution. Lipid nanoparticles enhanced the cytotoxicity of ATRA approximately 7.00-2.07-fold on HL-60 cell and 44.71-184.38-fold on HepG2 compared with ATRA solution. From Figure 41 and 42, It could be observed that ATRA-loaded lipid nanoparticles particularly in formulation with oleic acid could significantly decrease viability of HepG2 cells. This result indicated that the nature of the lipid matrix affected cell viability.

The observation is in agreement with many researchers who investigated the incorporation of ATRA in various colloidal carriers such as microemulsion, solid lipid nanoparticles (SLN), liposomes. Lim and coworkers carried out the antiproliferative effects of SLN powder formulation tested on HL-60, MCF-7 and KB. They found that the antiproliferative in the cells were not significantly different between SLN powder formulation and free ATRA (Lim et at. 2004 : 58). Kawakami and co-workers reported that incorporation of ATRA into cationic liposomes composed of DOTAP/cholesterol showed much higher toxicity effects on A594 cells and apoptosis inducing activity compared with free drug or ATRA incorporated in DSPC/cholesterol liposomes (Kawakami et al. 2006 : 517). Hwang and coworkers found that ATRA loaded in phospholipid-based microemulsion provided similar growth inhibitory effects on HL-60 and MCF-7 cell lines to that free ATRA (Hwang et al. 2004 : 181).

The increase of ATRA cytotoxicity or at least non-changed in anticancer activity when compared to the free drug solution has already been reported as described above. These data suggested that the anticancer activity of ATRA was not impaired by the lipid nanoparticles incorporation and heating process, ATRA might be released from colloidal system outside or inside of cells, and thus, binding to retinoic acid receptors and retinoid X receptors on the nuclear membrane of cells (Hwang et al. 2004 : 182).

Table 12 Inhibitory concentration of ATRA producing 50% of cell inhibition or dead.

Formulation	IC_{50}	
	HL-60 (ng/ml)	HepG2 (µg/ml)
Free drug		
ATRA	921.37	342.94
LE		
LE S-1mg/g	415.10	7.00
LE M-3mg/g	242.30	1.86
LE SO-3mg/g	390.21	5.27
LE MO-3mg/g	131.54	1.90
NLC		
NLC S-3mg/g	445.31	5.86
NLC M-3mg/g	331.08	7.67
NLC SO-3mg/g	389.00	2.93
NLC MO-3mg/g	371.04	2.48
Polymer coated-NLC		
NLC M-DPEG-3mg/g	290.45	4.34
NLC M-PPEG-3mg/g	177.18	5.09
NLC MO-DPEG-3mg/g	401.95	2.22
NLC MO-PPEG-3mg/g	309.64	1.89

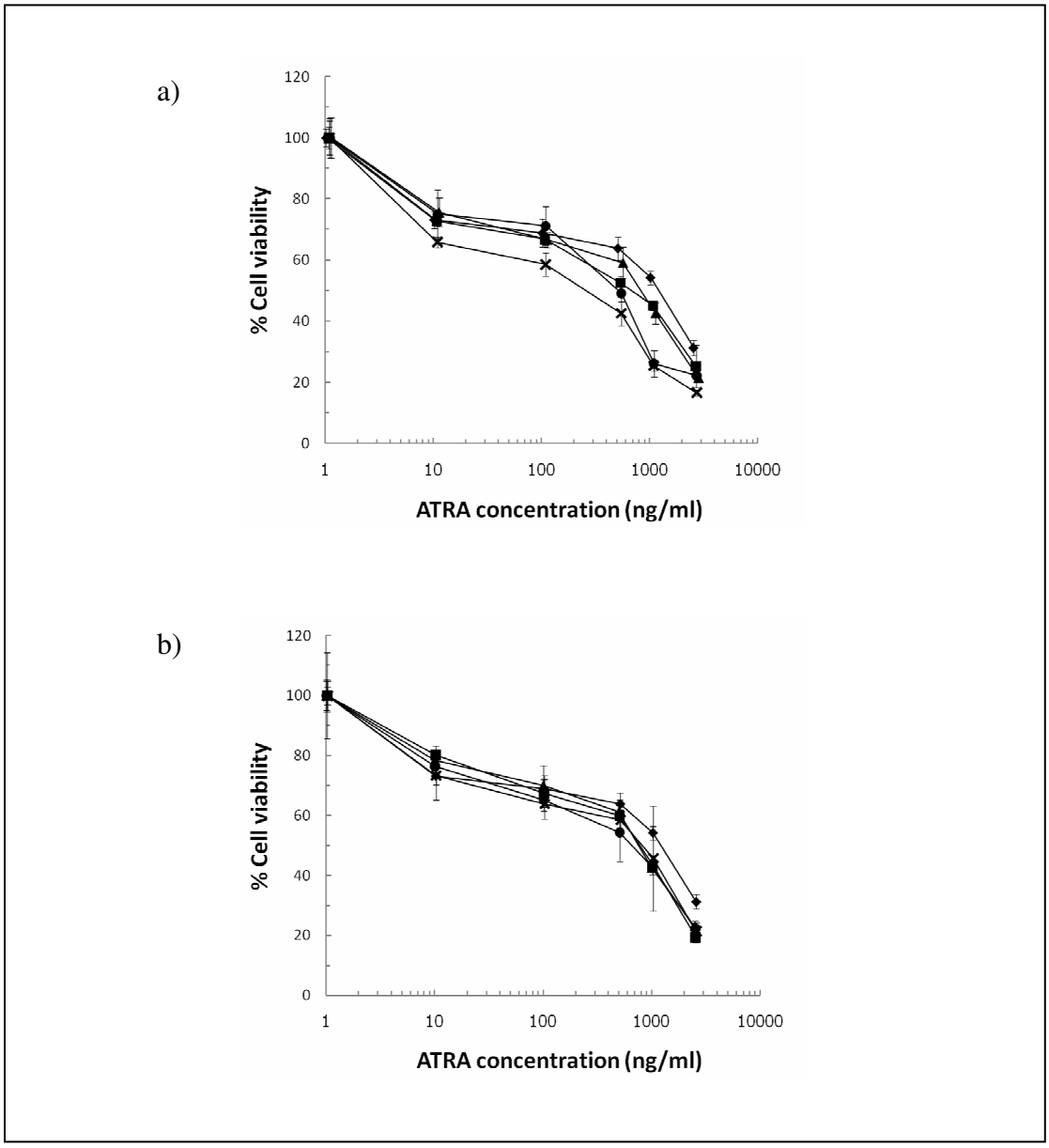


Figure 41 HL-60 Cytotoxicity of ATRA-loaded lipid nanoparticles composed of different oil phase compared with free ATRA; (a) ATRA-LE (b) ATRA-NLC.

Keys : ▲ Soybean oil (S)

● MCT (M)

■ Soybean oil:oleic acid (SO)

x MCT:oleic acid (MO)

♦ Free ATRA

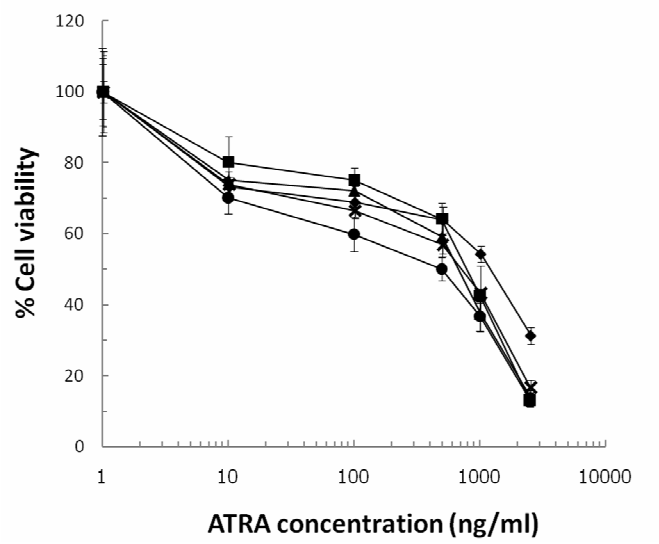


Figure 42 HL-60 Cytotoxicity of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration, compared with free ATRA.

Keys : ▲ ATRA-NLC-M-DPEG

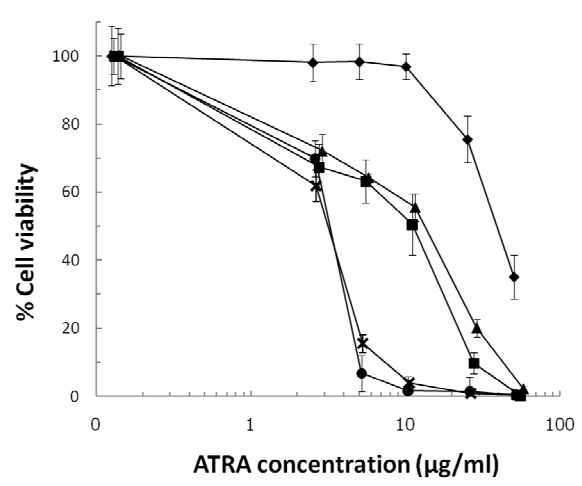
● ATRA-NLC-MO-DPEG

■ ATRA-NLC-M-PPEG

x ATRA-NLC-MO-PPEG

♦ Free ATRA

a)



b)

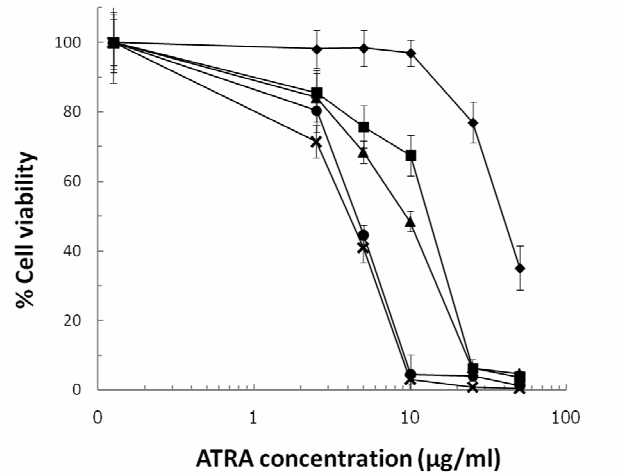


Figure 43 HepG2 Cytotoxicity of ATRA-loaded lipid nanoparticles composed of different oil phase compared with free ATRA; (a) ATRA-LE (b) ATRA-NLC.

Keys : ▲ Soybean oil (S)

● MCT (M)

■ Soybean oil:oleic acid (SO)

x MCT:oleic acid (MO)

♦ Free ATRA

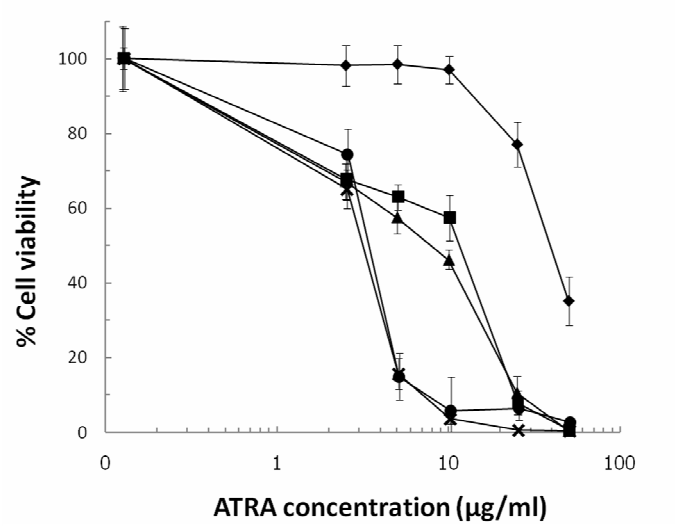


Figure 44 HepG2 cytotoxicity of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration, compared free ATRA.

Keys : ▲ ATRA-NLC-M-DPEG

● ATRA-NLC-MO-DPEG

■ ATRA-NLC-M-PPEG

x ATRA-NLC-MO-PPEG

♦ Free ATRA

CHAPTER 5
CONCLUSIONS

The lipid nanoparticles were successfully developed for delivery of anticancer drug, all-trans retinoic acid (ATRA). Three types of lipid nanoparticles including lipid emulsion (LE), nanostructured lipid carrier (NLC), and polymer coated nanostructured lipid carrier (polymer coated-NLC) were prepared by De novo emulsification method, and the lipid droplets size was reduced to the nanometer range by ultrasonication technique. Physicochemical characterization including particles size, polydispersity index (PDI), and zeta potential (ZP), stability study and *in vitro* drug release as well as physicochemical stability after being autoclaved and after stored at 4°C for 56 days were examined. Moreover, the anticancer efficacy of ATRA-loaded lipid nanoparticles on human acute promyelocytic leukemia cells (HL-60) and human hepatoma cells (HepG2) were also studied. The results of this study could be concluded as follow:

1. ATRA loaded-lipid emulsion (LE)

ATRA-loaded LEs was composed of 30% liquid lipid, 1.2% lecithin, 0.002% BHT, 8% polysorbate-80. The liquid lipid were varying from soybean oil (S), medium chain triglyceride (MCT, M), mixture of S and oleic acid (3:1); SO, and mixture of M and oleic acid (3:1); MO. The type of the oils and initial ATRA added affected the loading capacity of ATRA in LE. As the initial ATRA concentration from 1, 3, 5, 7 and 9 mg/g were performed, the incorporation efficiency of ATRA loaded of the formula S, M, SO and MO was higher than 80 % with 1, 3, 5 and 5 mg/g of the initial ATRA, respectively, indicating that oleic acid enhanced loading capacity of ATRA in LE.

These lipid emulsions had average sizes less than 200 nm (138.11 to 172.69 nm) with narrow PDI, and negative zeta potential, except for pure soybean oil

which showed slightly larger mean particle size (249 nm). The ZP values of formulated with S, and MCT gave -15.39 and -23.74 mV, respectively, which was slightly lower than the appropriate value of parenteral emulsions. LE formulated with oleic acid exhibited higher negative charge (-51.83 to -60.07 mV), indicating that oleic acid not only affects the incorporation efficiency but also stability of ATRA-loaded lipid nanoparticles.

In vitro drug release kinetics across a dialysis bag followed zero-order kinetics. The release of ATRA from loaded-LE formulations were about 1.60-11.38% at 48 h, indicating that lipid emulsions could be carriers for controlling the release of ATRA. ATRA was gradually released from emulsions during 48 h. The percent increase of the ATRA release was in the order of MO > SO > M > S. The higher release rate was observed in an oleic acid-contained formula (MO and SO), suggesting that the release of ATRA from LE was not affected by the initial drug concentration but by the type of oil.

