Study on Evaluation and Control of Release Property of Drug-encapsulated Liposomes by Ultrasound Irradiation

超音波照射による薬物内包リポソームの放出特性の評価と制御に関する研究

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Abstract

Liposomes are widely known as artificial cells whose lipid components exist in nature. They are formed in aqueous solutions when hydrated phospholipids arrange themselves in circular sheets with consistent head-tail orientation. Liposomes are microscopic, spherical vesicles with aqueous solutions core(s) which are enclosed by bilayer lipid membrane. There are many kinds of lipids that can compose liposomes, hence it is possible to obtain liposomes with different properties by adjusting the type and ratio of the components. Liposomes are not only used in simulation of physiological response, like photosynthesis and enzyme activity, by chemical modification, but also used as drug carriers and are researched in a wide area, such as engineering, medicine, pharmacology etc. As a drug delivery system, liposomes offer the possibility to delivery drugs to the right place, at the right time and with the proper amount of dose thus improve the efficacy, reduce toxicity and improve patient compliance and convenience. After fulfilling the blood longevity of liposomes by incorporating PEG into its lipid membrane, the controlled release of drug encapsulated liposomes by physical (like temperature and ultrasound) and chemical (like pH) stimuli are attracting a great deal of attention.

However, the sensitivity of PEGylated liposomes to ultrasound has been investigated, but the influences of ultrasound frequency and power on the release properties are remained unclear. Further, in the investigation on ultrasound induced release, researchers often use a commercial ultrasound probe with fixed frequency. Few of them put their interests on the frequency shifts of the ultrasound probe when it is acting on different objects which would probably influence the release efficiency greatly. Moreover, the traditional fluorescence method for evaluation the

release property of drug encapsulated liposomes has shown its inconvenience to those non-fluorescence substances measurement as well as on-line measurement.

In this study, liposomes composed of POPC, DPPE-PEG2000 and cholesterol with different molar ratios were prepared and acted as the research objects. And instead of drugs, a fluorescent substance (calcein, dissolved in NaOH solution) was encapsulated in the PEGyalated liposomes. Their release properties were investigated when the liposomes were exposed to low frequency ultrasound irradiation (<100kHz) by adjusting the input frequency and power of an ultrasound probe. According to the results, an efficiency control method was proposed. Further, a new evaluation method for liposome release property based on impedance measurement was presented and its validity was confirmed by comparing with the fluorescence intensity evaluation method. The proposed method has the potential to evaluate the release property of ionic substances. The general outline about this thesis is given as follows.

Chapter 1 provides a brief introduction on the background of liposome research, the objective and the organization of this dissertation.

Chapter 2 presents the basic principle of liposome formation and introduces the preparation methods of liposomes. Further, the morphologies of the prepared liposomes were studied by transmission electron microscopy. The result showed that the liposomes were prepared successfully and were multilamellar structured sphere whose size was around 100nm.

Chapter 3 investigates how the physical properties of liposomes, such as size distribution, zeta-potential and release profile are influenced by ultrasound irradiation, using commercial ultrasound equipments. And the basic conception and principle of dynamic light scattering, zeta potential and the traditional fluorescence evaluation method for release profile investigation of drug-encapsulated liposomes are introduced. Further, the release of calcein liposomes induced by temperature and ultrasound was studied, respectively. Results showed that the low frequency

ultrasound was effective to release the inner substances of liposmes while temperature was of much lower efficiency to the sample prepared with POPC, DPPE-PEG200 and cholesterol. The size distribution of the liposomes decreased when exposed to ultrasound. And their zeta-potential also decreased because the release of encapsulated calcein. It also showed that ultrasound irradiation promotes the release of liposomes inner substances and PEG influences ultrasound induced release in a mode of dose dependent fashion up to 10% PEG.

Chapter 4 gives a new idea on control of the release rate of the inner-substances from liposomes the ultrasonic irradiation. Two kinds of ultrasonic control methods are tested for investigation of the efficiency of the release property of the liposomes. One control method is to increase the input power by fixing its frequency just near the probe resonant frequency. Another method is to adjust the input frequency around the probe resonant frequencies but fixing the input power. As a conclusion, the release property of liposomes could be controlled efficiently by an ultrasonic probe in the way that the input frequency should be selected a little lower than its resonant frequency, so that the release speed of drugs from the encapsulated liposomes can be adjusted suitably by the input power.

Chapter 5 describes the development of a novel method for evaluation of the liposomes release property by measuring the electric impedance changes of liposome suspensions. To validate the proposed impedance measuring method, the calcein release rates were evaluated both by the impedance changes and the fluorescence intensity changes in calcein-liposome suspensions. With the comparison of these results obtained by the two methods, it is shown that the impedance method has much wider detecting concentration range than the fluorescence one. Furthermore, the impedance method can be efficiently used for evaluation of the release property on various ionic substances encapsulated within liposomes.

Chapter 6 provides conclusions and prospects of the thesis work.

論文の要旨

リポソームは生体由来の脂質で構成される人工細胞膜として知られており，液体中で脂質分子は自動的に二重層膜を形成し，水相溶液を内包した円球体となる．リポソームを構成する脂質の種類は数多くあり，その脂質の種類，割合を変化させることで，様々な機能性を付加することが可能である．そのため，光合成や酵素などの生体反応シミュレーションに用いられるだけでなく，薬物の運搬体(Drug Carrier)にも用いられ，生化学，工学，医学，薬学など幅広い分野で研究がなされている．特に生体親和性が高いため，薬物を特定部位に適切な量運搬し，副作用の軽減や薬物の効果を増大するドラッグ・デリバリーシステム（以下 DDS）の運搬体として注目されている．また，リポソーム膜に PEG を修飾することで長時間生体内に存在させることもできるため，DDS の研究において，PEG 修飾リポソームに物理的（超音波，温度等）刺激や化学的（pH 等）な刺激を与えカプセルに内包した薬剤を放出制御する研究が注目されている．

これまでの研究から PEG 修飾リポソームに超音波を加えると内包薬物が放出されることは確認されているが，超音波の周波数や出力の違いによる膜の放出特性に関しては不明な点が多い．また，超音波照射による薬物漏出研究では市販超音波装置をよく利用しているが，作用部位によってプローブ本体の共振周波数が変化するためリポソーム漏出制御の不安定の原因となる．さらに，今まで幅広く利用している伝統的な蛍光強度評価法は非蛍光性物質に対しての測定およびオンーライン測定にも非常に不便である．

本研究は，リポソームの材料として一般的な POPC, DPPE-PEG2000，コレステロールの比率を変えることにより，数種のリポソームを調製し，超音波を与えた場合の放出特性について解明することを目的とする．具体的には，内包薬物の代わりに蛍光物質であるカルセイン（Calcein）を用い，蛍光剤漏出時の蛍光強度を測ることにより，低周波

数超音波（＜100kHz）プローブの駆動周波数とパワーを変化させながらPEG修飾リポソームの内包薬物の放出特性について調査する．実験結果からPEG脂質の割合に対して超音波照射時の内包薬物の放出特性を解明し，さらに作用部位によらず安定した放出特性を可能とする制御法を提案する．また，イオン系物質に対してインピーダンス測定法に基づく新しい放出特性の評価方法を提案し，その有効性を蛍光強度評価法で確認するものである．本論文は緒論・結言を含め6章から構成されている．

第１章では，リポソームに関する研究の背景と本論文の構成について述べている．

第２章では，リポソームの形成原理と調整方法について説明している．また，調整したリポソームの脂質濃度を確認する方法及び，リポソームの外観を透過型電子顕微鏡（TEM）で観察する方法について説明した．TEM観察結果から，調製したリポソームは多重層膜構造の円球体であり，粒径は１００nm前後になっていることが分かった。

第３章では，超音波照射によるリポソームの物理的な特性（粒径，ゼータ電位，漏出率）評価の基礎実験を行う．観察方法は動的光散乱，ゼータ電位，蛍光強度法であり，本章で其々の基本原理について説明し，温度および超音波の影響でリポソーム漏出率を評価し，超音波照射前後のリポソーム平均粒径及びゼータ電位の変化を検討している．本研究で調整したリポソームに対しては温度変化による漏出率は低く，低周波数超音波照射でリポソームの内包物は多く漏れることが明らかになった．また，超音波照射後に粒径が小さくなることが確認され，ゼータ電位は内包されているカルセインの漏出による小さくなることも確認された．実験結果から，超音波照射はリポソームの内包物の漏出に大きく寄与することを確認できた．

第４章では，超音波照射による薬物の放出制御を目的に数種類のリポソームに対して超音波照射実験を行う．照射する超音波は超音波プローブを用いて行い，照射する超音波の周波数及び入力電圧の変化に伴う内包薬物の放出特性を観察し，薬物を効果的な

放出させる制御方法について考案する．リポソームへの超音波照射実験から，共振周波数付近で放出率の大きな変化が見られた．プローブの特性評価実験から，共振周波数より低く目に入力周波数を設定することで，薬物の放出制御に最も効果的であることが確認された．

第５章では，イオン系物質の漏出特性の評価するため，インピーダンス測定による新たなリポソーム漏出特性を評価する方法を提案する．このインピーダンス測定法の有効性を確信するためリポソームに内包したカルセインの漏出率を同時に蛍光強度で評価した．両方法で得られた結果を比較することでインピーダンス測定法の有効性が証明された．

第６章は，本研究の結論および展望であり，本論文で取り扱ってきた各章の内容と得られた結果について要約し，将来の展望について述べている．

Contents

1

INTRODUCTION

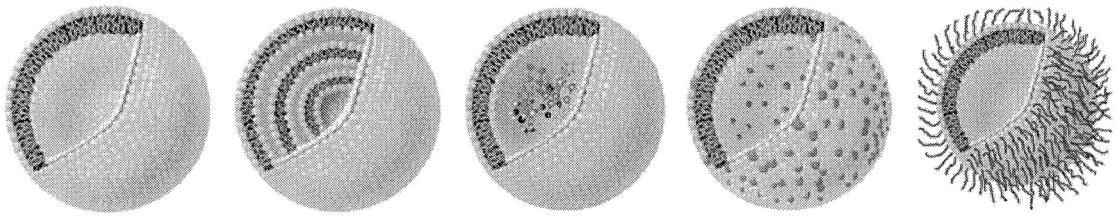
A drug delivery system (DDS) is the engineering of physical, chemical, and biological components into systems for delivering controlled amounts of a therapeutic agent over a prolonged period, thereby maintaining plasma or tissue drug levels at a constant level (http://www.answers.com). Such delivery systems offer numerous advantages compared to conventional dosage forms including improved efficacy, reduced toxicity and improved patient compliance and convenience (Uhrich K.E. et al., 1999). To date, varieties of delivery systems have been devised over the years, for example microspheres (Dongming Peng et al., 2007), nanoparticles (A. Aumelas, et al., 2007), nanocapsules (Sunghoon Kim, et al., 2008), niosomes (Maria Manconi, et al., 2002), micellular systems (Jiaping Lin, et al., 2009) and liposomes (Ashish Garg, et al., 2009). Among these, liposome system attracts more interests and has been under extensive investigation ever since shortly after their discovery by Bangham, et al. (A.D.Bangham, et al, 1965).

In this chapter, the background information of liposomes, such as history, merits and classification of liposomes, are to be introduced briefly as well as the scopes, objective and organization of this thesis.

1.1 Background

1.1.1 History of liposomes

Liposomes are formed in aqueous solutions when hydrated phospholipids arrange themselves in circular sheets with consistent head-tail orientation. They are microscopic, spherical vesicles with aqueous solutions core(s) which are enclosed by bilayer lipid membrane. It was first reported in the year of 1965 by Bangham and co-workers (A. D. Bangham, et al, 1965) who was studying the role of phospholipids in blood clotting. The word "liposome" was coined by Sessa and Weissmann (Grazia Sessa and Gerald Weissmann, 1968) according to Bangham's expression and is derived from the Greek: "lipo" referring to their fatty constitution and "soma" referring to their structure. From then on, the name of "liposome" is gaining favor and winning general acceptance. To given a intuitive impression of liposome, Fig 1.1 shows the several models of liposome where a is unilamellar liposome, b is multiplayer liposome, c is hydrophilic drugs encapsulated liposome, d is hydrophobic drugs encapsulated liposome and e is PEGylated liposome.



a b c d e

Fig. 1.1. Models of liposomes. a: unilamellar liposome; b: multilamellar liposome; c: hydrophilic drugs encapsulated liposome; d: hydrophobic drugs encapsulated liposome; e: PEGylated liposome (cited from http://www.encapsula.com/)

Since the first report of liposome in 1965, it has been over 40 years and progresses have been made in the study of liposomes. Table 1.1 lists the most important milestones in the process of liposome evolution from which one can get a global idea of liposome history.

Table 1.1 The most important milestones in the process of liposome evolution (http://www.liposome.org/pages/evolution.html)

Year	Events
1965	First description of closed lipid bilayer vesicles
1968	Introduction of the term "liposomes" to describe closed lipid bilayer vesicles
1971	Liposomes first used as delivery systems of drugs
1974	First patients to be injected with liposomes
1979	Liposomes first used as delivery systems of nucleic acids to cells
1980	First monoclonal antibody-targeted liposomes, termed "immunoliposomes" in 1981
1987	First sterically-stabilized long-circulating liposome system introduced
1987	First synthetic cationic liposomes delivery genes to cells
1992	First liposome-based non-viral vector gene therapy clinical trial on cystic fibrosis patients
1993	First liposome-based vaccine (against hepatitis A) is marketed
1995	The liposome encapsulated form of the anticancer drugs doxorubicin and daunorubicin approved for human use
1995	First long-circulating immunoliposomes
1997	First liposome-based DNA vaccine

1.1.2 Merits of liposomes

The idea of using liposomes for drug delivery has been aroused since the early 1970s (Gregoriadis G, et al, 1971). The goals are to protect the body from unwanted side effects of various drugs and, when made to be targeted to specific tissue such as a tumor and inflammation sites, to protect the encapsulated drugs from rapid degradation hence achieve desired concentrations of these drugs at a target site. Liposomes continue to arouse great interest due to its unique properties, mainly their biocompatibility and vesicular structure which can encapsulate a wider range of drugs or diagnosis agents by hydrophilic molecules in its aqueous cores, hydrophobic molecules in its lipid membranes, and amphiphilic molecules in both phases, as reviewed by Gulati, et al. (Monica Gulati, et al, 1998), see Fig. 1.2.

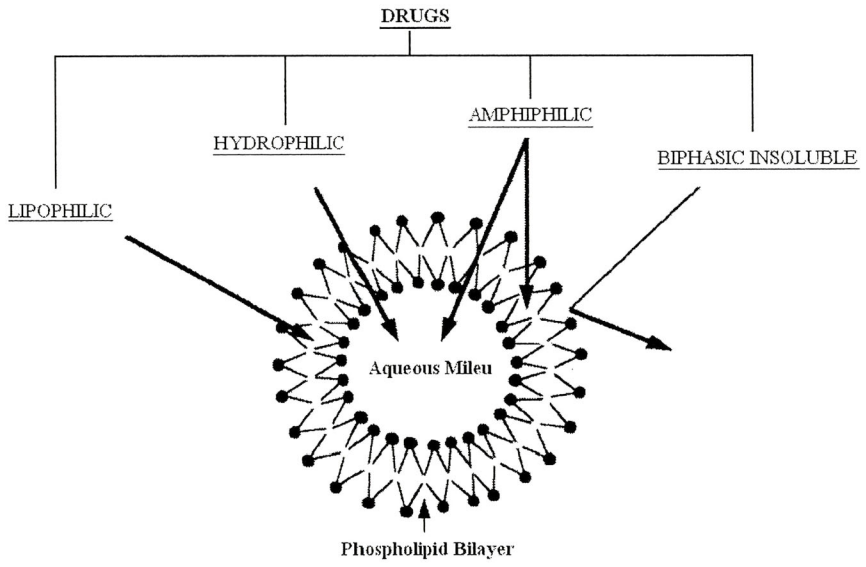


Fig. 1.2 Types of drugs and site of their incorporation into liposomal vesicle. Cite from: M. Gulati, et al., Internatial Jounal of Pharmaceutics 165(1998) 129-168

Moreover, liposomes have several characteristics which also contribute to their increasing use as drug delivery devices, namely:

- They are biodegradable and biocompatible
- Site specific drug delivery can be achieved
- Liposomes can protect the encapsulated drug from metabolic degradation and reduce the systemic toxicity of the drug.
- Liposomes can be employed as sustained release vehicles.
- Liposomes can enhance cutaneous/percutaneous drug absorption.
- Liposomes can be administered via practically all routes of drug delivery.