The LE formulations were effective in protecting ATRA under irradiation of UVA light for 6 h. The ATRA content in all formulations of lipid emulsion was significantly higher than that in IPA solutions. The protective effect increased with the increase of ATRA concentration in formulation. The initial ATRA concentration of 5 mg/g showed the highest intact ATRA content in LE thus the highest photo-protective efficacy. There were no significant difference between type of oil used in LEs and photo-protective efficacy.

These LEs possessed good physicochemical stability as no pH change after storage at 4°C for 56 days and no ATRA crystals were found in all formulations both after being freshly prepared and after autoclaving. After being kept at 4°C for 56 days, the crystallization of ATRA was found only in LE-SO-5 mg/g, and LE-MO-5 mg/g, but there was no phase separation in all formulations. The percentage yield of ATRA was remained more than 90% during the storage at 4°C. These results reveals the good stability of those all LEs under 4°C storage condition.

The cytotoxicity of ATRA loaded-LE on human carcinoma cell lines, acute promyelocytic leukemia cells (HL-60) and human hepatoma cells (HepG2)

assess by MTT assay showed that HepG2 cells was more sensitive to ATRA than the HL-60 cells. ATRA loaded-LEs could inhibit the proliferation of HL-60 and HepG2 cell lines with a dose dependent approach in the range of 1-2,500 ng/ml and 0.1-50 µg/ml, respectively, and formulations with oleic acid gave significantly decrease viability of HepG2 cells compared with other formulations. All ATRA loaded-LEs had higher cytotoxicity than the free drug solution.

2. ATRA loaded-nanostructured lipid carriers (NLC)

In order to screen the suitable ratio of liquid lipid to solid lipid in oil phase of NLC, incorporation efficiency of ATRA loaded-NLC was examined by varying the proportion of liquid lipid (S, M, SO, MO) : solid lipid (CP) i.e. 5:1, 3:3, 1:5. The ratio M:CP (3:3) gave much more loading capacity and possessed good physical appearance. Hence, liquid lipids with the equal ratio of solid lipid (S:CP, M:CP, SO:CP and MO:CP = 1:1) were chosen to be oil phase for the NLCs formulation and characterization.

The loading capacity of initial ATRA concentration from 1, 3, 5, 7, 9 and 11 mg/g was examined. NLCs formulation showed advantages on ATRA loading capacity over LE from the percentage yield of ATRA loaded of the formula S:CP, M:CP, SO:CP and MO:CP was higher than 80 % with 3, 5, 9 and 9 mg/g of the initial ATRA, respectively. These results indicated that oleic acid affected the ATRA loading capacity in LE. The greatly enhanced in loading capacity of ATRA into NLC with the addition of oleic acid in the formulation was explored in the present study.

ATRA loaded-NLC had average sizes less than 200 nm (141.80 to 172.95 nm) with narrow PDI, and negative zeta potential which was in an acceptable range of iv-injection. The ZP values of NLC formulated with pure oil, S and M was about -32.23 to -34.92 mV, respectively. In the same direction of LEs, NLC formulated with O gave NLC a higher negative charge (-55.23 to -57.38 mV).

In contrast with LE, *in vitro* ATRA release kinetics across a dialysis bag of NLCs was only about 4.06 to 4.34% and no significant difference in ATRA release rate from all NLCs in accordance with composition of oil phase, implied that the

release of ATRA from LE affected neither by initial drug concentration nor the type of oil. However, these results supported that ATRA incorporated into both LEs and NLCs was more favorable to persist in the lipid nanoparticles systems. Moreover, no burst release of drug was found, indicating that lipid nanoparticles could prolong the release of ATRA.

The photo-protective property of NLC also exhibited as same as of those LEs. When compared with ATRA in IPA solution in the same initial drug concentration (1, 3, 5, 7 and 9 mg/g of initial ATRA concentration), the decrease in the drug content in IPA solution was significantly faster than in NLC. An initial ATRA concentration of 9 mg/g showed the highest intact ATRA content in NLC and no significant difference between type of oil used in LEs and photo-protective efficacy. The results obtained from NLC contributed that the higher the incorporation amount of ATRA in lipid nanoparticles, the better photo-protection of ATRA-loaded lipid nanoparticles.

From physicochemical stability studies, no ATRA crystals were found in all ATRA loaded-NLC formulations after being freshly prepared and after being autoclaved. But after being stored in 4°C for 56 days, the crystallization of ATRA was found only in oleic-NLC with high loaded ATRA concentration i.e. NLC-SO-7 mg/g, NLC-SO-9 mg/g and NLC-MO-9 mg/g. There was no phase separation in all formulations in all conditions. The percentage yield of ATRA was remained more than 90% during the storage at 4°C. All results exhibited good stability of those all NLCs under 4°C storage condition similar to LEs.

There is no significant difference between the cytotoxicity effect of ATRA loaded-NLC as compared with LE. Both human carcinoma cell lines, the growth of HL-60 and HepG2 cells were inhibited with a dose dependent approach in the range of 1-2,500 ng/ml and 0.1-50 µg/ml, respectively, in the same pattern with those LEs. Therefore, it is verified that the production process of these nanolipid carriers did not change the anticancer efficacy of ATRA, but, could also rose up thus activity.

3. ATRA loaded in polymer coated-nanostructured lipid carriers (polymer coated-NLC)

Since, modification of nanoparticles surfaces by hydrophilic polymer coating such as PEG could prevent opsonization and thus prolonged the circulation of nanoparticles in blood stream leading to drug targeting by EPR effect. In this study, DSPE-PEG_{2000}) (DPEG) and PLC-g-mPEG with 95% degree of deacetylation (PPEG) were used for coating of the lipid nanoparticles droplets.

LE formulation composed with pure soybean oil gave bigger particle sizes and less negative ZP, although this was in acceptable range but demonstrated the less physical stability trend of the system when compared with MCT or mixture of MCT and oleic acid. The recrystallization of ATRA was detected in LE- but not in NLC formulations consideration on the same type of oil and the same ATRA loaded concentration (SO and MO at 5mg/g ATRA concentration). These results revealed that NLC possessed better physical stability than LE. Moreover, higher loading capacity of ATRA was achieved with NLC. For this reason, NLCs consisting of two types of oil matrix, M:CP (1:1) and MO:CP (1:1) at 3 mg/g concentration were selected in addition polymer coated studied.

The loading capacity of initial ATRA concentration from 5, 7 and 9, 11 mg/g was examined for polymer coated-NLC with M- and MO-oil matrix respectively. It was found that the ATRA loading capacity was not significantly different between the formulation presence of polymers, DPEG and PPEG, and the uncoated-NLCs.

The presence of DPEG and PPEG polymer increased the particle sizes of NLC. The particles sizes of PPEG-coated–NLCs were increased (160.39-172.69 nm) when compared with uncoated NLCs formulation, suggested the formation of coating layer on the surfaces of such lipid nanoparticles. The presence of DPEG and PPEG polymer dramatically affected the zeta potential of NLC formulated with MCT from -34.92 mV to -57.02 and -56.92 respectively.

The drug release profiles from polymer coated-NLC formulations were sustained over 48 h. No significant difference in ATRA release rate from DPEG- and PPEG coated NLCs was found as compared with uncoated M- and MO-NLC.

The photo-protective efficacy of polymer coated-NLC was not different as compared with NLC. No significant difference between type of oil used in polymer coated-NLCs and photo-protective efficacy was found. No ATRA crystals were found after being freshly prepared, autoclaved and stored in 4°C for 56 days, either DPEG- or PPEG-coated-NLC formulations. Phase separation was also not found in all conditions. The percentage yield of ATRA was remained more than 90% during the storage at 4°C. All results demonstrated the good stability of those polymer coated-NLCs under 4°C storage condition and ordinary NLCs.

There are no significant difference between the cytotoxicity effect of DPEG- coated or PPEG coated-NLC in accordance with NLC with the same types of oil. Therefore, the application of DPEG or PPEG on NLC surfaces proposing did not affect anticancer efficacy of ATRA as compared with those NLCs.

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APPENDIX

APPENDIX A

Validation characteristics for analysis of ATRA

1. System suitability tests

1.1. Tailing factor (Asymmetry factor)

Tailing factor, *T*, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. This test was performed by collecting data from replicate injections of standard solution. The test was determined by the equation

$$T = \frac{W_{0.05}}{2f}$$

In which $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

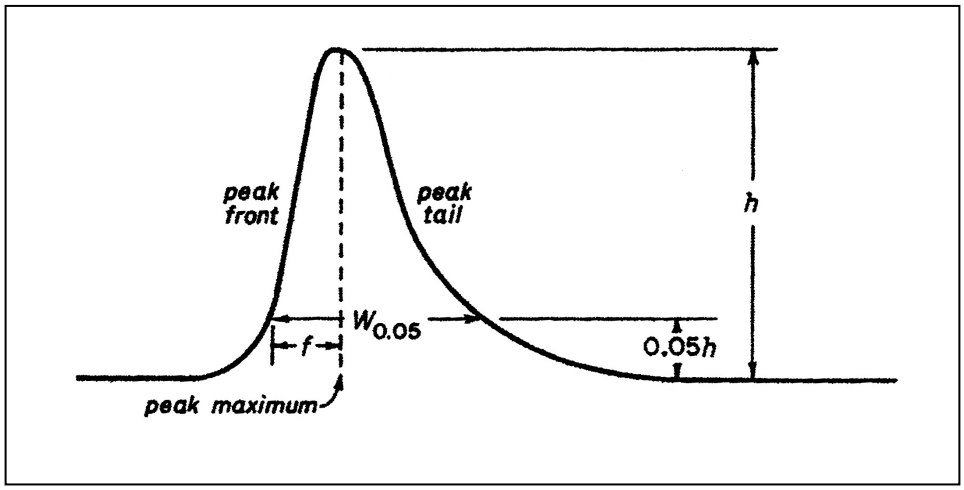


Figure 45 Asymetrical chromatographic peak

Source: The United States Pharmacopeial Convention, The United States Pharmacopeia 30th revision (2007) : 252.

Tailing factor, *T*, should be close to 1, not less than 0.5 and not more than 2. The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak.

1.2. Resolution

The separation of two components in the mixture, the resolution, *R*, was determined by the following equation

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

In which t_2 and t_1 are the retention times of the two components, and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline as shown in the following figure:

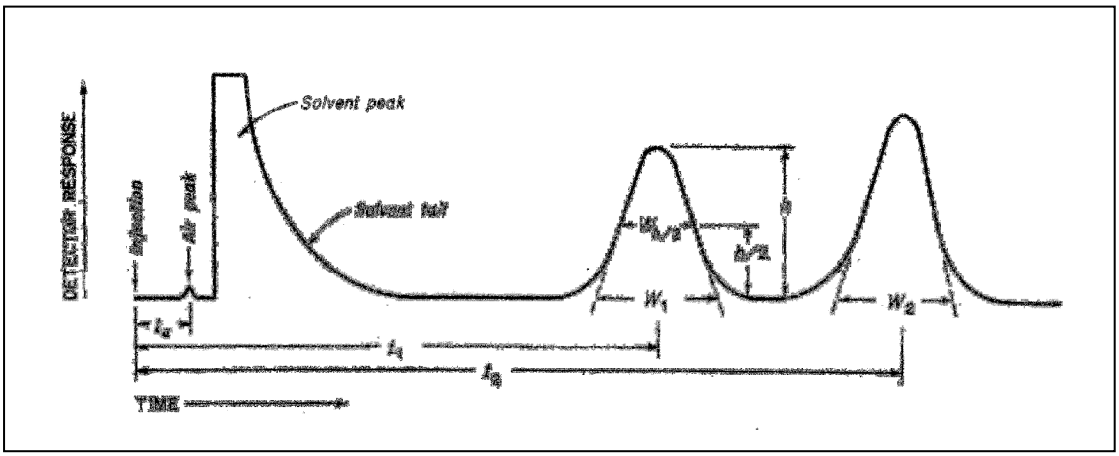


Figure 46 Chromatographic separation of two substances

Source: The United States Pharmacopeial Convention, The United States Pharmacopeia 30th revision (2007) : 252.

Resolution, *R*, should be more than 2 for reliable quantitation.

1.3. Repeatability

Repeatability expresses the precision under the same operating condition. The repeatability is displayed as the %RSD and determined by multiple injections of a homogeneous sample under the analytical conditions.

2. Accuracy

Accuracy is the measure of how to close the experimental value is to the true value. Accuracy study was performed by the addition of know amounts of drug by weight or volume (dissolved in diluent) to the placebo formulation (soybean oil medium, MCT medium and lipid emulsion base), called “spiked sample” (a sample to which a known amount of the analyte has been deliberately added). The mixtures were subsequently analyzed by HPLC method in which previously described. The percentage recovery (%R) is calculated as follows:

$$\%R = [(CS-CU)/CA] \times 100$$

Where CS is the concentration of drug measured in the spiked sample; CU is the concentration of drug measured in the unspiked sample; CA is the concentration of drug added (measured value, not determined by method) in spiked sample.

3. Precision

The precision was determined by analyzing three sets of the five standard solutions of model drugs. Peak area ratios of model drugs to the corresponding internal standards were compared and the percentage coefficient of variation (%CV) for each concentration was determined.

4. Linearity

Linearity was determined by calculating a regression line by method of least squares of area under the curve and concentration of model drugs. The coefficient of determination (R^2) were performed, under most circumstances, R^2 is ≥ 0.999. Intercept and slope were indicated.

5. Limit of detection

The limit of detection (LOD) is the lowest concentration in a sample that can be determined, not quantified. It is expressed as a concentration at a specified signal:noise ratio, usually 3:1. LOD may also be calculated based on the standard deviation of the response and the slope of the calibration curve at levels approximating, the LOD according to the formula:

$$LOD = 3.3(SD/S)$$

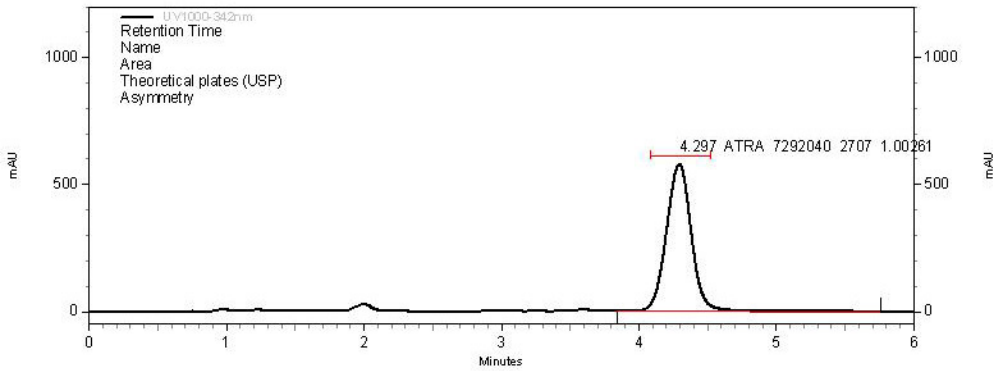
Where SD is the standard deviation of the response; S is the slope of the calibration curve.

6. Limit of quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal:noise ratio 10:1. LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve(s) at levels approximating ,the LOQ according to the formula:

$$LOQ = 10(SD/S)$$

Where SD is the standard deviation of the response; S is the slope of the calibration curve.



UV1000-342nm Results (System (1/6/2006 4:52:30 PM) (Reprocessed))

Name	Retention Time	Area	Height	Asymmetry	Theoretical plates (USP)	Plates / meter (USP)
ATRA	4.297	7292040	580185	1.00261	2707	18048.28
Totals						
		7292040	580185			

Figure 47 Tailing factor of assay method of ATRA.

The tailing factor (asymmetry factor) should be less than 2.0. to indicated that peak symmetry was acceptable and hence precision became reliable.

Table 13 The repeatability of assay method of ATRA.

Injection No.	AUC
1	7209269
2	7205241
3	7211422
4	7207557
5	7208474
6	7211155
7	7210442
8	7210568
9	7209137
10	7206677
Average	7208994.2
%RSD	0.03

%RSD should be less than 2.0 for ensure that method used were precise for quantitative analysis in the study

Table 14 The accuracy of assay method of ATRA.

ATRA Conc. (g)	IPA Vol. (ml)	ATRA Conc. (µg/ml)	AUC	Average	%RSD	Conc. Yield (µg/ml)	% Recovery
0.0001016	100	1.02	138853	138441.67	0.34	1.01	99.08
			137926				
			138546				
0.0001016	10	10.16	1115536	1116334.33	0.31	10.15	99.89
			1113343				
			1120124				
0.000254	10	25.40	2750131	2752112.67	0.32	25.44	100.17
			2761823				
			2744384				
0.00127	25	50.80	5472109	5469896.00	0.04	50.85	100.10
			5469856				
			5467723				
0.000762	10	76.20	8165886	8171691.00	0.23	76.11	99.88
			8192754				
			8156433				
0.00254	25	101.60	10892672	10879883.67	0.12	101.43	99.83
			10880363				
			10866616				

% Recovery should be close to 100% for ensure that the experimental value is to the true value

Table 15 The precision of assay method of ATRA.

Average AUC	Calculated concentration from calibration curve (µg/ml)					
	No.1	No.2	No.3	Average	SD	%RSD
122791.67	0.84	0.83	0.86	0.85	0.01	1.62
1218503.67	10.75	10.98	11.10	10.95	0.18	1.63
3011116.00	26.97	27.59	27.86	27.48	0.46	1.66
5914116.00	53.24	54.49	55.00	54.24	0.91	1.68
8962021.33	80.81	82.72	83.50	82.34	1.38	1.68
11877636.00	107.19	109.73	110.76	109.23	1.84	1.68

%RSD should be less than 2.0 for ensure that method used were precise for quantitative analysis in the study

Table 16 The linearity of assay method of ATRA.

Concentration of ATRA (µg/ml)	Area under The Curve (AUC)
1.01	135067.75
10.11	1106377.24
25.28	2734123.08
50.56	5448583.32
75.84	8161734.58
101.12	10828336.17

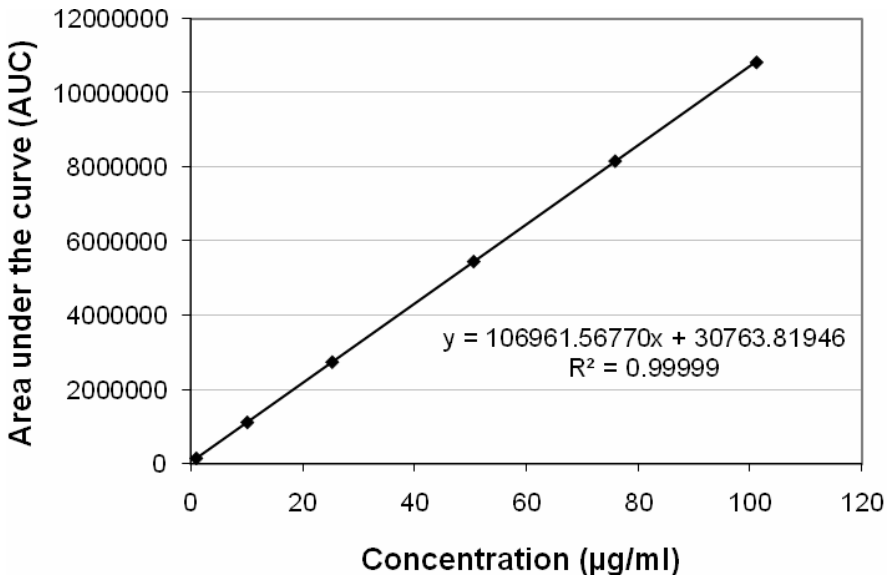


Figure 48 The calibration curve of ATRA (by High Performance Liquid Chromatography, HPLC).

The coefficient of determination (R^2) should be more than 0.999 for acceptable of quantitative analysis

Table 17 Limit of detection (LOD) and Limit of quantitation (LOQ) of ATRA assay method

Calibration curve	Conc. of ATRA (µg/ml)	Area under The Curve (AUC)	Calibration equation	Regression parameters		
				Slope	Intercept	R^2
No.1	1.01	141566.67	y = 110531.07467x + 29879.49360	110531.07467	29879.49360	0.99999
	10.08	1155567.33				
	25.20	2803149.00				
	50.40	5605391.67				
	75.60	8370276.33				
	100.80	11182725.33				
No.2	1.00	141226.02	y = 107944.06537x + 32763.81946	107944.06537	32763.81946	1.00000
	10.05	1117385.79				
	25.12	2744318.74				
	50.24	5455873.66				
	75.36	8167428.59				
	100.48	10878983.51				
No.3	1.01	135067.75	y = 106961.56770x + 30763.81946	106961.56770	30763.81946	0.99999
	10.11	1106377.24				
	25.28	2734123.08				
	50.56	5448583.32				
	75.84	8161734.58				
	101.12	10828336.17				
Mean ± SD				108478.90±1843.88	31135.71±1477.69	0.99999±0.00001
Limit of detection (LOD) = 3.3(SD/S) = [3.3x(1477.69/108478.90)] = 0.045 µg/ml						
Limit of quantitation (LOQ) = 10(SD/S) = [10x(1477.69/108478.90)] = 0.136 µg/ml						

SD = SD of intercept from calibration curve ; S = slope of calibration curve

APPENDIX B

Physicochemical Properties Data of ATRA-Lipid Nanoparticles

Table 18 The solubility of ATRA in various solvents at 25°C

Formula	Solubility (mg/g)				
	Rep.1	Rep.2	Rep.3	Average	SD
O	12.172	12.111	12.251	12.178	0.070
M:O (1:3)	11.309	11.184	11.144	11.212	0.086
S:M:O (0.5:0.5:3)	11.262	11.134	11.141	11.179	0.072
S:O (1:3)	10.458	10.494	10.474	10.475	0.018
M:O (1:1)	9.122	8.875	9.115	9.037	0.140
S:M:O (1:1:2)	8.966	9.017	8.988	8.990	0.026
S:O (1:1)	8.360	8.274	8.261	8.298	0.054
M:O (3:1)	7.466	7.320	7.384	7.390	0.074
S:M:O (1.5:1.5:1)	6.779	6.799	6.729	6.769	0.037
S:O (3:1)	6.107	6.046	5.940	6.031	0.085
M	3.072	3.060	3.054	3.062	0.009
S	1.455	1.412	1.550	1.473	0.070
W	0.029	0.028	0.030	0.029	0.001

Table 19 Effect of initial ATRA concentration on ATRA content and percentage yield of ATRA incorporated in the lipid emulsion formulations (LEs) composed with different oil phases.

Formulation	Initial conc of ATRA (mg/g)	ATRA recoveried (mg/g)					Percentage yield				
		Rep.1	Rep.2	Rep.3	AVG	SD	Rep.1	Rep.2	Rep.3	AVG	SD
LE-S	1	0.85	0.85	0.87	0.86	0.01	94.09	93.95	94.68	94.24	0.39
	3	1.20	1.21	1.20	1.20	0.01	43.93	44.03	43.81	43.92	0.11
	5	1.45	1.44	1.46	1.45	0.01	32.18	32.00	32.56	32.25	0.29
	7	1.45	1.48	1.44	1.46	0.02	23.37	22.15	23.53	23.02	0.76
	9	1.42	1.41	1.42	1.41	0.01	17.67	17.33	17.71	17.57	0.21
LE-M	1	0.91	0.92	0.91	0.91	0.01	100.26	99.93	99.52	99.91	0.37
	3	2.76	2.69	2.64	2.70	0.06	102.40	99.25	98.03	99.89	2.25
	5	1.90	2.00	1.86	1.92	0.07	42.23	44.46	41.01	42.57	1.75
	7	1.93	1.85	1.89	1.89	0.04	30.50	29.88	29.24	29.87	0.63
	9	1.78	1.69	1.78	1.75	0.06	21.91	20.10	20.78	20.93	0.92
LE-SO	1	0.91	0.87	0.93	0.90	0.03	99.92	97.13	101.79	99.61	2.35
	3	2.60	2.74	2.72	2.69	0.08	95.95	104.11	102.76	100.94	4.37
	5	4.53	4.49	4.45	4.49	0.04	100.78	100.25	99.23	100.08	0.79
	7	5.17	5.21	5.02	5.13	0.10	82.27	82.36	79.68	81.44	1.52
	9	4.12	4.10	4.13	4.12	0.01	51.54	50.57	51.50	51.20	0.55
LE-MO	1	0.90	0.95	0.94	0.93	0.02	97.28	99.72	100.78	99.26	1.80
	3	2.67	2.52	2.69	2.63	0.10	99.06	95.45	100.73	98.42	2.70
	5	4.75	4.78	4.70	4.74	0.04	102.29	100.67	96.30	99.75	3.10
	7	3.64	3.37	4.11	3.70	0.37	57.31	53.39	65.17	58.62	6.00
	9	2.84	3.07	3.01	2.97	0.12	34.80	37.74	37.08	36.54	1.54

Table 20 Effect of liquid lipid (M) and solid lipid (CP) ratios in oil phase on ATRA content and the percentage yield of ATRA incorporated in the NLC formulations

Formulation	Initial conc of ATRA (mg/g)	ATRA recoveried (mg/g)					Percentage yield				
		Rep.1	Rep.2	Rep.3	AVG	SD	Rep.1	Rep.2	Rep.3	AVG	SD
NLC-M:CP (5:1)	1	0.83	0.79	0.78	0.80	0.03	83.40	79.22	77.91	80.18	2.87
	3	2.45	2.55	2.35	2.45	0.10	81.59	84.93	78.45	81.66	3.24
	5	4.01	3.87	3.91	3.93	0.07	80.20	77.48	78.21	78.63	1.41
	7	3.98	4.22	4.02	4.07	0.13	56.80	60.34	57.43	58.19	1.89
	9	4.04	4.16	4.25	4.15	0.10	44.92	46.20	47.20	46.11	1.14
NLC-M:CP (3:3)	1	0.88	0.86	0.84	0.86	0.02	87.28	85.92	83.99	85.73	1.65
	3	2.46	2.51	2.49	2.49	0.02	82.13	83.70	82.86	82.90	0.78
	5	3.97	4.02	4.20	4.06	0.12	79.46	80.44	83.92	81.27	2.34
	7	3.80	3.83	3.67	3.77	0.09	54.33	54.77	52.40	53.83	1.26
	9	4.06	3.86	3.89	3.94	0.11	45.12	42.87	43.23	43.74	1.21
NLC-M:CP (1:5)	1	0.81	0.79	0.84	0.81	0.02	81.24	78.95	83.30	81.16	2.18
	3	1.66	1.68	1.80	1.71	0.07	55.32	55.93	59.96	57.07	2.52
	5	2.63	2.52	2.70	2.61	0.09	52.46	50.38	53.96	52.27	1.80
	7	3.30	3.23	3.48	3.34	0.12	47.16	46.18	49.64	47.66	1.79
	9	3.62	3.84	3.88	3.78	0.14	40.25	42.67	43.09	42.00	1.53

Table 21 Effect of initial ATRA concentration on ATRA content and the percentage yield of ATRA incorporated in the NLC formulations composed with different oil phases.

Formulation	Initial conc of ATRA (mg/g)	ATRA recoveried (mg/g)					Percentage yield				
		Rep.1	Rep.2	Rep.3	AVG	SD	Rep.1	Rep.2	Rep.3	AVG	SD
NLC-S	1	0.82	0.83	0.83	0.83	0.01	81.55	81.81	83.30	82.22	0.95
	3	2.55	2.54	2.42	2.50	0.07	84.80	84.39	80.58	83.26	2.33
	5	3.55	3.47	3.44	3.49	0.06	70.79	69.32	68.70	69.60	1.08
	7	3.55	3.45	3.17	3.39	0.20	50.63	49.29	45.28	48.40	2.78
	9	3.96	3.79	3.63	3.80	0.16	43.89	42.11	40.39	42.13	1.75
	11	3.65	3.63	3.92	3.74	0.16	33.18	32.98	35.62	33.93	1.47
NLC-M	1	0.89	0.90	0.92	0.90	0.02	85.55	84.76	90.83	87.04	3.30
	3	2.68	2.57	2.64	2.63	0.05	88.99	85.71	87.03	87.24	1.65
	5	4.15	4.23	4.50	4.29	0.18	82.64	83.42	89.64	85.23	3.84
	7	4.54	4.89	4.82	4.75	0.19	64.72	69.65	68.84	67.74	2.64
	9	5.04	5.18	4.70	4.97	0.25	55.99	57.54	52.15	55.23	2.77
	11	5.40	5.53	5.40	5.44	0.08	48.88	49.81	48.94	49.21	0.52
NLC-SO	1	0.82	0.88	0.86	0.86	0.03	82.48	88.09	85.62	85.40	2.81
	3	2.63	2.46	2.67	2.59	0.11	87.65	82.15	88.83	86.21	3.57
	5	4.21	4.41	4.23	4.28	0.11	83.87	88.04	84.44	85.45	2.26
	7	5.82	5.64	5.85	5.77	0.11	83.16	80.44	83.64	82.41	1.73
	9	7.39	7.49	7.77	7.55	0.19	82.12	83.13	86.28	83.84	2.17
	11	7.42	7.90	7.20	7.50	0.36	67.31	71.74	65.34	68.13	3.28
NLC-MO	1	0.84	0.82	0.86	0.84	0.02	82.97	82.01	86.32	83.77	2.26
	3	2.61	2.60	2.53	2.58	0.05	86.57	86.52	83.96	85.68	1.49
	5	4.15	4.17	4.25	4.19	0.05	82.70	83.00	84.63	83.45	1.04
	7	5.85	6.06	5.92	5.94	0.10	83.59	86.18	84.33	84.70	1.33
	9	7.48	7.55	7.32	7.45	0.12	82.92	83.61	81.28	82.60	1.20
	11	7.45	7.70	7.74	7.63	0.16	67.52	69.74	70.25	69.17	1.45

Table 22 Effect of initial ATRA concentration on the ATRA content and the percentage yield of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase.