1.1.3 Classification

There are a number of possible classification schemes for liposomes based on certain distinguishing characteristics. It can be categorized in the basis of size and lamellarity (the number of phospholipid membrane layers), preparation method, function and modifier, etc. These different types of liposomes are discussed below under these headings.

Size and lamellarity

Perez-Soler divides them on the basis of their size and lamellarity (Perez-Soler R., et al, 1989). As shown in Fig. 1.3, liposomes are usually classified into five categories: small unilamellar vesicles (SUVs, 25-50 nm), large unilamellar vesicles (LUVs, 100 nm–1 μm), multilamellar vesicles (MLVs, 0.1–15 μm), giant unilamellar vesicles (GUVs, 1-200 μm), and multi-vesicular vesicles (MVVs, 1.6-10.5 μm) (Agustina Gómez-Hens and Juan Manel Fernández-Romero, 2005).

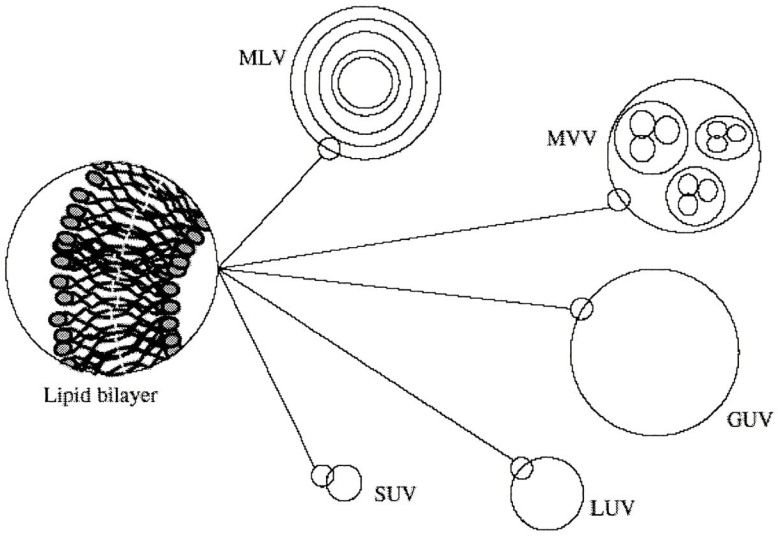


Fig. 1.3 Categories of liposomes by size and lamellarity: MLV, multilamellar vesicle; MVV, multivesicular vesicle; GUV, giant ulinamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle. Cited from (Agustina Gómez-Hens and Juan Manel Fernández-Romero, 2005)

Preparation method

There are methods for liposome preparation, such as hand-shaken, sonication, reverse-phase evaporation, freeze-dried rehydration and detergent depletion method. Hence the corresponding liposomes are named as hand-shaken liposomes, sonication liposomes, reverse-phase evaporation (REV) liposomes, freeze-dried rehydrated liposomes and detergent depletion liposomes.

Function and modifier

Liposomes could be divided by its function and modifiers. As shown in Fig. 1.4, the circles represent the membrane of liposomes and the four ellipses (a) in each circle are drugs encapsulated in the liposome. The first liposome (No. 1) is traditional "plain" liposome with no modifiers incorporated in the lipid membrane, also called unmodified liposome. The second

liposome (No. 2) is modified with special targeting ligand (b) in its surface, called targeted liposome or immunoliposome. The third liposome (No. 3) is magnetic liposome which encapsulated magnetic particles (c) together with drugs and is sensitive for external magnetic field and use as a contrast agent for magnetic resonance imaging. The forth (No. 4) is long circulating liposome, also called stealth liposomes, with protecting polymer (usually PEG) (d) incorporated in its membrane and allowing for prolonged circulation in blood. The fifth is contrast liposome for imaging purpose loaded with heavy metal atoms (e). The sixth is cell penetrating liposome with cell-penetrating peptide, CCP, (f) attached in its surface and allowing enhancement of uptake by cells. The seventh is DNA carrying liposome, or lipoplex, with DNA (g) complexed the liposome via surface positive charge. The eighth is a multifunction liposome combining the properties of the former liposomes (No. 1-7).

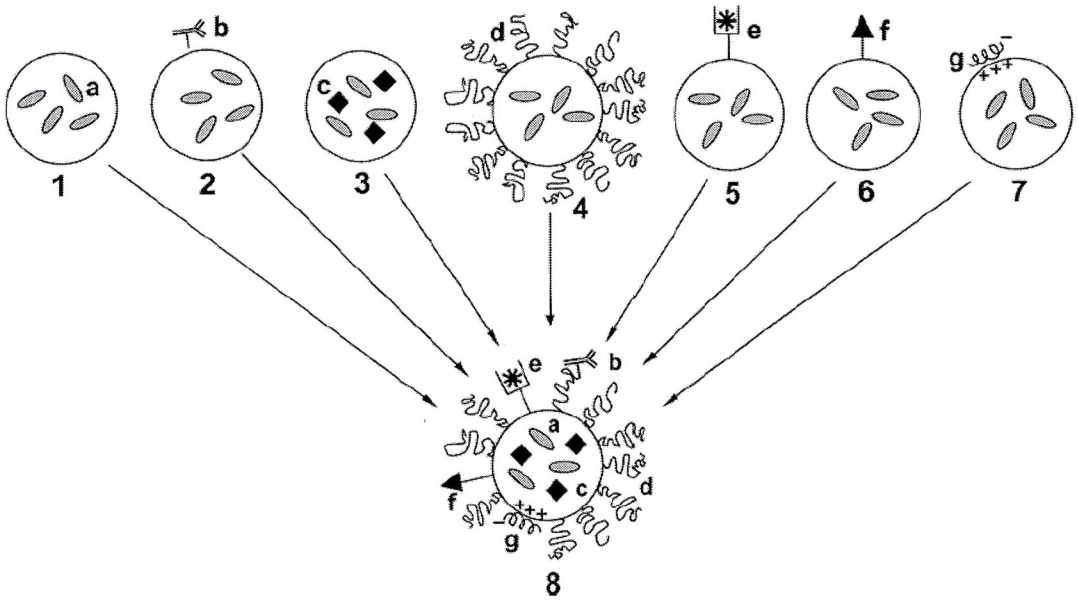


Fig. 1.4 schematic structures of multifunctional liposomes

Cited from (Vladimir P. Trochilin, 2006)

Trigger mechanism

Liposomes could also be classified by the way how the encapsulated substances are to be released, such as light active liposomes, themal active liposomes, ultrasound active lipsomes, pH active lipsomes, to name a few.

1.1.4 Controlled release

As summarized by Tomas L. Andresen et al (Thomas L. Andresen, et al, 2005), there are three basic requirements which need to be fulfilled if liposomes are to be successful in delivering drugs: (i) prolonged blood circulation, (ii) sufficient cite accumulation, (iii) controlled drug release or uptake by cells with a release profile matching the pharmacodynamics of the drugs.