Formulation	Initial conc of ATRA (mg/g)	ATRA recoveried (mg/g)					Percentage yield				
		Rep.1	Rep.2	Rep.3	AVG	SD	Rep.1	Rep.2	Rep.3	AVG	SD
NLC-M-DPEG	5	4.25	4.15	4.09	7.45	0.12	84.94	82.97	81.86	83.26	1.56
	7	4.68	4.59	4.33	7.45	0.12	66.85	65.60	61.77	64.74	2.65
NLC-MO-DPEG	9	7.48	7.59	7.35	7.45	0.12	82.95	84.22	81.60	82.92	1.31
	11	7.34	7.28	7.63	7.45	0.12	66.72	65.96	69.27	67.32	1.73
NLC-M-PPEG	5	4.20	4.38	4.18	7.45	0.12	84.04	87.54	83.62	85.07	2.15
	7	4.60	4.51	4.85	7.45	0.12	65.69	64.33	69.21	66.41	2.52
NLC-MO-PPEG	9	7.30	7.29	7.54	7.45	0.12	81.10	80.92	83.77	81.93	1.60
	11	7.49	7.55	7.31	7.45	0.12	68.09	68.64	66.36	67.70	1.19

Table 23 Mean particles size, particles size distribution (PDI) and surface charge (zeta potential, ZP) of ATRA loaded-lipid nanoparticles composed of different oil phase.

Formulation	Size (nm)		PDI		ZP (mV)	
	AVG	SD	AVG	SD	AVG	SD
LE						
LE S-1mg/g	249.00	8.80	0.07	0.03	-15.39	2.58
LE M-1mg/g	135.00	3.64	0.05	0.04	-23.76	2.64
LE M-3mg/g	162.00	4.09	0.06	0.04	-23.74	2.32
LE SO-1mg/g	148.67	13.01	0.04	0.03	-49.46	3.86
LE SO-3mg/g	149.11	4.37	0.06	0.03	-48.87	2.82
LE SO-5mg/g	148.11	6.51	0.07	0.02	-51.83	2.82
LE MO-1mg/g	135.78	9.15	0.07	0.03	-51.91	7.43
LE MO-3mg/g	138.11	5.42	0.06	0.03	-56.28	4.03
LE MO-5mg/g	149.78	7.34	0.12	0.05	-60.07	9.20
NLC						
NLC S-1mg/g	168.10	1.21	0.13	0.02	-32.23	2.06
NLC S-3mg/g	170.73	1.50	0.14	0.03	-32.42	2.87
NLC M-1mg/g	151.03	2.44	0.15	0.03	-34.53	1.21
NLC M-3mg/g	151.65	2.42	0.17	0.05	-34.92	3.87
NLC M-5mg/g	153.17	1.19	0.16	0.03	-33.90	2.25
NLC SO-1mg/g	162.68	0.75	0.11	0.04	-57.02	1.55
NLC SO-3mg/g	167.10	3.01	0.13	0.04	-57.38	4.39
NLC SO-5mg/g	169.50	1.55	0.12	0.03	-56.63	1.44
NLC SO-7mg/g	170.80	4.67	0.14	0.03	-55.93	1.04
NLC SO-9mg/g	172.95	2.14	0.13	0.04	-56.33	1.96
NLC MO-1mg/g	141.80	1.36	0.14	0.03	-55.43	2.64
NLC MO-3mg/g	143.72	2.12	0.13	0.04	-55.45	1.30
NLC MO-5mg/g	145.93	0.92	0.13	0.01	-55.80	1.48
NLC MO-7mg/g	148.13	0.79	0.16	0.02	-55.48	1.70
NLC MO-9mg/g	152.60	1.86	0.14	0.03	-55.23	1.56
Polymer coated-NLC						
NLC M-DPEG-3mg/g	170.35	2.15	0.15	0.03	-57.02	1.55
NLC M-mPEG-3mg/g	172.69	2.40	0.17	0.04	-56.92	2.15
NLC MO-DPEG-3mg/g	162.89	1.94	0.15	0.03	-61.82	1.61
NLC MO-mPEG-3mg/g	160.39	2.27	0.16	0.05	-61.20	1.99

Table 24 The cumulative drug released of lipid emulsion (LE) formulations composed of different oil phase.

Formulations	% Cumulative drug released	Time (h)						
		1	2	4	8	12	24	48
LE S-1mg/g	Rep.1	0.04	0.06	0.11	0.36	0.45	0.74	1.34
	Rep.2	0.06	0.10	0.13	0.40	0.55	0.89	1.75
	Rep.3	0.05	0.08	0.20	0.40	0.52	0.82	1.71
	AVG	0.05	0.08	0.15	0.39	0.51	0.82	1.60
	SD	0.01	0.02	0.04	0.03	0.05	0.08	0.23
LE M-1mg/g	Rep.1	0.05	0.12	0.27	0.60	0.89	1.69	3.58
	Rep.2	0.04	0.10	0.25	0.59	0.81	1.60	3.27
	Rep.3	0.12	0.17	0.32	0.65	0.93	1.83	3.03
	AVG	0.07	0.13	0.28	0.61	0.88	1.71	3.29
	SD	0.04	0.04	0.04	0.03	0.06	0.12	0.28
LE M-3mg/g	Rep.1	0.42	0.54	0.86	1.16	1.29	1.94	2.97
	Rep.2	0.67	0.87	1.14	1.54	1.67	2.29	3.15
	Rep.3	0.55	0.77	0.82	1.35	1.67	2.65	3.11
	AVG	0.55	0.73	0.94	1.35	1.55	2.29	3.08
	SD	0.12	0.17	0.18	0.19	0.22	0.35	0.09
LE SO-1mg/g	Rep.1	0.22	0.54	0.93	1.65	2.33	3.77	6.01
	Rep.2	0.18	0.45	0.94	1.71	2.59	4.33	6.97
	Rep.3	0.19	0.47	1.00	1.95	3.08	5.68	9.56
	AVG	0.20	0.49	0.96	1.77	2.67	4.59	7.52
	SD	0.02	0.05	0.04	0.16	0.38	0.98	1.84
LE SO-3mg/g	Rep.1	0.20	0.41	0.80	1.45	2.43	5.08	7.36
	Rep.2	0.28	0.42	0.97	1.71	2.44	4.81	7.53
	Rep.3	0.19	0.38	0.95	1.47	2.33	4.06	6.35
	AVG	0.22	0.40	0.91	1.54	2.40	4.65	7.08
	SD	0.05	0.02	0.09	0.14	0.06	0.53	0.64

Table 24 (Continue)

Formulations	% Cumulative drug released	Time (h)						
		1	2	4	8	12	24	48
LE SO-5mg/g	Rep.1	0.25	0.45	0.90	1.84	2.40	4.42	6.40
	Rep.2	0.52	0.72	1.39	2.24	3.60	5.52	7.70
	Rep.3	0.45	0.70	1.25	2.14	3.17	5.02	7.14
	AVG	0.41	0.62	1.18	2.07	3.06	4.99	7.08
	SD	0.14	0.15	0.25	0.21	0.61	0.55	0.65
LE MO-1mg/g	Rep.1	0.25	0.56	1.17	2.44	3.80	7.09	11.37
	Rep.2	0.27	0.56	1.17	2.35	4.03	7.23	10.29
	Rep.3	0.30	0.64	1.37	2.90	4.02	7.60	12.48
	AVG	0.28	0.59	1.24	2.56	3.95	7.31	11.38
	SD	0.02	0.05	0.12	0.30	0.13	0.26	1.10
LE MO-3mg/g	Rep.1	0.38	0.79	1.45	2.71	4.07	7.91	14.26
	Rep.2	0.44	0.90	1.61	2.93	4.21	7.62	13.20
	Rep.3	0.38	0.71	1.28	2.27	3.28	6.00	10.30
	AVG	0.40	0.80	1.45	2.64	3.85	7.17	12.59
	SD	0.03	0.10	0.17	0.34	0.50	1.03	2.05
LE MO-5mg/g	Rep.1	0.32	0.72	1.61	3.12	4.31	7.91	12.45
	Rep.2	0.28	0.59	1.34	2.63	3.58	8.05	11.89
	Rep.3	0.23	0.54	1.11	2.08	2.47	5.83	10.31
	AVG	0.28	0.62	1.35	2.61	3.45	7.26	11.55
	SD	0.05	0.09	0.25	0.52	0.92	1.24	1.11

Table 25 The cumulative drug released of nanostructured lipid carrier (NLC) formulations composed of different oil phase.

Formulations	% Cumulative drug released	Time (h)						
		1	2	4	8	12	24	48
NLC S-1mg/g	Rep.1	0.18	0.32	0.48	0.85	1.48	2.59	4.03
	Rep.2	0.17	0.30	0.46	0.93	1.35	2.41	3.95
	Rep.3	0.20	0.35	0.52	1.00	1.49	2.70	4.49
	AVG	0.18	0.32	0.48	0.93	1.44	2.57	4.16
	SD	0.01	0.03	0.03	0.08	0.08	0.15	0.29
NLC S-3mg/g	Rep.1	0.19	0.26	0.48	0.85	1.22	2.61	4.18
	Rep.2	0.14	0.23	0.50	0.93	1.33	2.43	4.25
	Rep.3	0.18	0.30	0.56	1.06	1.53	2.59	4.49
	AVG	0.17	0.26	0.51	0.95	1.36	2.54	4.31
	SD	0.03	0.04	0.04	0.11	0.15	0.10	0.16
NLC M-1mg/g	Rep.1	0.17	0.25	0.54	0.98	1.38	2.48	4.02
	Rep.2	0.18	0.25	0.55	1.09	1.50	2.60	4.29
	Rep.3	0.15	0.24	0.51	0.97	1.22	2.26	3.86
	AVG	0.17	0.25	0.53	1.01	1.36	2.44	4.06
	SD	0.02	0.01	0.02	0.06	0.14	0.17	0.22
NLC M-3mg/g	Rep.1	0.17	0.35	0.64	1.19	1.67	2.75	4.20
	Rep.2	0.45	0.25	0.52	0.99	1.53	2.61	4.34
	Rep.3	0.18	0.30	0.57	1.08	1.48	2.57	4.50
	AVG	0.26	0.30	0.58	1.09	1.56	2.65	4.34
	SD	0.16	0.05	0.06	0.10	0.10	0.10	0.15
NLC M-5mg/g	Rep.1	0.23	0.29	0.64	1.11	1.85	2.81	4.30
	Rep.2	0.19	0.25	0.44	0.88	1.44	2.58	3.98
	Rep.3	0.19	0.25	0.45	0.87	1.43	2.53	3.98
	AVG	0.20	0.26	0.51	0.95	1.57	2.64	4.09
	SD	0.02	0.02	0.11	0.14	0.24	0.15	0.19

Table 25 (Continue)

Formulations	% Cumulative drug released	Time (h)						
		1	2	4	8	12	24	48
NLC SO-1mg/g	Rep.1	0.17	0.26	0.54	1.01	1.78	2.69	4.28
	Rep.2	0.15	0.24	0.52	0.98	1.56	2.43	4.00
	Rep.3	0.17	0.24	0.49	0.99	1.67	2.52	4.17
	AVG	0.16	0.25	0.52	1.00	1.67	2.54	4.15
	SD	0.01	0.01	0.03	0.01	0.11	0.13	0.14
NLC SO-3mg/g	Rep.1	0.09	0.27	0.54	1.03	1.49	2.70	4.29
	Rep.2	0.14	0.26	0.53	1.03	1.46	2.62	4.11
	Rep.3	0.17	0.27	0.48	0.91	1.33	2.29	4.35
	AVG	0.13	0.26	0.52	0.99	1.43	2.54	4.25
	SD	0.04	0.01	0.03	0.07	0.09	0.21	0.13
NLC SO-5mg/g	Rep.1	0.21	0.28	0.56	1.08	1.76	2.45	4.11
	Rep.2	0.19	0.26	0.55	0.92	1.55	2.29	3.97
	Rep.3	0.22	0.28	0.61	1.18	1.87	2.52	4.20
	AVG	0.21	0.27	0.57	1.06	1.72	2.42	4.10
	SD	0.01	0.01	0.04	0.13	0.16	0.12	0.11
NLC SO-7mg/g	Rep.1	0.17	0.22	0.47	0.82	1.44	2.39	3.89
	Rep.2	0.17	0.25	0.53	1.16	1.78	2.70	4.21
	Rep.3	0.16	0.24	0.47	0.96	1.68	2.52	4.15
	AVG	0.17	0.24	0.49	0.98	1.63	2.54	4.08
	SD	0.00	0.02	0.04	0.17	0.18	0.15	0.17
NLC SO-9mg/g	Rep.1	0.17	0.25	0.54	1.00	1.37	2.48	4.00
	Rep.2	0.22	0.29	0.67	1.14	1.56	2.77	4.32
	Rep.3	0.21	0.26	0.55	1.00	1.46	2.51	4.05
	AVG	0.20	0.27	0.59	1.05	1.47	2.58	4.12
	SD	0.02	0.02	0.08	0.08	0.09	0.16	0.17

Table 25 (Continue)

Formulations	% Cumulative drug released	Time (h)						
		1	2	4	8	12	24	48
NLC MO-1mg/g	Rep.1	0.16	0.26	0.57	0.99	1.63	2.34	4.01
	Rep.2	0.16	0.25	0.47	0.84	1.44	2.24	3.98
	Rep.3	0.17	0.27	0.57	1.03	1.80	2.55	4.29
	AVG	0.16	0.26	0.54	0.95	1.63	2.38	4.09
	SD	0.01	0.01	0.06	0.10	0.18	0.16	0.17
NLC MO-3mg/g	Rep.1	0.12	0.25	0.50	1.01	1.50	2.66	4.21
	Rep.2	0.16	0.26	0.54	1.02	1.47	2.69	4.33
	Rep.3	0.17	0.28	0.52	0.98	1.39	2.36	4.18
	AVG	0.15	0.27	0.52	1.00	1.45	2.57	4.24
	SD	0.02	0.02	0.02	0.02	0.06	0.19	0.08
NLC MO-5mg/g	Rep.1	0.13	0.25	0.48	0.99	1.58	2.49	4.01
	Rep.2	0.16	0.24	0.55	1.03	1.76	2.50	4.23
	Rep.3	0.13	0.22	0.50	0.99	1.73	2.53	4.22
	AVG	0.14	0.24	0.51	1.00	1.69	2.51	4.15
	SD	0.02	0.02	0.04	0.02	0.10	0.02	0.13
NLC MO-7mg/g	Rep.1	0.15	0.23	0.54	0.97	1.36	2.47	3.99
	Rep.2	0.18	0.26	0.56	1.23	1.61	2.68	4.24
	Rep.3	0.18	0.26	0.55	1.12	1.59	2.63	4.23
	AVG	0.17	0.25	0.55	1.11	1.52	2.59	4.15
	SD	0.01	0.01	0.01	0.13	0.14	0.11	0.14
NLC MO-9mg/g	Rep.1	0.16	0.26	0.57	1.03	1.68	2.58	4.18
	Rep.2	0.14	0.25	0.49	0.96	1.48	2.44	4.01
	Rep.3	0.15	0.26	0.57	1.12	1.74	2.58	4.22
	AVG	0.15	0.25	0.54	1.04	1.63	2.53	4.14
	SD	0.01	0.01	0.05	0.08	0.14	0.08	0.12

Table 26 The cumulative drug released of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Formulations	% Cumulative drug released	Time (h)						
		1	2	4	8	12	24	48
NLC M-DPEG-3mg/g	Rep.1	0.18	0.25	0.57	1.11	1.71	2.61	4.22
	Rep.2	0.17	0.26	0.59	1.05	1.77	2.60	4.22
	Rep.3	0.14	0.24	0.44	0.96	1.67	2.42	4.00
	AVG	0.16	0.25	0.53	1.04	1.71	2.54	4.15
	SD	0.02	0.01	0.08	0.08	0.05	0.11	0.13
NLC M-PPEG-3mg/g	Rep.1	0.15	0.26	0.48	0.99	1.46	2.51	4.19
	Rep.2	0.15	0.25	0.46	0.86	1.44	2.25	3.97
	Rep.3	0.16	0.30	0.54	1.08	1.49	2.62	4.38
	AVG	0.15	0.27	0.49	0.97	1.46	2.46	4.18
	SD	0.01	0.02	0.04	0.11	0.02	0.19	0.20
NLC MO-DPEG-3mg/g	Rep.1	0.20	0.32	0.54	1.02	1.51	2.71	4.34
	Rep.2	0.19	0.24	0.51	0.96	1.38	2.45	3.92
	Rep.3	0.20	0.26	0.52	0.98	1.50	2.67	4.20
	AVG	0.19	0.28	0.52	0.99	1.46	2.61	4.15
	SD	0.01	0.04	0.01	0.03	0.07	0.14	0.21
NLC MO-PPEG-3mg/g	Rep.1	0.14	0.28	0.53	1.14	1.43	2.68	4.20
	Rep.2	0.12	0.26	0.50	1.12	1.39	2.52	4.09
	Rep.3	0.13	0.25	0.50	0.12	1.39	2.57	4.00
	AVG	0.13	0.26	0.51	0.79	1.40	2.59	4.10
	SD	0.01	0.01	0.02	0.58	0.03	0.08	0.10

Table 27 The effect of light on the chemical stability of ATRA in isopropyl alcohol solution (IPA) in different amount of initial ATRA concentration.

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
IPA-1mg/g	Rep.1	100.00	91.46	73.55	54.96	32.30	24.28
	Rep.2	100.00	74.62	70.81	52.39	38.28	28.94
	Rep.3	100.00	74.22	71.33	53.35	38.38	27.54
	AVG	100.00	80.10	71.90	53.56	36.32	26.92
	SD	0.00	9.84	1.45	1.30	3.48	2.39
IPA-3mg/g	Rep.1	100.00	91.30	82.92	71.70	53.37	42.70
	Rep.2	100.00	92.87	87.75	78.28	56.23	43.96
	Rep.3	100.00	90.23	86.64	75.06	54.83	43.01
	AVG	100.00	91.47	85.77	75.01	54.81	43.22
	SD	0.00	1.33	2.53	3.29	1.43	0.66
IPA-5mg/g	Rep.1	100.00	93.68	90.41	82.08	68.45	56.69
	Rep.2	100.00	90.89	84.06	76.68	66.84	53.46
	Rep.3	100.00	93.81	90.63	80.84	69.41	55.75
	AVG	100.00	92.79	88.37	79.87	68.23	55.30
	SD	0.00	1.65	3.73	2.83	1.30	1.66
IPA-7mg/g	Rep.1	100.00	96.34	93.19	85.98	73.46	64.30
	Rep.2	100.00	95.68	90.28	83.61	73.79	62.23
	Rep.3	100.00	93.13	88.01	80.92	68.16	60.99
	AVG	100.00	95.05	90.49	83.50	71.80	62.50
	SD	0.00	1.70	2.59	2.53	3.16	1.68
IPA-9mg/g	Rep.1	100.00	95.15	91.23	81.86	75.99	65.90
	Rep.2	100.00	96.03	92.61	85.73	77.14	66.12
	Rep.3	100.00	97.01	93.48	87.14	78.39	68.67
	AVG	100.00	96.06	92.44	84.91	77.17	66.90
	SD	0.00	0.93	1.14	2.73	1.20	1.54

Table 28 The effect of light on the chemical stability of ATRA loaded-lipid emulsions (LE) composed of different oil phase in different amount of initial ATRA concentration.

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
LE S-1mg/g	Rep.1	100.00	98.03	92.03	84.64	74.81	62.09
	Rep.2	100.00	95.43	91.22	82.65	74.09	59.67
	Rep.3	100.00	95.65	90.77	82.55	73.72	60.39
	AVG	100.00	96.37	91.34	83.28	74.21	60.72
	SD	0.00	1.44	0.64	1.18	0.56	1.24
LE M-1mg/g	Rep.1	100.00	92.15	85.26	79.75	76.20	61.83
	Rep.2	100.00	95.73	92.91	84.91	78.63	62.12
	Rep.3	100.00	91.52	83.70	74.66	75.11	57.73
	AVG	100.00	93.13	87.29	79.78	76.65	60.56
	SD	0.00	2.27	4.93	5.12	1.80	2.45
LE M-3mg/g	Rep.1	100.00	97.16	91.67	85.39	85.02	76.24
	Rep.2	100.00	96.08	89.00	84.96	84.02	74.61
	Rep.3	100.00	96.47	91.46	86.08	79.27	75.64
	AVG	100.00	96.57	90.71	85.48	82.77	75.50
	SD	0.00	0.55	1.49	0.56	3.07	0.82
LE SO-1mg/g	Rep.1	100.00	99.63	94.14	86.54	69.97	60.48
	Rep.2	100.00	100.18	93.15	85.44	81.27	64.67
	Rep.3	100.00	98.35	94.68	85.98	76.30	50.90
	AVG	100.00	99.39	93.99	85.99	75.85	58.69
	SD	0.00	0.94	0.77	0.55	5.66	7.06

Table 28 (Continue)

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
LE SO-3mg/g	Rep.1	100.00	98.37	94.20	89.44	88.56	75.16
	Rep.2	100.00	96.69	94.21	90.35	86.50	75.07
	Rep.3	100.00	94.92	95.40	89.42	83.07	75.65
	AVG	100.00	96.66	94.60	89.74	86.04	75.29
	SD	0.00	1.73	0.69	0.53	2.77	0.31
LE SO-5mg/g	Rep.1	100.00	96.82	94.73	90.87	88.37	81.05
	Rep.2	100.00	96.37	90.60	91.45	92.12	86.11
	Rep.3	100.00	98.28	96.47	91.63	84.51	86.90
	AVG	100.00	97.16	93.93	91.32	88.33	84.69
	SD	0.00	1.00	3.01	0.40	3.81	3.18
LE MO-1mg/g	Rep.1	100.00	94.26	87.67	79.38	75.46	58.97
	Rep.2	100.00	92.27	88.28	75.30	78.24	59.97
	Rep.3	100.00	91.61	88.22	77.50	73.12	59.91
	AVG	100.00	92.71	88.06	77.40	75.61	59.61
	SD	0.00	1.38	0.33	2.04	2.56	0.56
LE MO-3mg/g	Rep.1	100.00	97.35	95.20	91.50	91.35	73.16
	Rep.2	100.00	98.04	95.62	91.13	80.01	72.46
	Rep.3	100.00	98.44	96.50	89.37	81.59	73.72
	AVG	100.00	97.94	95.77	90.67	84.32	73.11
	SD	0.00	0.56	0.66	1.14	6.14	0.63
LE MO-5mg/g	Rep.1	100.00	99.65	98.34	93.68	91.58	83.92
	Rep.2	100.00	97.24	94.08	89.77	90.80	86.67
	Rep.3	100.00	97.21	93.49	90.10	91.02	87.30
	AVG	100.00	98.04	95.30	91.18	91.13	85.96
	SD	0.00	1.40	2.65	2.17	0.40	1.80

Table 29 The effect of light on the chemical stability of ATRA loaded-nanostructured lipid carrier (NLC) composed of different oil phase in different amount of initial ATRA concentration.

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
NLC S-1mg/g	Rep.1	100.00	97.19	94.24	83.12	70.42	61.85
	Rep.2	100.00	95.03	91.00	80.84	68.92	59.00
	Rep.3	100.00	94.10	90.15	80.23	67.08	59.25
	AVG	100.00	95.44	91.80	81.40	68.81	60.03
	SD	0.00	1.59	2.16	1.52	1.67	1.58
NLC S-3mg/g	Rep.1	100.00	98.24	95.39	89.60	80.09	70.63
	Rep.2	100.00	96.84	93.75	88.47	77.31	68.97
	Rep.3	100.00	97.05	93.97	88.80	79.71	71.49
	AVG	100.00	97.38	94.37	88.96	79.04	70.36
	SD	0.00	0.75	0.89	0.58	1.51	1.28
NLC M-1mg/g	Rep.1	100.00	94.14	90.24	84.39	71.49	60.61
	Rep.2	100.00	92.14	88.59	82.17	69.22	59.38
	Rep.3	100.00	92.01	89.04	82.55	68.88	57.75
	AVG	100.00	92.76	89.29	83.04	69.86	59.25
	SD	0.00	1.19	0.85	1.19	1.42	1.44
NLC M-3mg/g	Rep.1	100.00	102.38	94.58	88.62	78.71	69.37
	Rep.2	100.00	96.40	91.64	87.31	77.14	68.41
	Rep.3	100.00	97.82	93.20	86.40	75.49	66.23
	AVG	100.00	98.87	93.14	87.44	77.11	68.01
	SD	0.00	3.12	1.47	1.12	1.61	1.61
NLC M-5mg/g	Rep.1	100.00	97.76	95.40	91.78	86.01	83.58
	Rep.2	100.00	95.13	93.10	89.63	84.61	81.21
	Rep.3	100.00	95.22	93.71	89.51	84.00	81.00
	AVG	100.00	96.04	94.07	90.31	84.87	81.93
	SD	0.00	1.49	1.19	1.28	1.03	1.43

Table 29 (Continue)

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
NLC SO-1mg/g	Rep.1	100.00	94.00	88.91	77.98	66.15	57.95
	Rep.2	100.00	96.93	93.47	80.11	68.01	58.80
	Rep.3	100.00	95.22	92.06	79.09	67.44	58.24
	AVG	100.00	95.38	91.48	79.06	67.20	58.33
	SD	0.00	1.47	2.33	1.07	0.95	0.43
NLC SO-3mg/g	Rep.1	100.00	97.88	93.69	87.03	77.58	68.63
	Rep.2	100.00	96.45	93.47	87.68	77.96	68.57
	Rep.3	100.00	96.83	93.94	87.22	77.61	67.20
	AVG	100.00	97.05	93.70	87.31	77.72	68.13
	SD	0.00	0.74	0.24	0.33	0.21	0.81
NLC SO-5mg/g	Rep.1	100.00	98.63	96.98	92.17	90.46	85.02
	Rep.2	100.00	97.71	93.64	90.27	87.17	83.12
	Rep.3	100.00	98.55	97.11	93.81	91.28	86.95
	AVG	100.00	98.30	95.91	92.08	89.63	85.03
	SD	0.00	0.51	1.97	1.77	2.17	1.92
NLC SO-7mg/g	Rep.1	100.00	98.37	96.81	94.59	90.14	88.94
	Rep.2	100.00	99.19	97.76	95.82	91.56	89.57
	Rep.3	100.00	97.27	95.84	92.77	88.78	86.02
	AVG	100.00	98.27	96.81	94.39	90.16	88.18
	SD	0.00	0.96	0.96	1.53	1.39	1.89
NLC SO-9mg/g	Rep.1	100.00	96.03	95.32	93.54	89.88	86.21
	Rep.2	100.00	98.28	97.10	94.73	91.75	88.38
	Rep.3	100.00	96.69	95.14	94.90	89.71	86.27
	AVG	100.00	97.00	95.85	94.39	90.45	86.95
	SD	0.00	1.15	1.09	0.74	1.13	1.24

Table 29 (Continue)

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
NLC MO-1mg/g	Rep.1	100.00	97.21	91.48	81.50	67.25	59.01
	Rep.2	100.00	94.19	87.15	78.17	64.14	55.53
	Rep.3	100.00	95.77	90.92	80.69	66.77	58.20
	AVG	100.00	95.72	89.85	80.12	66.05	57.58
	SD	0.00	1.51	2.35	1.74	1.68	1.82
NLC MO-3mg/g	Rep.1	100.00	96.80	92.60	88.55	77.03	65.48
	Rep.2	100.00	97.86	97.85	89.60	79.51	72.22
	Rep.3	100.00	95.89	94.52	88.44	79.44	69.54
	AVG	100.00	96.85	94.99	88.86	78.66	69.08
	SD	0.00	0.99	2.66	0.64	1.41	3.39
NLC MO-5mg/g	Rep.1	100.00	97.92	94.50	92.78	87.84	83.74
	Rep.2	100.00	98.86	96.12	93.41	88.27	84.61
	Rep.3	100.00	95.20	92.69	89.02	85.55	81.98
	AVG	100.00	97.33	94.44	91.74	87.22	83.45
	SD	0.00	1.90	1.72	2.38	1.46	1.34
NLC MO-7mg/g	Rep.1	100.00	97.92	96.05	94.64	91.47	87.03
	Rep.2	100.00	96.02	95.44	93.41	90.88	85.93
	Rep.3	100.00	96.66	94.89	92.80	91.06	86.21
	AVG	100.00	96.87	95.46	93.62	91.14	86.39
	SD	0.00	0.97	0.58	0.94	0.30	0.57
NLC MO-9mg/g	100.00	98.02	96.19	94.23	92.39	87.87	100.00
	100.00	94.18	93.63	91.18	88.70	83.92	100.00
	100.00	95.08	94.71	92.48	89.13	84.19	100.00
	100.00	95.76	94.85	92.63	90.07	85.33	100.00
	0.00	2.01	1.29	1.53	2.02	2.21	0.00

Table 30 The effect of light on the chemical stability of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Formulation	% ATRA Recovery	Exposure time (h)					
		0.0	0.5	1.0	2.0	4.0	6.0
NLC M-DPEG-3mg/g	Rep.1	100.00	97.98	95.72	90.68	84.28	76.36
	Rep.2	100.00	96.22	94.17	88.10	83.01	73.41
	Rep.3	100.00	97.05	95.01	89.91	84.99	75.29
	AVG	100.00	97.08	94.97	89.57	84.10	75.02
	SD	0.00	0.88	0.78	1.33	1.00	1.49
NLC M-PPEG-3mg/g	Rep.1	100.00	97.71	95.38	91.78	84.51	75.84
	Rep.2	100.00	95.57	93.18	87.19	81.21	72.00
	Rep.3	100.00	96.00	94.24	88.62	80.68	71.52
	AVG	100.00	96.43	94.27	89.20	82.13	73.12
	SD	0.00	1.13	1.10	2.35	2.08	2.37
NLC MO-DPEG-3mg/g	Rep.1	100.00	98.15	93.89	91.89	83.92	78.02
	Rep.2	100.00	98.88	92.58	92.43	84.29	79.14
	Rep.3	100.00	96.60	92.21	89.76	82.07	75.29
	AVG	100.00	97.88	92.89	91.36	83.43	77.48
	SD	0.00	1.17	0.88	1.41	1.19	1.98
NLC MO-PPEG-3mg/g	Rep.1	100.00	98.72	96.77	91.37	85.01	75.95
	Rep.2	100.00	97.87	95.20	90.29	84.88	75.38
	Rep.3	100.00	95.31	93.86	88.49	82.64	71.75
	AVG	100.00	97.30	95.28	90.05	84.18	74.36
	SD	0.00	1.77	1.46	1.45	1.33	2.28

Table 31 The change in pH of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration, at initial and after stability studies.