After the long circulation liposomes and immunoliposomes have been developed, the first two requirements have been solved, though it is far from satisfactory. One of the single biggest challenges, then, facing drug delivery for liposomes (and indeed other drug carriers) is to initiate and produce release of the encapsulated drug only at the diseased site and at controllable rates. There are two stages for targeted delivery of DDS, i.e., site-specific targeting and site-specific triggering (Thomas L. Andresen et. al., 2005). Drug carries with proper size will accumulate to the site in a passive mode because the vascular interstice of a solid tumor is much larger than the normal tissue (Rakesh K. Jain, 1998; Seong Hoon Jang et al., 2003), while antibodies or receptors are also incorporated into the liposomes membrane or the covering polymer layer to obtain active targeting (Eric Forssen and Michael Willis, 1998; Rong Deng and Joseph P. Balthasar, 2005; Kimberley Laginha et al., 2005; Tomotaka Kobayashi et al., 2007). In the other hand, pH- (Oleg V. Gerasimov et al., 1999; Debra T. Auguste et al., 2006; Kazue Hiraka et al.,

2008), redox- (Xin Guo and Francis C. Szoka JR., 2003), thermo- (Kenji Kono et al., 2001; Martin Hossann et al., 2007), light- (Oleg V. Gerasimov et al., 1999; Xin-Ming Liu et al., 2005; Lauri Paasonen et al., 2007), enzyme- (Paul Meers, 2001), electric fields (S. Murdan, 2003) and magnetic fields (M. Babincova et al., 2002) triggered liposomes as active triggering system are developed to improve drug release efficiency.

1.1.5 Ultrasound induced release

Ultrasound is well established as a medical technology for a wide range of applications: imaging, flow analysis, physiotherapy, tumor and fibroid ablation, kidney-stone shattering, and others (Avi Schroeder et al., 2009). In recent years, however, research interests in biomedical ultrasound have shifted gradually from pure diagnostic imaging towards the therapeutic application of ultrasound energy (Myhr G, 2007). Starting with the pioneer works of Kost and Langer on sonopheresis (J. Kost and R. Langer, 1992), efforts of a number of research groups have been foused on using ultraound as an effective drug delivery modality. An advantage of ultrasound to this application is that it is non-invasive, can penetrate deep in the interior of the body, and can be carefully controlled via a number of parameters including frequency, power density, duty cycles, and time of application (Zhong-gao gao, et al., 2005). In addition, ultrasound can be focused on targeted sites and moreover has been shown to increase the permeability of blood–tissue barriers and cell membranes (Shao-Ling Huang, et al., 2004).

In this study, the controlled release of the inner-substances from liposomes by the ultrasonic irradiation will be investigated.

1.2 Aim and organization of this thesis

The research on ultrasound induced release of drug encapsulated liposomes has been attracting more and more interests, but the influences of ultrasound frequency and power on the release properties are remained unclear. In the investigation on ultrasound induced release, researchers often use a commercial ultrasound probe with fixed frequency. Few of them put their interests on the influence of frequency shifts when the ultrasound probe is acting on different objects which would probably influence the release efficiency greatly. Moreover, the traditional fluorescence method for evaluation the release property of drug encapsulated liposomes has shown its inconvenience to those non-fluorescence substances and on-line measurement.

This thesis shows our efforts on solving the above aspects and focuses on the study of controlled release of drug encapsulated lipososmes by ultrasound irradiation. In particular, this work focuses on three points. First is to prepare Pegylated calcein-encapsulated liposomes and characterize their morphologies by transmission electron microscopy (TEM). And do some fundamental studies on how ultrasound influences the properties of liposomes as size distribution, zeta position, and release profiles, using commercial ultrasound equipments, Second, this thesis is going to study on how the encapsulated drugs could be released efficiently by changing the frequency and power of a piezoelectric actuator. Third, it is going to develop an electric impedance based evaluation method for ionic drug release while the samples are exposed to ultrasound. The entire work is summarized as follows:

Chapter 1 provides a brief introduction on the background of liposome research, the objective and scopes and organization of this dissertation.

Chapter 2 presents the basic principle of liposome formation and introduces the preparation methods of liposomes. Further, the morphology of the prepared liposomes was studied by transmission electron microscopy. The result showed that the liposomes were multilamellar structured sphere whose size was around 100nm. It indicates that the liposomes were prepared successfully.

Chapter 3 investigates how the physical properties of liposomes, such as size distribution, zeta-potential and release profile are influenced by ultrasound irradiation, using commercial ultrasound equipments. And the basic conception and principle of dynamic light scattering, zeta potential and the traditional fluorescence evaluation method for release profile investigation of drug-encapsulated liposomes are introduced. Further, the release of calcein liposomes induced by temperature and ultrasound was studied, respectively. Results showed that the low frequency ultrasound was effective to release the inner substances of liposmes while temperature was of much lower efficiency to the sample prepared with POPC, DPPE-PEG200 and cholesterol. The size distribution of the liposomes decreased when exposed to ultrasound. And their zeta-potential also decreased because the release of encapsulated calcein. It also showed that ultrasound irradiation promotes the release of liposomes inner substances and PEG influences ultrasound induced release in a mode of dose dependent fashion up to 10% PEG.

Chapter 4 gives the information of ultrasound on controlled release and studies how to control the release rate of the inner-substances from liposomes by low frequency ultrasonic irradiation. Two kinds of ultrasonic control methods are tested for investigation of the efficiency of the release property of the liposomes. One control method is to increase the input power by fixing its frequency at a frequency near the probe resonant frequencies. Another method is to adjust the input frequency around the probe resonant frequencies but fixing the input power. As a conclusion, the release property of liposomes could be controlled efficiently by an ultrasonic

probe in the way that the input frequency should be selected a little lower than its resonant frequency, so that the release speed of drugs from the encapsulated liposomes can be adjusted suitably by the input power.

Chapter 5 describes the development of a novel method for evaluation of the liposomes release property by measuring the electric impedance changes of liposome suspensions. To validate the proposed impedance measuring method, the calcein release rates were evaluated both by the impedance changes and the fluorescence intensity changes in calcein-liposome suspensions. With the comparison of these results obtained by the two methods, it is shown that the impedance method has much wider detecting concentration range than the fluorescence one. Furthermore, the impedance method can be efficiently used for evaluation of the release property on various ionic substances encapsulated within liposomes.

Chapter 6 provides conclusions and prospects of the thesis work.

2

PREPARATION OF LIPOSOMES

Liposomes are formed in aqueous solutions when hydrated phospholipids arrange themselves in circular sheets with consistent head-tail orientation. They are microscopic, spherical vesicles with aqueous solutions core(s) which are enclosed by bilayer lipid membrane similar in composition to the cell membranes found in biological systems. Liposomes are of great interest to the pharmaceutical industry as drug-delivery vectors due to the fact that they can encapsulate hydrophobic compounds in the lipid bilayer, and at the same time hydrophilic compounds in their inner aqueous volume. In this chapter, the basic principle of liposome formation and the preparation procedures of liposomes will be introduced. Further, the morphology of the liposomes is studied by transmission electron microscopy (TEM).

2.1 Basic principle of liposome formation

Molecules are roughly divided into polar and nonpolar according to the symmetry and distribution of their electronic clouds. Polar molecules are soluble in polar solvents and insoluble in nonpolar solvents, and vice versa. This is the chemical principle of "like dissolves like". However, there is a class of molecules called amphiphile in nature. They usually have a

polar head attached to a long hydrophobic tail in a same molecule and hence possess both hydrophilic (*water-loving*) and lipophilic (*fat-liking*) properties.