Formulation	pH	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
LE S-1mg/g	Rep.1	8.00	7.98	7.98	7.95	7.94	6.83
	Rep.2	8.00	7.99	7.97	7.96	7.96	7.01
	AVG	8.00	7.99	7.98	7.96	7.95	6.92
	SD	0.00	0.01	0.01	0.01	0.01	0.13
LE M-1mg/g	Rep.1	8.00	7.99	7.98	7.97	7.96	7.15
	Rep.2	8.00	7.99	7.99	7.98	7.96	6.94
	AVG	8.00	7.99	7.99	7.98	7.96	7.05
	SD	0.00	0.00	0.01	0.01	0.00	0.15
LE M-3mg/g	Rep.1	8.00	7.98	7.96	7.96	7.95	7.12
	Rep.2	8.01	7.99	7.98	7.97	7.96	6.96
	AVG	8.01	7.99	7.97	7.97	7.96	7.04
	SD	0.01	0.01	0.01	0.01	0.01	0.11
LE SO-1mg/g	Rep.1	7.99	7.99	7.98	7.96	7.94	6.43
	Rep.2	8.00	7.99	7.98	7.95	7.95	6.36
	AVG	8.00	7.99	7.98	7.96	7.95	6.40
	SD	0.01	0.00	0.00	0.01	0.01	0.05
LE SO-3mg/g	Rep.1	8.01	7.99	7.97	7.96	7.96	6.47
	Rep.2	8.00	7.99	7.98	7.96	7.95	6.14
	AVG	8.01	7.99	7.98	7.96	7.96	6.31
	SD	0.01	0.00	0.01	0.00	0.01	0.23

Table 31 (Continue)

Formulation	pH	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 14	Day 14	Day 14	
LE SO-5mg/g	Rep.1	8.01	7.99	7.99	7.97	7.95	6.85
	Rep.2	8.00	7.99	7.98	7.96	7.95	6.48
	AVG	8.01	7.99	7.99	7.97	7.95	6.67
	SD	0.01	0.00	0.01	0.01	0.00	0.26
LE MO-1mg/g	Rep.1	8.00	7.99	7.98	7.98	7.96	7.14
	Rep.2	8.02	8.01	8.00	7.98	7.97	6.86
	AVG	8.01	8.00	7.99	7.98	7.97	7.00
	SD	0.01	0.01	0.01	0.00	0.01	0.20
LE MO-3mg/g	Rep.1	8.01	8.00	7.99	7.98	7.97	6.88
	Rep.2	8.02	8.01	7.99	7.98	7.98	7.07
	AVG	8.02	8.01	7.99	7.98	7.98	6.98
	SD	0.01	0.01	0.00	0.00	0.01	0.13
LE MO-5mg/g	Rep.1	8.00	7.99	7.99	7.98	7.97	6.88
	Rep.2	8.02	8.01	8.00	7.98	7.98	7.10
	AVG	8.01	8.00	8.00	7.98	7.98	6.99
	SD	0.01	0.01	0.01	0.00	0.01	0.16

Table 32 The change in pH of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration, at initial and after stability studies.

Formulation	pH	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
NLC S-1mg/g	Rep.1	8.00	7.95	7.87	7.78	7.68	6.85
	Rep.2	8.01	7.97	7.87	7.79	7.70	7.11
	AVG	8.01	7.96	7.87	7.79	7.69	6.98
	SD	0.01	0.01	0.00	0.01	0.01	0.18
NLC S-3mg/g	Rep.1	8.02	7.93	7.85	7.79	7.65	6.85
	Rep.2	8.01	7.94	7.87	7.79	7.67	7.11
	AVG	8.02	7.94	7.86	7.79	7.66	6.98
	SD	0.01	0.01	0.01	0.00	0.01	0.18
NLC M-1mg/g	Rep.1	8.01	7.97	7.93	7.90	7.88	7.25
	Rep.2	8.02	7.96	7.94	7.91	7.88	6.94
	AVG	8.02	7.97	7.94	7.91	7.88	7.10
	SD	0.01	0.01	0.01	0.01	0.00	0.22
LE M-3mg/g	Rep.1	8.01	7.96	7.94	7.91	7.84	7.17
	Rep.2	8.01	7.96	7.95	7.92	7.83	6.99
	AVG	8.01	7.96	7.95	7.92	7.84	7.08
	SD	0.00	0.00	0.01	0.01	0.01	0.13
LE M-5mg/g	Rep.1	8.02	7.98	7.92	7.91	7.86	7.20
	Rep.2	8.02	7.97	7.92	7.89	7.85	7.01
	AVG	8.02	7.98	7.92	7.90	7.86	7.11
	SD	0.00	0.01	0.00	0.01	0.01	0.13

Table 32 (Continue)

Formulation	pH	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
LE SO-1mg/g	Rep.1	8.00	7.95	7.86	7.77	7.58	6.40
	Rep.2	8.01	7.95	7.87	7.76	7.57	6.55
	AVG	8.01	7.95	7.87	7.77	7.58	6.48
	SD	0.01	0.00	0.01	0.01	0.01	0.11
LE SO-3mg/g	Rep.1	8.00	7.95	7.87	7.73	7.56	6.34
	Rep.2	8.01	7.96	7.87	7.75	7.55	6.57
	AVG	8.01	7.96	7.87	7.74	7.56	6.46
	SD	0.01	0.01	0.00	0.01	0.01	0.16
LE SO-5mg/g	Rep.1	8.00	7.93	7.84	7.72	7.57	6.55
	Rep.2	8.00	7.93	7.84	7.73	7.58	6.38
	AVG	8.00	7.93	7.84	7.73	7.58	6.47
	SD	0.00	0.00	0.00	0.01	0.01	0.12
LE SO-7mg/g	Rep.1	8.00	7.92	7.82	7.73	7.55	6.30
	Rep.2	8.01	7.93	7.83	7.74	7.54	6.67
	AVG	8.01	7.93	7.83	7.74	7.55	6.49
	SD	0.01	0.01	0.01	0.01	0.01	0.26
LE SO-9mg/g	Rep.1	8.00	7.94	7.83	7.76	7.56	6.47
	Rep.2	8.00	7.93	7.81	7.75	7.55	6.61
	AVG	8.00	7.94	7.82	7.76	7.56	6.54
	SD	0.00	0.01	0.01	0.01	0.01	0.10

Table 32 (Continue)

Formulation	pH	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
LE MO-1mg/g	Rep.1	8.03	7.96	7.86	7.74	7.77	7.36
	Rep.2	8.02	7.96	7.87	7.75	7.79	7.02
	AVG	8.03	7.96	7.87	7.75	7.78	7.19
	SD	0.01	0.00	0.01	0.01	0.01	0.24
LE MO-3mg/g	Rep.1	8.01	7.95	7.88	7.81	7.78	7.30
	Rep.2	8.02	7.95	7.90	7.84	7.80	6.94
	AVG	8.02	7.95	7.89	7.83	7.79	7.12
	SD	0.01	0.00	0.01	0.02	0.01	0.25
LE MO-5mg/g	Rep.1	8.00	7.93	7.90	7.80	7.77	7.08
	Rep.2	8.02	7.96	7.91	7.82	7.79	7.15
	AVG	8.01	7.95	7.91	7.81	7.78	7.12
	SD	0.01	0.02	0.01	0.01	0.01	0.05
LE MO-7mg/g	Rep.1	8.02	7.93	7.90	7.80	7.79	6.94
	Rep.2	8.01	7.96	7.91	7.82	7.78	7.33
	AVG	8.02	7.95	7.91	7.81	7.79	7.14
	SD	0.01	0.02	0.01	0.01	0.01	0.28
LE MO-9mg/g	Rep.1	8.00	7.93	7.90	7.80	7.77	7.08
	Rep.2	8.02	7.96	7.91	7.82	7.78	7.26
	AVG	8.01	7.95	7.91	7.81	7.78	7.17
	SD	0.01	0.02	0.01	0.01	0.01	0.13

Table 33 The change in pH of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Formulation	pH	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
NLC M-DPEG-3mg/g	Rep.1	8.00	7.96	7.94	7.87	7.83	7.20
	Rep.2	8.00	7.95	7.95	7.88	7.84	6.99
	AVG	8.00	7.96	7.95	7.88	7.84	7.10
	SD	0.00	0.01	0.01	0.01	0.01	0.15
NLC M-PPEG-3mg/g	Rep.1	8.00	7.97	7.93	7.86	7.82	7.21
	Rep.2	8.01	7.98	7.94	7.87	7.84	6.93
	AVG	8.01	7.98	7.94	7.87	7.83	7.07
	SD	0.01	0.01	0.01	0.01	0.01	0.20
NLC MO-DPEG-3mg/g	Rep.1	8.00	7.98	7.96	7.84	7.80	7.33
	Rep.2	8.01	7.98	7.95	7.83	7.78	6.96
	AVG	8.01	7.98	7.96	7.84	7.79	7.15
	SD	0.01	0.00	0.01	0.01	0.01	0.26
NLC MO-PPEG-3mg/g	Rep.1	8.00	7.97	7.94	7.87	7.80	7.22
	Rep.2	8.01	7.97	7.93	7.85	7.79	7.02
	AVG	8.01	7.97	7.94	7.86	7.80	7.12
	SD	0.01	0.00	0.01	0.01	0.01	0.14

Table 34 The change in percentage yield of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration, at initial and after stability studies.

Formulation	% ATRA content	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
LE S-1mg/g	Rep.1	100.00	98.63	96.78	94.90	91.98	68.63
	Rep.2	100.00	98.10	96.00	93.86	92.84	62.38
	AVG	100.00	98.36	96.39	94.38	92.41	65.51
	SD	0.00	0.37	0.55	0.73	0.60	4.42
LE M-1mg/g	Rep.1	100.00	97.69	95.85	94.45	92.97	70.21
	Rep.2	100.00	98.44	96.53	93.43	91.32	66.80
	AVG	100.00	98.06	96.19	93.94	92.00	68.50
	SD	0.00	0.53	0.48	0.72	0.95	2.41
LE M-3mg/g	Rep.1	100.00	97.66	95.67	93.40	91.22	63.94
	Rep.2	100.00	97.10	94.89	93.12	91.79	69.55
	AVG	100.00	97.38	95.28	93.26	91.50	66.75
	SD	0.00	0.40	0.55	0.20	0.40	3.97
LE SO-1mg/g	Rep.1	100.00	99.15	97.14	94.62	93.05	69.69
	Rep.2	100.00	98.38	96.59	93.44	91.74	66.35
	AVG	100.00	98.77	96.86	94.03	92.39	68.02
	SD	0.00	0.55	0.39	0.84	0.93	2.36
LE SO-3mg/g	Rep.1	100.00	97.67	97.20	94.91	94.42	70.02
	Rep.2	100.00	98.81	96.82	95.37	92.69	67.94
	AVG	100.00	98.24	97.01	95.14	93.55	68.98
	SD	0.00	0.80	0.27	0.33	1.22	1.47

Table 34 (Continue)

Formulation	% ATRA content	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 14	Day 14	Day 14	
LE SO-5mg/g	Rep.1	100.00	99.65	99.03	95.25	93.42	69.75
	Rep.2	100.00	98.58	97.02	95.46	92.16	65.39
	AVG	100.00	99.12	98.02	95.35	92.79	67.57
	SD	0.00	0.76	1.42	0.14	0.89	3.08
LE MO-1mg/g	Rep.1	100.00	98.95	95.03	95.40	91.48	67.36
	Rep.2	100.00	98.58	96.29	94.24	92.70	65.07
	AVG	100.00	98.76	95.66	94.82	92.09	66.22
	SD	0.00	0.27	0.89	0.82	0.86	1.61
LE MO-3mg/g	Rep.1	100.00	99.10	97.84	95.87	93.85	69.91
	Rep.2	100.00	98.53	96.83	95.19	93.31	65.03
	AVG	100.00	98.81	97.34	95.53	93.58	67.47
	SD	0.00	0.40	0.71	0.48	0.38	3.45
LE MO-5mg/g	Rep.1	100.00	97.55	97.82	94.38	92.57	65.65
	Rep.2	100.00	99.30	95.70	95.13	92.27	69.50
	AVG	100.00	98.42	96.70	94.76	92.92	67.58
	SD	0.00	1.24	1.59	0.53	0.50	2.72

Table 35 The change in percentage yield of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration, at initial and after stability studies.

Formulation	% ATRA content	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
NLC S-1mg/g	Rep.1	100.00	98.64	96.22	92.10	92.10	70.68
	Rep.2	100.00	99.08	95.73	95.01	92.85	67.45
	AVG	100.00	98.86	95.97	93.56	92.48	69.07
	SD	0.00	0.32	0.34	2.06	0.53	2.28
NLC S-3mg/g	Rep.1	100.00	99.00	95.75	91.90	90.72	71.33
	Rep.2	100.00	98.51	96.62	93.19	91.81	69.14
	AVG	100.00	98.76	96.18	92.55	91.27	70.24
	SD	0.00	0.35	0.62	0.91	0.77	1.54
NLC M-1mg/g	Rep.1	100.00	99.39	96.28	94.38	91.38	69.45
	Rep.2	100.00	99.07	96.04	93.77	92.69	72.03
	AVG	100.00	99.23	96.16	94.08	92.03	70.74
	SD	0.00	0.23	0.17	0.44	0.93	1.82
LE M-3mg/g	Rep.1	100.00	99.00	96.69	94.67	91.68	71.54
	Rep.2	100.00	99.16	96.47	94.46	93.89	69.27
	AVG	100.00	99.08	96.58	94.56	92.79	70.41
	SD	0.00	0.11	0.16	0.15	1.56	1.61
LE M-5mg/g	Rep.1	100.00	98.81	95.59	92.48	91.96	71.23
	Rep.2	100.00	98.98	94.47	93.87	92.07	67.67
	AVG	100.00	98.89	95.03	93.17	92.01	69.45
	SD	0.00	0.12	0.79	0.99	0.08	2.52

Table 35 (Continue)

Formulation	% ATRA content	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
LE SO-1mg/g	Rep.1	100.00	98.54	96.61	95.13	93.20	73.22
	Rep.2	100.00	98.57	95.78	94.85	93.69	69.77
	AVG	100.00	98.55	96.19	94.99	93.45	71.50
	SD	0.00	0.02	0.59	0.20	0.35	2.44
LE SO-3mg/g	Rep.1	100.00	98.79	97.08	95.41	93.14	68.09
	Rep.2	100.00	99.36	97.94	95.96	92.78	71.93
	AVG	100.00	99.08	97.51	95.69	92.96	70.01
	SD	0.00	0.40	0.61	0.38	0.25	2.72
LE SO-5mg/g	Rep.1	100.00	99.09	97.48	96.07	93.74	69.41
	Rep.2	100.00	98.68	96.98	95.80	94.19	74.91
	AVG	100.00	98.89	97.23	95.93	93.96	72.16
	SD	0.00	0.29	0.36	0.20	0.32	3.89
LE SO-7mg/g	Rep.1	100.00	99.04	97.03	94.71	91.03	68.53
	Rep.2	100.00	98.76	97.20	96.15	93.82	73.86
	AVG	100.00	98.90	97.12	95.43	92.43	71.19
	SD	0.00	0.20	0.13	1.02	1.97	3.77
LE SO-9mg/g	Rep.1	100.00	98.42	95.71	95.71	91.49	66.91
	Rep.2	100.00	98.63	96.48	96.48	93.17	70.80
	AVG	100.00	98.53	96.10	96.10	92.33	68.86
	SD	0.00	0.15	0.54	0.54	1.19	2.75

Table 35 (Continue)

Formulation	% ATRA content	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
LE MO-1mg/g	Rep.1	100.00	98.74	97.43	94.93	93.14	71.70
	Rep.2	100.00	99.20	96.65	95.15	92.26	75.84
	AVG	100.00	98.97	97.04	95.04	92.70	73.77
	SD	0.00	0.33	0.55	0.16	0.62	2.93
LE MO-3mg/g	Rep.1	100.00	99.22	97.34	94.17	93.17	71.10
	Rep.2	100.00	99.24	97.24	94.93	92.32	70.02
	AVG	100.00	99.23	97.29	94.55	92.75	70.56
	SD	0.00	0.02	0.07	0.54	0.61	0.77
LE MO-5mg/g	Rep.1	100.00	98.95	97.09	94.91	91.82	72.56
	Rep.2	100.00	99.08	96.61	95.50	92.41	74.63
	AVG	100.00	99.02	96.85	95.20	92.11	73.59
	SD	0.00	0.09	0.34	0.42	0.42	1.46
LE MO-7mg/g	Rep.1	100.00	99.12	96.84	95.50	93.08	65.54
	Rep.2	100.00	98.99	95.71	94.45	92.59	73.02
	AVG	100.00	99.05	96.28	94.98	92.83	69.28
	SD	0.00	0.09	0.80	0.75	0.35	5.29
LE MO-9mg/g	Rep.1	100.00	98.92	95.66	93.56	91.64	66.85
	Rep.2	100.00	98.84	96.37	94.94	93.31	72.86
	AVG	100.00	98.88	96.01	94.25	92.47	69.85
	SD	0.00	0.05	0.50	0.97	1.18	4.25

Table 36 The change in percentage yield of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Formulation	% ATRA content	At initial (Day 0)	After Stability studies				
			Stored at 4C for 56 days				Autoclaving
			Day 14	Day 28	Day 42	Day 56	
NLC M-DPEG-3mg/g	Rep.1	100.00	98.33	95.99	93.88	91.51	72.03
	Rep.2	100.00	98.82	95.17	93.66	92.49	70.61
	AVG	100.00	98.58	95.58	93.77	92.00	71.32
	SD	0.00	0.35	0.58	0.15	0.69	1.01
NLC M-PPEG-3mg/g	Rep.1	100.00	98.78	94.32	94.25	90.47	71.25
	Rep.2	100.00	99.34	95.84	92.04	92.85	67.59
	AVG	100.00	99.06	95.08	93.15	91.66	69.42
	SD	0.00	0.40	1.07	1.56	1.69	2.59
NLC MO-DPEG-3mg/g	Rep.1	100.00	99.11	96.33	93.36	92.03	69.18
	Rep.2	100.00	98.67	95.91	92.63	90.20	67.15
	AVG	100.00	98.89	96.12	93.00	91.11	68.16
	SD	0.00	0.31	0.29	0.51	1.29	1.44
NLC MO-PPEG-3mg/g	Rep.1	100.00	98.59	95.81	92.69	89.58	68.88
	Rep.2	100.00	99.30	96.28	93.26	92.56	72.12
	AVG	100.00	98.95	96.04	92.98	91.07	70.50
	SD	0.00	0.50	0.33	0.40	2.11	2.29

Table 37 The change in particles size and zeta potential of ATRA–loaded LEs composed with different oil phase in different amount of initial ATRA concentration

Day	Formulation	Size (nm)		PDI		ZP (mV)	
		AVG	SD	AVG	SD	AVG	SD
14	LE S-1mg/g	251.75	1.51	0.16	0.04	251.75	1.51
	LE M-1mg/g	136.72	1.20	0.12	0.03	136.72	1.20
	LE M-3mg/g	160.33	1.72	0.12	0.05	160.33	1.72
	LE SO-1mg/g	154.38	1.33	0.12	0.03	154.38	1.33
	LE SO-3mg/g	152.80	2.51	0.14	0.05	152.80	2.51
	LE SO-5mg/g	153.67	1.67	0.14	0.03	153.67	1.67
	LE MO-1mg/g	141.00	1.10	0.12	0.01	141.00	1.10
	LE MO-3mg/g	140.25	1.50	0.13	0.02	140.25	1.50
	LE MO-5mg/g	151.30	1.94	0.13	0.03	151.30	1.94
28	LE S-1mg/g	254.40	3.18	0.24	0.03	254.40	3.18
	LE M-1mg/g	135.70	0.78	0.12	0.03	135.70	0.78
	LE M-3mg/g	163.38	2.59	0.12	0.05	163.38	2.59
	LE SO-1mg/g	158.98	0.99	0.14	0.03	158.98	0.99
	LE SO-3mg/g	157.37	1.73	0.14	0.04	157.37	1.73
	LE SO-5mg/g	156.87	1.58	0.12	0.05	156.87	1.58
	LE MO-1mg/g	140.85	1.32	0.14	0.03	140.85	1.32
	LE MO-3mg/g	140.27	1.09	0.14	0.02	140.27	1.09
	LE MO-5mg/g	153.33	1.27	0.17	0.03	153.33	1.27
42	LE S-1mg/g	251.32	3.87	0.26	0.05	251.32	3.87
	LE M-1mg/g	135.72	0.72	0.25	0.34	135.72	0.72
	LE M-3mg/g	163.10	1.93	0.15	0.06	163.10	1.93
	LE SO-1mg/g	160.67	1.40	0.14	0.03	160.67	1.40
	LE SO-3mg/g	160.75	2.00	0.14	0.03	160.75	2.00
	LE SO-5mg/g	161.87	1.25	0.14	0.03	161.87	1.25
	LE MO-1mg/g	139.98	0.90	0.14	0.03	139.98	0.90
	LE MO-3mg/g	145.22	2.48	0.13	0.05	145.22	2.48
	LE MO-5mg/g	154.02	1.13	0.13	0.03	154.02	1.13

Table 37 (Continue)

Day	Formulation	Size (nm)		PDI		ZP (mV)	
		AVG	SD	AVG	SD	AVG	SD
56	LE S-1mg/g	255.40	3.34	0.22	0.05	255.40	3.34
	LE M-1mg/g	136.17	0.64	0.12	0.04	136.17	0.64
	LE M-3mg/g	162.77	3.08	0.13	0.06	162.77	3.08
	LE SO-1mg/g	165.47	1.08	0.14	0.03	165.47	1.08
	LE SO-3mg/g	164.57	2.21	0.16	0.05	164.57	2.21
	LE SO-5mg/g	166.48	1.04	0.13	0.03	166.48	1.04
	LE MO-1mg/g	145.07	0.71	0.15	0.05	145.07	0.71
	LE MO-3mg/g	146.07	2.39	0.14	0.03	146.07	2.39
	LE MO-5mg/g	155.35	0.82	0.13	0.03	155.35	0.82
Autoclaving	LE S-1mg/g	564.05	15.14	0.21	0.03	564.05	15.14
	LE M-1mg/g	484.80	35.36	0.07	0.05	484.80	35.36
	LE M-3mg/g	406.95	13.81	0.06	0.06	406.95	13.81
	LE SO-1mg/g	627.63	16.64	0.18	0.05	627.63	16.64
	LE SO-3mg/g	670.52	16.97	0.23	0.06	670.52	16.97
	LE SO-5mg/g	658.58	23.39	0.18	0.05	658.58	23.39
	LE MO-1mg/g	455.60	14.03	0.16	0.05	455.60	14.03
	LE MO-3mg/g	457.13	21.51	0.19	0.05	457.13	21.51
	LE MO-5mg/g	460.00	19.56	0.15	0.04	460.00	19.56

Table 38 The change in particles size and zeta potential of ATRA–loaded NLCs composed with different oil phase in different amount of initial ATRA concentration

Day	Formulation	Size (nm)		PDI		ZP (mV)	
		AVG	SD	AVG	SD	AVG	SD
14	NLC S-1mg/g	170.30	1.61	0.15	0.01	170.30	1.61
	NLC S-3mg/g	171.62	1.45	0.12	0.03	171.62	1.45
	NLC M-1mg/g	151.53	0.70	0.15	0.04	151.53	0.70
	NLC M-3mg/g	151.47	1.75	0.15	0.03	151.47	1.75
	NLC M-5mg/g	153.68	1.52	0.14	0.04	153.68	1.52
	NLC SO-1mg/g	160.23	1.85	0.17	0.05	160.23	1.85
	NLC SO-3mg/g	168.87	3.00	0.12	0.05	168.87	3.00
	NLC SO-5mg/g	169.48	1.41	0.13	0.02	169.48	1.41
	NLC SO-7mg/g	174.10	1.28	0.13	0.03	174.10	1.28
	NLC SO-9mg/g	176.10	0.87	0.15	0.03	176.10	0.87
	NLC MO-1mg/g	142.03	0.65	0.14	0.02	142.03	0.65
	NLC MO-3mg/g	145.87	2.65	0.14	0.06	145.87	2.65
	NLC MO-5mg/g	145.25	0.72	0.15	0.02	145.25	0.72
	NLC MO-7mg/g	148.83	1.23	0.13	0.02	148.83	1.23
	NLC MO-9mg/g	153.13	1.29	0.14	0.03	153.13	1.29
28	NLC S-1mg/g	171.85	2.89	0.14	0.02	171.85	2.89
	NLC S-3mg/g	172.39	2.79	0.11	0.05	172.39	2.79
	NLC M-1mg/g	152.10	1.46	0.15	0.01	152.10	1.46
	NLC M-3mg/g	151.10	3.35	0.13	0.06	151.10	3.35
	NLC M-5mg/g	153.58	1.15	0.15	0.03	153.58	1.15
	NLC SO-1mg/g	164.65	0.73	0.15	0.04	164.65	0.73
	NLC SO-3mg/g	167.30	1.63	0.14	0.05	167.30	1.63
	NLC SO-5mg/g	168.15	1.21	0.13	0.03	168.15	1.21
	NLC SO-7mg/g	175.57	1.54	0.13	0.03	175.57	1.54
	NLC SO-9mg/g	177.63	2.27	0.15	0.03	177.63	2.27
	NLC MO-1mg/g	142.92	0.65	0.15	0.02	142.92	0.65
	NLC MO-3mg/g	151.37	1.69	0.13	0.06	151.37	1.69
	NLC MO-5mg/g	146.02	1.37	0.14	0.02	146.02	1.37
	NLC MO-7mg/g	150.52	1.06	0.12	0.02	150.52	1.06
	NLC MO-9mg/g	154.65	1.58	0.14	0.02	154.65	1.58

Table 38 (Continue)

Day	Formulation	Size (nm)		PDI		ZP (mV)	
		AVG	SD	AVG	SD	AVG	SD
42	NLC S-1mg/g	172.37	1.58	0.14	0.02	172.37	1.58
	NLC S-3mg/g	173.98	2.18	0.09	0.05	173.98	2.18
	NLC M-1mg/g	151.78	1.22	0.16	0.03	151.78	1.22
	NLC M-3mg/g	153.85	3.87	0.14	0.06	153.85	3.87
	NLC M-5mg/g	153.23	2.22	0.15	0.03	153.23	2.22
	NLC SO-1mg/g	166.77	2.35	0.17	0.05	166.77	2.35
	NLC SO-3mg/g	172.33	2.07	0.16	0.04	172.33	2.07
	NLC SO-5mg/g	173.02	2.55	0.16	0.04	173.02	2.55
	NLC SO-7mg/g	177.02	1.41	0.15	0.03	177.02	1.41
	NLC SO-9mg/g	180.27	1.83	0.14	0.04	180.27	1.83
	NLC MO-1mg/g	143.82	1.18	0.14	0.03	143.82	1.18
	NLC MO-3mg/g	149.78	2.79	0.13	0.02	149.78	2.79
	NLC MO-5mg/g	146.35	0.53	0.13	0.03	146.35	0.53
	NLC MO-7mg/g	151.57	1.46	0.15	0.03	151.57	1.46
	NLC MO-9mg/g	154.67	0.97	0.15	0.02	154.67	0.97
56	NLC S-1mg/g	174.65	1.87	0.14	0.03	174.65	1.87
	NLC S-3mg/g	176.48	2.29	0.15	0.04	176.48	2.29
	NLC M-1mg/g	152.52	1.63	0.13	0.02	152.52	1.63
	NLC M-3mg/g	152.63	2.90	0.15	0.03	152.63	2.90
	NLC M-5mg/g	153.57	0.85	0.14	0.03	153.57	0.85
	NLC SO-1mg/g	174.20	1.52	0.14	0.03	174.20	1.52
	NLC SO-3mg/g	174.22	3.71	0.16	0.04	174.22	3.71
	NLC SO-5mg/g	176.33	1.54	0.14	0.04	176.33	1.54
	NLC SO-7mg/g	180.45	1.99	0.16	0.05	180.45	1.99
	NLC SO-9mg/g	183.78	1.26	0.16	0.05	183.78	1.26
	NLC MO-1mg/g	144.68	1.91	0.13	0.02	144.68	1.91
	NLC MO-3mg/g	145.52	2.30	0.13	0.06	145.52	2.30
	NLC MO-5mg/g	147.78	1.00	0.13	0.03	147.78	1.00
	NLC MO-7mg/g	152.10	2.10	0.14	0.02	152.10	2.10
	NLC MO-9mg/g	154.27	1.20	0.14	0.02	154.27	1.20