However, it is not the case that any amphiphiles could form liposomes. The geometry of the molecules greatly influences the final structure of an aggregation. There are three basic molecular shapes of amphiphile, i.e., a cone, a cylinder, and an inverted cone, as shown in Fig.2.1

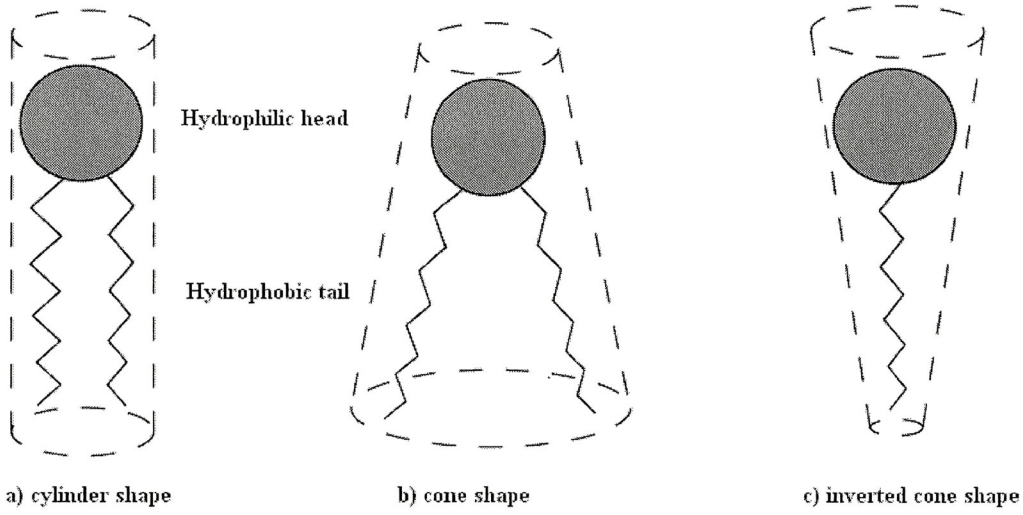


Fig. 2.1 Three basic molecular shapes of amphiphile: a cone, a cylinder, an inverted cone.

Israelachvili and colleagues (Israelachvili, et al., 1977) showed that molecular and aggregate structures can be predicted reasonably well in terms of the packing parameter $v/(a_0 \cdot l_c)$, v being the hydrocarbon volume, a_0 the optimum surface area per molecule, and l_c the critical hydrocarbon chain length. An amphiphile with a packing parameter of $v/(a_0 \cdot l_c) < 0.5$ possesses a cone-shaped structure and tends to form spherical or cylindrical micelle aggregates. Cylindrical molecules have $v/(a_0 \cdot l_c) \approx 1$ spontaneously form planar or vesicular bilayers. Inverted micelle aggregates are the result of inverted cone-shaped amphiphiles with $v/(a_0 \cdot l_c) > 1$.

Phosphatidylcholines are a class of phospholipids with a zwitterionic headgroup composed of a negative phosphate group esterified with glycerol and a positive choline group. The glycerol moiety is further esterified with two saturated and/or unsaturated fatty acid chains which form the lipophilic molecule part. The resulting packing parameter $v/(a_0 \cdot l_c) \approx 1$ suggests that phosphatidylcholines should form bilayer aggregates above the critical aggregate concentration.

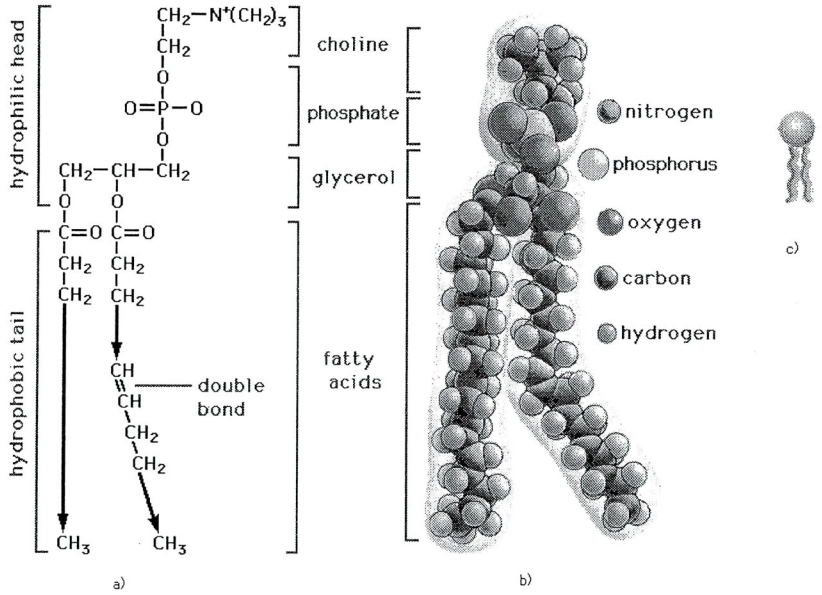


Fig. 2.2 Molecule structure (a), Space-filling model (b) and Symbol (c) of POPC
Cited from: http//www.agen.ufl.edu

This class of amphiphilic molecules, like phospholipids, is the main components of biological membranes. Figure 2.2 shows the molecule structure, space-filling model and symbol of POPC, a kind of phospholipid. As shown Fig.2.2, the phospholipid molecule has a hydrophilic head and two hydrophobic tails. The amphiphilic nature of these molecules defines the way in which they form membranes. They arrange themselves into bilayers, by positioning their polar groups towards the surrounding aqueous medium, and their lipophilic chains towards the inside of the

bilayer, defining a non-polar region between two polar ones. Fig.2.3 shows the schematic structure of a phospholipid bilayer.

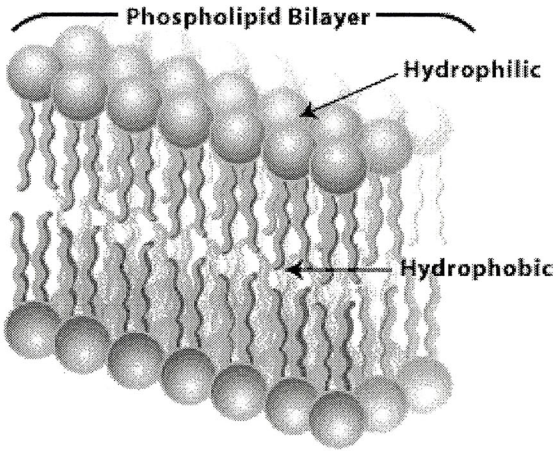


Fig. 2.3 Schematic diagram of bilayer
Cited from: http//www.bioteach.ubc.ca

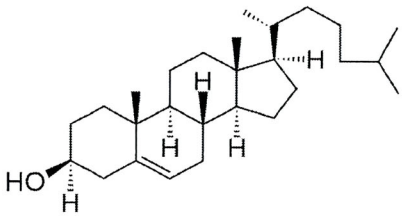
The formation of liposomes is a procedure that minimizes the free energy of the system. The main driving force for the formation of liposomes is hydration. It obeys the second law of thermodynamics in spite of the self-assembly of ordered bilayers of liposome membrane. From the view point of water molecules, the increase of entropy occurs when the hydrophobic regions of the amphiphiles are removed from water and the ordered structure of the water molecules around this region of the molecule is lost.

2.2 Modification of lipid membrane

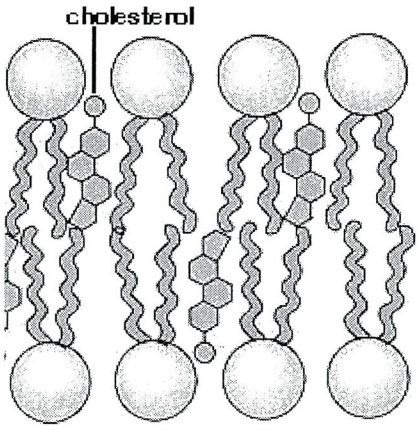
Cholesterol (http://courses.washington.edu/)

Actual biological membranes contain cholesterol as well as phospholipids. Figure 2.4 shows the molecule structure of cholesterol and how its molecules are aligned with the phospholipid

molecules on both sides of the bilayer. The hydroxyl group is positioned where it can interact with the lower oxygens on the phospholipids. The ringed portion of cholesterol, which is rigid and flat, lies next to the first portions of the fatty acid chains. The cholesterol's linear tail, which is quite flexible, lies among the lower portions of the fatty acid chains.



a)



b)

Fig. 2.4 Molecule structure of cholesterol (a) and position of cholesterol in lipid bilayer (b. Cited from http://www.cytochemistry.net).

Cholesterol influences the physical properties of the membrane, especially its fluidity. For example, positioned between the phospholipids, the cholesterol prevents their interaction and possible crystallization. Also, the rigid, planar portion of the molecule tends to block many of the motions of the first portions of the fatty acid chains. This makes the intermediate portion of

the bilayer more rigid and less permeable to small, polar molecules. Lower down, near the center of the bilayer, the flexible tail of the cholesterol molecule allows more movement, making the central part of the bilayer the most fluid.