Table 38 (Continue)

Day	Formulation	Size (nm)		PDI		ZP (mV)	
		AVG	SD	AVG	SD	AVG	SD
Autoclaving	NLC S-1mg/g	480.42	7.79	0.14	0.03	480.42	7.79
	NLC S-3mg/g	484.88	23.00	0.16	0.05	484.88	23.00
	NLC M-1mg/g	467.10	14.64	0.17	0.03	467.10	14.64
	NLC M-3mg/g	463.97	26.38	0.46	0.42	463.97	26.38
	NLC M-5mg/g	468.80	9.37	0.15	0.03	468.80	9.37
	NLC SO-1mg/g	447.63	18.20	0.15	0.04	447.63	18.20
	NLC SO-3mg/g	453.08	27.63	0.15	0.05	453.08	27.63
	NLC SO-5mg/g	449.45	3.09	0.13	0.02	449.45	3.09
	NLC SO-7mg/g	451.38	22.67	0.17	0.05	451.38	22.67
	NLC SO-9mg/g	447.80	20.54	0.16	0.03	447.80	20.54
	NLC MO-1mg/g	341.93	6.64	0.14	0.04	341.93	6.64
	NLC MO-3mg/g	341.22	13.97	0.14	0.04	341.22	13.97
	NLC MO-5mg/g	349.32	8.51	0.15	0.02	349.32	8.51
	NLC MO-7mg/g	349.83	6.40	0.15	0.03	349.83	6.40
	NLC MO-9mg/g	354.38	6.08	0.15	0.03	354.38	6.08

Table 39 The change in particles size and zeta potential of ATRA–loaded DPEG or PPEG coated NLCs composed of different oil phase at 3 mg/g of initial ATRA concentration.

Day	Formulation	Size (nm)		PDI		ZP (mV)	
		AVG	SD	AVG	SD	AVG	SD
14	NLC M-DPEG-3mg/g	170.35	2.15	0.15	0.03	170.35	2.15
	NLC M-PPEG-3mg/g	172.69	2.40	0.17	0.04	172.69	2.40
	NLC MO-DPEG-3mg/g	162.89	1.94	0.15	0.03	162.89	1.94
	NLC MO-PPEG-3mg/g	160.39	2.27	0.16	0.05	160.39	2.27
28	NLC M-DPEG-3mg/g	172.63	2.63	0.16	0.02	172.63	2.63
	NLC M-PPEG-3mg/g	171.84	2.10	0.18	0.02	171.84	2.10
	NLC MO-DPEG-3mg/g	160.35	2.68	0.17	0.03	160.35	2.68
	NLC MO-PPEG-3mg/g	159.82	2.10	0.17	0.04	159.82	2.10
42	NLC M-DPEG-3mg/g	172.41	2.02	0.15	0.05	172.41	2.02
	NLC M-PPEG-3mg/g	174.29	1.26	0.18	0.06	174.29	1.26
	NLC MO-DPEG-3mg/g	159.02	3.60	0.15	0.05	159.02	3.60
	NLC MO-PPEG-3mg/g	157.75	3.78	0.17	0.05	157.75	3.78
56	NLC M-DPEG-3mg/g	170.84	2.44	0.13	0.03	170.84	2.44
	NLC M-PPEG-3mg/g	173.48	2.04	0.17	0.05	173.48	2.04
	NLC MO-DPEG-3mg/g	163.32	3.23	0.17	0.05	163.32	3.23
	NLC MO-PPEG-3mg/g	162.65	2.61	0.19	0.04	162.65	2.61
Autoclaving	NLC M-DPEG-3mg/g	172.50	2.08	0.12	0.03	172.50	2.08
	NLC M-PPEG-3mg/g	173.66	2.54	0.14	0.04	173.66	2.54
	NLC MO-DPEG-3mg/g	159.02	3.60	0.19	0.04	159.02	3.60
	NLC MO-PPEG-3mg/g	157.25	3.09	0.17	0.05	157.25	3.09

Table 40 Cytotoxicity on HL-60 cells of ATRA in solution and ATRA-loaded lipid nanoparticles composed of different oil phase.

Free drug								
ATRA	ATRA concentration (ng/ml)		1	10	102	512	1024	2560
	% cells viability	AVG	100.00	73.17	68.83	63.82	54.24	31.25
		SD	2.98	2.88	4.60	3.78	2.22	2.43
LE								
LE S-1mg/g	ATRA concentration (ng/ml)		1	11	113	566	1133	2832
	% cells viability	AVG	100.00	75.63	66.61	59.06	42.59	21.42
		SD	6.64	4.79	2.54	5.23	3.42	1.15
LE M-3mg/g	ATRA concentration (ng/ml)		1	11	110	550	1100	2750
	% cells viability	AVG	100.00	75.07	71.22	49.20	26.17	22.25
		SD	6.36	7.87	6.28	2.87	4.29	1.55
LE SO-3mg/g	ATRA concentration (ng/ml)		1	11	108	541	1081	2703
	% cells viability	AVG	100.00	72.68	66.73	52.48	45.07	25.19
		SD	3.54	1.95	2.35	2.33	1.62	6.93
LE MO-3mg/g	ATRA concentration (ng/ml)		1	11	109	546	1093	2732
	% cells viability	AVG	100.00	65.85	58.54	42.52	25.37	16.54
		SD	5.47	1.78	3.87	4.02	1.53	1.10

Table 40 (Continue)

NLC								
NLC S-3mg/g	ATRA concentration (ng/ml)		1	10	100	501	1002	2506
	% cells viability	AVG	100.00	78.51	69.96	61.23	42.46	22.93
		SD	4.85	1.81	6.73	2.21	1.44	1.85
NLC M-3mg/g	ATRA concentration (ng/ml)		1	10	101	505	1010	2526
	% cells viability	AVG	100.00	76.28	65.13	54.33	42.69	22.11
		SD	5.50	1.41	3.76	9.77	2.34	1.33
NLC SO-3mg/g	ATRA concentration (ng/ml)		1	10	102	509	1019	2547
	% cells viability	AVG	100.00	80.18	67.30	59.86	43.27	19.35
		SD	14.38	3.09	4.61	5.28	2.13	1.77
NLC MO-3mg/g	ATRA concentration (ng/ml)		1	10	103	514	1028	2571
	% cells viability	AVG	100.00	73.30	63.80	58.51	45.73	21.28
		SD	4.97	8.21	5.08	3.29	17.51	2.86
Polymer coated-NLC								
NLC M-DPEG-3mg/g	ATRA concentration (ng/ml)		1	10	101	505	1010	2525
	% cells viability	AVG	100.00	75.02	72.11	59.01	37.44	13.90
		SD	9.49	2.46	4.52	5.43	5.20	1.99
NLC M-PPEG-3mg/g	ATRA concentration (ng/ml)		1	10	101	504	1007	2518
	% cells viability	AVG	100.00	80.16	75.08	64.19	42.57	13.04
		SD	7.72	7.31	3.46	4.50	1.95	1.94
NLC MO-DPEG-3mg/g	ATRA concentration (ng/ml)		1	10	100	501	1003	2507
	% cells viability	AVG	100.00	70.23	59.89	50.13	36.81	13.36
		SD	12.37	4.65	4.93	3.20	4.27	1.87
NLC MO-PPEG-3mg/g	ATRA concentration (ng/ml)		1	10	102	510	1020	2551
	% cells viability	AVG	100.00	73.98	66.56	56.95	43.25	16.67
		SD	11.40	0.87	1.86	2.67	7.65	2.10

Table 41 Cytotoxicity on HepG2 cells of ATRA in solution and ATRA-loaded lipid nanoparticles composed of different oil phase.

Free drug								
ATRA	ATRA concentration (ng/ml)		0.1	2.5	5.0	10.1	25.2	50.4
	% cells viability	AVG	100.000	98.090	98.381	96.957	76.950	35.092
		SD	8.716	5.502	5.187	3.676	5.907	6.429
LE								
LE S-1mg/g	ATRA concentration (ng/ml)		0.1	2.9	5.8	11.6	29.0	57.9
	% cells viability	AVG	100.000	72.102	64.284	55.643	20.140	2.251
		SD	6.560	5.041	1.174	4.061	2.588	0.892
LE M-3mg/g	ATRA concentration (ng/ml)		0.1	2.8	5.5	11.1	27.7	55.3
	% cells viability	AVG	100.000	67.378	63.254	50.597	9.812	0.284
		SD	8.122	6.790	6.309	9.017	3.115	0.088
LE SO-3mg/g	ATRA concentration (ng/ml)		0.1	2.6	5.2	10.4	26.0	52.1
	% cells viability	AVG	100.000	69.967	6.800	1.610	1.491	0.418
		SD	5.115	5.026	1.449	1.757	0.352	0.370
LE MO-3mg/g	ATRA concentration (ng/ml)		0.1	2.7	5.3	10.6	26.5	53.0
	% cells viability	AVG	100.000	61.867	15.512	3.855	0.813	0.422
		SD	0.000	4.620	2.565	0.772	0.508	0.148

Table 41 (Continue)

NLC								
NLC S-3mg/g	ATRA concentration (ng/ml)		0.1	2.5	5.0	10.0	25.1	50.2
	% cells viability	AVG	100.000	84.150	68.518	48.487	6.334	4.743
		SD	11.657	7.019	3.206	2.895	2.445	0.736
NLC M-3mg/g	ATRA concentration (ng/ml)		0.1	2.5	5.0	10.1	25.2	50.4
	% cells viability	AVG	100.000	85.515	75.712	67.489	6.199	3.605
		SD	7.887	6.385	6.080	5.811	1.285	0.953
NLC SO-3mg/g	ATRA concentration (ng/ml)		0.1	2.5	5.0	10.1	25.2	50.3
	% cells viability	AVG	100.000	80.435	44.680	4.569	4.017	1.257
		SD	8.049	3.814	4.278	1.037	0.874	0.295
NLC MO-3mg/g	ATRA concentration (ng/ml)		0.1	2.5	5.0	10.0	25.0	50.1
	% cells viability	AVG	100.000	71.433	40.903	3.112	0.729	0.392
		SD	0.000	4.639	4.174	1.306	0.275	0.331
Polymer coated-NLC								
NLC M-DPEG-3mg/g	ATRA concentration (ng/ml)		0.1	2.5	5.0	10.0	25.1	50.2
	% cells viability	AVG	100.000	67.133	57.495	46.155	10.578	0.425
		SD	8.254	4.776	4.314	2.592	4.388	0.202
NLC M-PPEG-3mg/g	ATRA concentration (ng/ml)		0.1	2.5	5.1	10.1	25.4	50.7
	% cells viability	AVG	100.000	67.706	62.904	57.386	7.880	0.581
		SD	2.942	5.551	3.454	6.028	3.185	0.345
NLC MO-DPEG-3mg/g	ATRA concentration (ng/ml)		0.1	2.6	5.2	10.3	25.8	51.6
	% cells viability	AVG	100.000	74.225	14.933	5.797	6.366	2.733
		SD	5.613	2.926	2.911	2.100	0.448	0.963
NLC MO-PPEG-3mg/g	ATRA concentration (ng/ml)		0.1	2.6	5.1	10.2	25.5	51.1
	% cells viability	AVG	100.000	64.993	15.625	3.535	0.535	0.342
		SD	4.273	3.545	1.506	1.773	0.126	0.306

APPENDIX C

LIST OF ABBREVIATIONS

%	percent
%RSD	coefficient of variation
%v/v	percent volume by volume
%w/w	percent weight by weight
μg	microgram
μl	microliter
μm	micrometer
>	more than
<	less than
≤	less than or equal to
±	plus or minus
°C	degree Celsius
APL	acute promyelocytic leukemia
ATRA	all-*trans* retinoic acid
AUC	Area under the curve
BHT	butylated hydroxyl toluene
cm	centimeter
cm^2	square centimeter
cp	centipoises
CP	cetylpalmitate
DMSO	dimethyl sulfoxide
DPEG	1,2-distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethyleneglycol)-2000] (Ammonium Salt) (DSPE-PEG2000)
EO	ethylene oxide
EPR	enhanced permeability and retention effects

FFA	free fatty acid
ft	foot
g	gram
h	hour
HepG2	human hepatocellular carcinoma
HL-60	human acute promyelocytic leukemia
HLB	hydrophilic-lipophilic balance
HPLC	high pressure liquid chromatography
IMDM	Iscoved's Modified Dulbecco's Medium
IPA	isopropyl alcohol
i.m.	intramuscular
i.v.	intravenous
kg	kilogram
lbs	pounds
LCT	long chain triglyceride
LE	lipid emulsions
MCT	medium chain triglyceride
mg	milligram
min	minute
ml	milliliter
MPS	Mononuclear phagocyte system
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
mV	millivolt
MWCO	molecular weight cut-off
N	normality
NaOH	sodium hydroxide
ng	nanogram
NLC	nanostructured lipid carriers
nm	nanometer

O	oleic acid
O/W	oil in water
PC	phosphatidylcholine
PCS	photon correlation spectroscopy
PDI	polydispersity index
PEG	poly(ethylene glycol)
PG	phosphatidylglycerol
pH	The negative logarithm of the hydrogen ion concentration
pKa	Acid dissociation constant
PO	propylene oxide
ppm	part per million
PPEG	N-phthaloylchitosan-grafted poly (ethylene glycol) methyl ether (PLC-g-mPEG)
psi	pounds per square inch
qs.	add to
R^2	coefficient of determination
RARs	retinoic acid receptors
RES	reticuloendothelial system
rpm	round per minute
RXRs	retinoid X receptors
S	soybean oil
s.c.	subcutaneous
SD	standard deviation
sec	second
SLN	solid lipid nanoparticles
sq.	square
t½	half life
UVA	ultraviolet A
UV-VIS	UV visible

W/O	water in oil
W/O/W	water in oil in water
ZP	zeta potential

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