PEGylation

An important limitation to the use of conventional liposomes or unmodified liposomes in biological application is their rapid removal from blood by the mononuclear phagocytic system (MPS) (Daleke DL, et al, 1990; Allen TM, et al, 1991; Lasic DD, et al, 1996; Dü zgü nes N, et al, 1999), since for the body defense system, conventional liposomes usually represent foreign particles. To avoid fast clearance from blood, the synthetic polymer, such as poly (ethylene glycol) (PEG) or other hydrophilic polymers grafted lipids have been incorporated into liposomes' membrane to create so-called "stealth" particles which make it "invisible" to macrophagocyte, and hence significantly increased its half-life in the blood, maintain a required level of drug in blood, and realize the accumulation of liposomes to the specific site in a passive or active passion (A.L. Klibanov, et al, 1990).

As a protecting polymer, PEG provides a very attractive combination of properties: excellent solubility in aqueous solutions, high flexibility of its polymer chain, very low toxicity, immunogenicity, and antigenicity, lack of accumulation in the RES cells, minimum influences on specific biological properties of modified pharmaceuticals (S. Zalipsky, 1995; S.N.J. Pang, 1993; G.M.Powell, 1980; T.Yamaoka, et al., 1994). And it is also important that PEG is not biodegradable and subsequently does not form any toxic metabolites. PEG molecules with the molecular weight below 40kDa are readily excretable form the body via kidneys (Vladimir P. Torchilin, 2006). Figure 2.5 shows the molecule structure of DPPE-PEG2000 (upper) and position of DPPE-PEG2000 in lipid bilayer (right-bottom).

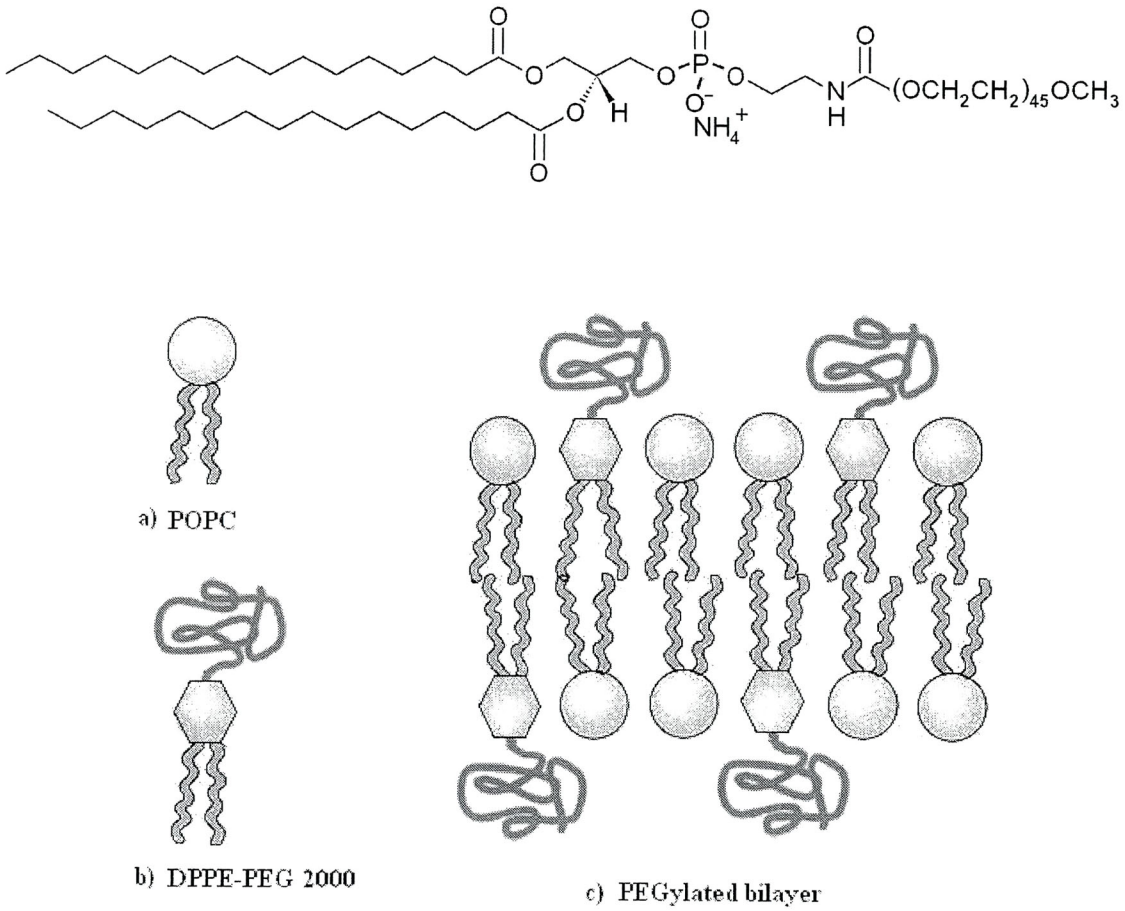


Fig.2.5. Molecule structure of DPPE-PEG2000 (upper) and position of DPPE-PEG2000 in lipid bilayer (right-bottom).

Other modifiers

There are many other chemicals and reagents could be incorporated into the liposome membrane to modify its properties or functions. Monoclonal antibodies, for example, have the advantage over most of other tumoritropics to possess a high degree of specificity. When its fragments are coupled onto liposomes, active targeting to malignant cells could be achieved

2.3 Preparation methods of liposomes

The methods for liposome preparation have been summarized by many researchers (Sharif Mohammad Shaheen, et al., 2006; V.P. Torchilin and V. Weissig, 2003; Crommelin D.J.A , 1999). In the following, the main methods will be introduced.

1) Hand-shaken method

In order to produce liposomes lipid molecules must be introduced into an aqueous environment. When dry lipid film is hydrated the lamellae swell and grow into myelin figures. Only mechanical agitation provided by vortexing, shaking, swirling or pipetting causes myelin figures (thin lipid tubules) to break and reseal the exposed hydrophobic edges resulting in the formation of liposomes. Large multilamellar liposomes can be made by hand-shaken method.

2) Sonication method

This method is probably the most widely used method for the preparation of small unilamellar vesicles. There are two sonication techniques:

- Probe sonication: The tip of a sonicator is directly immersed into the lipsome dispersion. The energy input into lipid dispersion is very high in this method. The dissipation of energy at the tip results in local overheating and therefore the vessel must be immersed into ice/water bath. During the sonication up to one hour more than 5% of the lipids can be de-esterify. Also, with the probe sonicator, titanium will slough off and contaminate the solution.
- Bath sonication: the liposome dispersion in a tube is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier in this method compare

to sonication the dispersion directly using the tip. Material being sonicated can be kept in a sterile container, unlike the probe units, or under an inert atmosphere.

3) Reverse-phase evaporation method

Historically this method provided a breakthrough in liposome technology, since it allowed for the first time the preparation of liposomes with a high aqueous space-to-lipid ratio and able to entrap a large percentage of the aqueous material presented. Reverse-phase evaporation is based on the formation of inverted micelles. These inverted micelles are formed upon sonication of a mixture of a buffered aqueous phase, which contains the water soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. The slow removal of the organic solvent leads to transformation of these inverted micelles into a gel-like and viscous state. At a critical point in this procedure, the gel state collapses and some of the inverted micelles disintegrate. The excess of phospholipids in the environment contributes to the formation of a complete bilayer around the remaining micelles, which results in formation of liposomes. Liposomes made by reverse phase evaporation method can be made from various lipid formulations and have aqueous volume-to-lipid ratios that are four times higher than multilamellar liposomes or hand-shaken liposome.

4) Freeze-dried rehydration method

Freeze-dried liposomes are formed preformed liposomes. Very high encapsulation efficiencies even for macromolecules can be achieved using freeze-dried hydration method. During the dehydration the lipid bilayers and the materials to be encapsulated into the liposomes are brought into close contact. Upon reswelling the chances for encapsulation of the adhered molecules are much higher. The rehydration is a very important step and it should be done very carefully. The aqueous phase should be added in very small portions with a micropipette to the

dried materials. After eacj addotopm the tube should be vortexed thoroughly. As a general rule, the total volume used for rehydration must be smaller than the starting volume of the liposome dispersion.

5) Detergent depletion method

The detergent depletion method is used for preparation of a variety of liposomes and proteoliposome formulations. Detergent can be depleted from a mixed detergent-lipid micelles by various techniques which leads to the formation of very homogenous liposomes. In practice all lipids below their phase transition temperature can be used with this preparation method. Not all detergents are suited for this method and only a few detergents can be used for detergent depletion method. The most popular detergent are sodium cholate, alkyl(thio)glucoside, and alkyloxypolyethylenes. Mixed micelles are prepared by adding the concentrated detergent solution to multilamellar lipsomes (the final concentration of the detergent should be well above the critical micelle concentration (CMC) of the detergent). Equilibrium of the mixed micelles in the aqueous phase takes quite some time and the equilibrium does not happen during a short period time. The use of different detergents results in different size distributions of the vesicles formed. Faster depletion rates produces smaller size liposomes. The use of detergents also results in different ratios of large unilamellar vesicles/ oligolamella vesicles/ multilamellar vesicles.

6) Freezing-thawing extrusion method

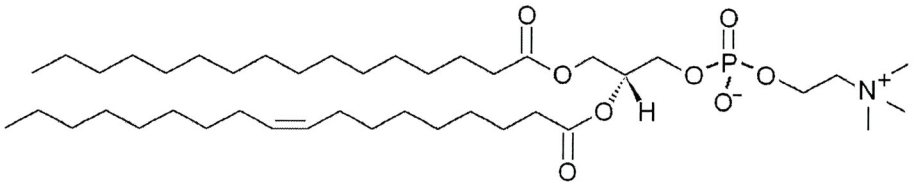
Liposomes formed by the hand-shaken method are vortexed with the solute to be entrapped until the entire film is suspended and the resulting MLVs are frozen in a dry ice/ethanol bath (about −78.5℃), thawed in lukewarm water (about 37℃). After several freezing-thawing cycles, the sample is extruded several times through a polycarbonate membrane.

In this paper, freezing-thawing extrusion method and reverse phase evaporation method were employed to prepare liposomes.

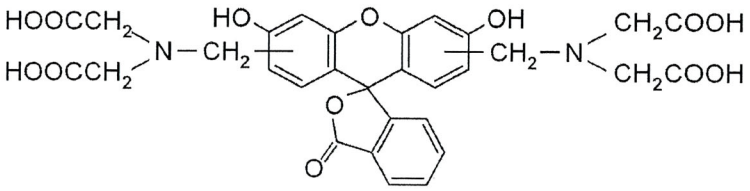
2.4 Preparation of liposomes

2.4.1 Starting materials

1-palmitoyl-2-oleogy-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DPPE-PEG2000) was purchased from AVT. Cholesterol and 3,3'-Bis[N,N-bis(carboxymethyl)-amino-methyl]fluorescein (Calcein) were from WAKO Pure Chemical Industries, Ltd. Sodium hydroxide (NaOH) was from SIGMA. All other chemicals used for the present investigation were of analytical grade. Fig. 2.6 shows the molecule structures of POPC and Calcein. As for the molecule structures of DPPE-PEG2000 and Cholesterol please refer to Fig.2.2 and Fig. 2.4.



a). POPC



b). Calcein

Fig. 2.6 Chemical structure of POPC and Calcein

2.4.2 Liposome preparation

Liposomes used in this study were prepared by two methods, i.e., Freezing-thawing method and Reverse phase evaporation method. The procedures are given in the following.

Freezing-thawing method

Liposomes used in this study were prepared following the next steps:

Dissolving and rotary evaporation: the lipid used for preparations was composed of POPC, DPPE and cholesterol at molar ratios of 9:1:10. First, the lipid mixture was dissolved in chloroform in a round-bottom flask. Subsequently, chloroform was removed by a rotary evaporation (REN-1, Iwaki. Glass Co. Ltd., Iwaki, Japan) in a water bath about 37℃. Thirdly, diethyl ether was added into the flask to re-dissolve the lipid mixture. Finally, the rotary evaporation was carried out for one more time to remove the solvent of diethyl ether until a thin fine lipid film was formed on the inside wall of the flask.

Freeze-drying: to completely get rid of the solvent, it was freeze-dried for 2 hours with freeze dryer (FRD-50M, IWAKI, Japan).

Freezing-thawing: calcein solution (calcein: NaOH=1:4 at molar ratio) of 100mM was added into the flask to dissolve the lipid film. It was mixed by a test tube mixer (TM-250, IWAKI, Japan) for several minutes until there was no obvious lipid film left on the wall of the flask. Then it was froze in a dry ice/ethanol bath (about -78.5℃, 5min) and thawed in a water bath (about 37℃, 5min) for several cycles.

Reverse phase evaporation method

The lipids were dissolved in chloroform in a round-bottom flask and dried by a rotary evaporator in a water bath of 37℃, respectively. Diethyl ether was added into the flask to

re-dissolve the lipid mixture. The solvent was removed again by rotary evaporation until a thin fine lipid film was formed on the inside wall of the flask. The film was then dissolved in 3ml Diethylether and 1ml calcein solution, prepared in 50mM tris-buffer with 100mM NaCl, was added. The mixture was stirred with ultrasound in a water bath and the Diethylether was completely removed by rotary evaporation.

Extrusion

The vesicle suspension was then extruded several times through polycarbonate membrane (pore size 100nm,Venstin®) at room temperature.

Gel Filtration

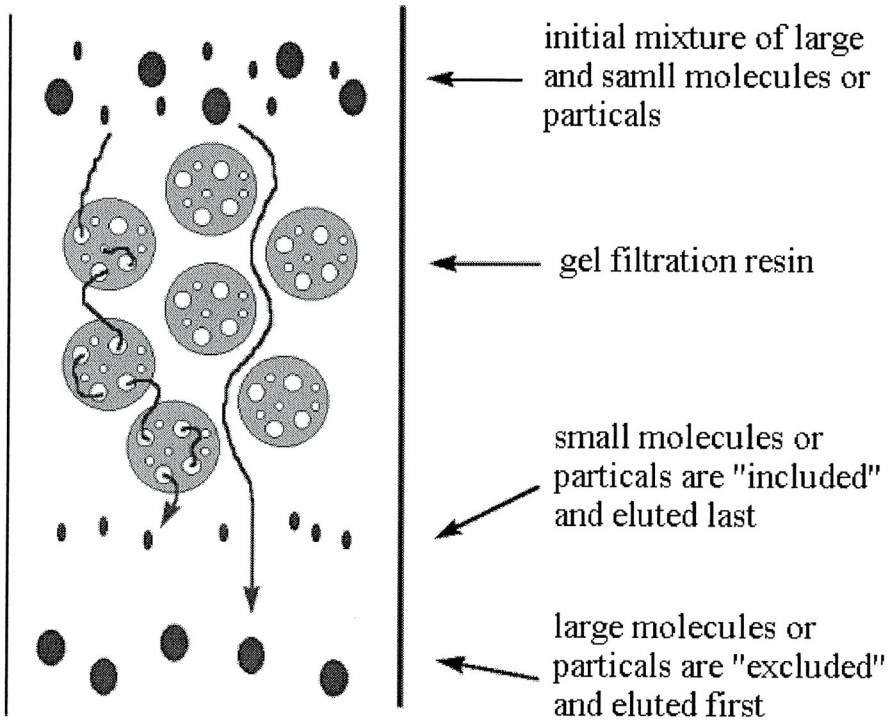


Fig. 2.7 Schematic of gel filtration

Cited from: http://slohs.slcsd.org

Gel filtration is often used in group separation mode to remove small molecules, such as free labels, from a group of larger molecules or particles. As shown in Fig. 2.7, the medium in the gel column is porous particles and large size particles, like liposomes, do not penetrate into the pores of the beads. They percolate through the inter-bead spaces. While the unentraped drugs will percolate in and out the beads which slow down their speed to pass through the column.

In this study, the liposome suspension was eluted through a Sephadex-4B column (Φ10×200mm) to remove the non-encapsulated calcein, using de-ionized water or tris-buffer for different samples. The column was pre-eluted with de-ionized water or tris-buffer thoroughly. The liposomes encapsulated calcein is yellow and the calcein solution of high concentration is brown, it is very easy to get rid of the un-encapsulated calcein.

2.4.3 Lipid concentration measurement

The total lipid concentration of the obtained liposomes was of great importance because not all the lipid used to prepared liposomes were withdrawed through all the preparation procedure, though the initial amount of lipids were accurately measured. It was measured using Wako phospholipids C test kit (Wako, Japan). The Wako phospholipids C is an enzymatic colorimetric method using N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS). This reagent has high specificityis not interfered with ascorbic acidbilirubin in serum. The principle of DAOS method is as following,

As shown in the chemical reactions equation (Fig. 2.8), phospholipids are hydrolyzed to free choline by phospholipase D. The liberated choline is subsequently oxidized to betaine by choline oxidase with the simultaneous production of hydrogen peroxide. The hydrogen peroxide which is produced quantitatively, oxidatively couples 4-aminoantipyrine DAOS to yield a chromogen with maximum absorption at λ=600nm.

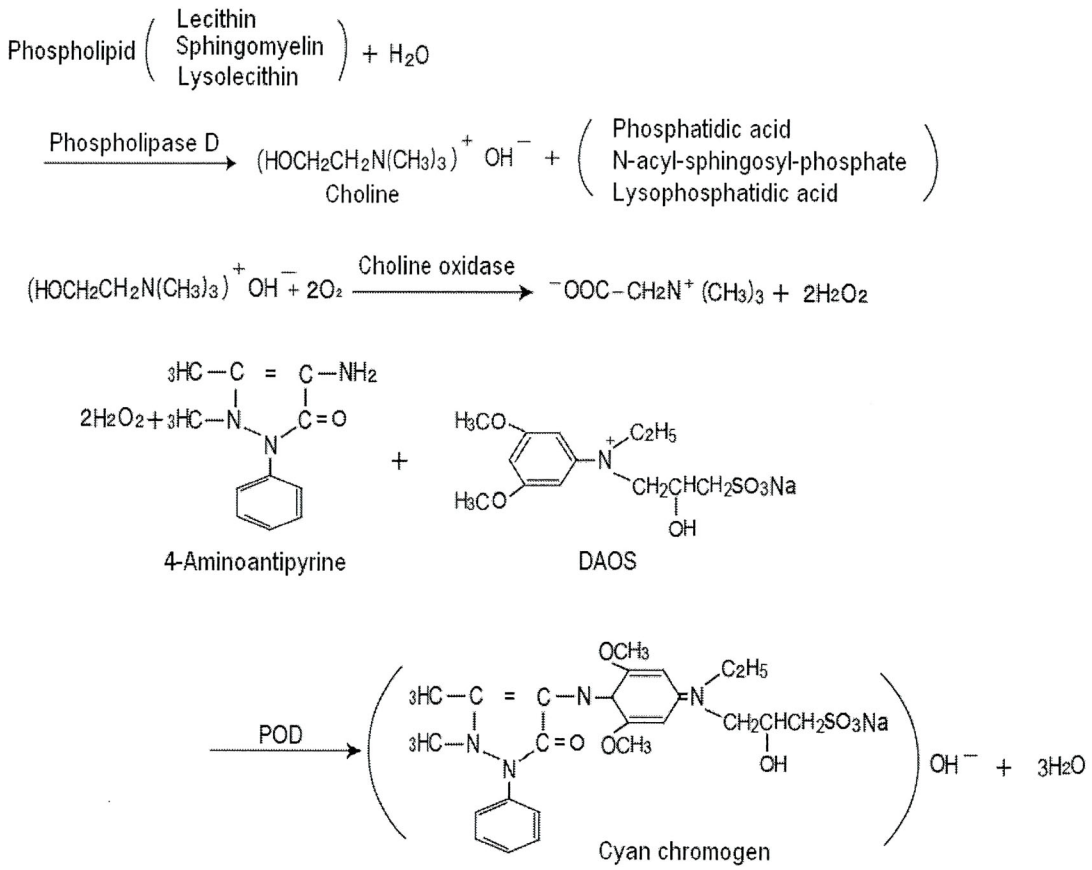


Fig.2.8 Chemical reaction equation of DOAS method

The absorption at 600nm was measured by an UV Spectrophotometer. A calibration line of relationship between lipid concentration and absorption need to be first determined for each bottle of phosphate C reagent (shown in Fig.2.9) by detecting the absorption of standard samples. While it is linear fitted, the criterion equation could be achieved,

$$Y = AX \qquad (2.1)$$

Y is the absorption at 600nm, X is the lipid concentration and A is the fitting parameter. Thus the lipid concentration of liposome could be obtained according to equation (2.1) after the absorption of liposomes are measured. The lipid concentrations of calcein-liposome solutions used in this paper were diluted with Tris-buffer or de-ionized water according to the needs of experiments.

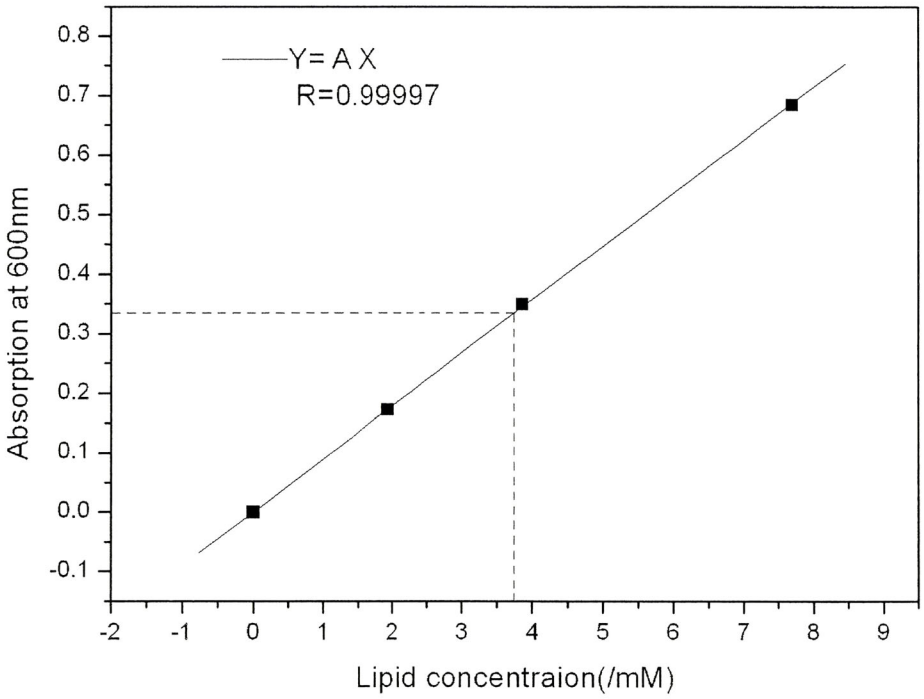


Fig. 2.9 Relationship between lipid concentration and absorption at 600nm.

2.5 Morphology observation by Transmission Electron Microscopy (TEM)

A conventional optical microscopy has made it possible for people to explore the unknown microcosm. Unfortunately, the resolution in optical microscopy has been known to be limited by the so-called Rayleigh criterion which states that two point-like objects cannot be resolved if they are much closer than about half a wavelength. As a consequence, conventional optical microscopy is restricted to a resolution of about 200 nm, and this is not enough for many important observations. While a TEM operating at 100 kV with an electron wavelength of 0.0037 nm can expect to achieve a resolution not much better than 0.1 nm (Crispin Hetherington, 2004), and theoretically, the higher the operating voltage is the higher resolution would be

obtained. The mean sizes of liposomes used in our research are nearly 100nm, and the thickness of the lipid membrane is about 4-5nm, thus a TEM is needed to study the morphology of the liposomes.

2.5.1 Basic principle of TEM

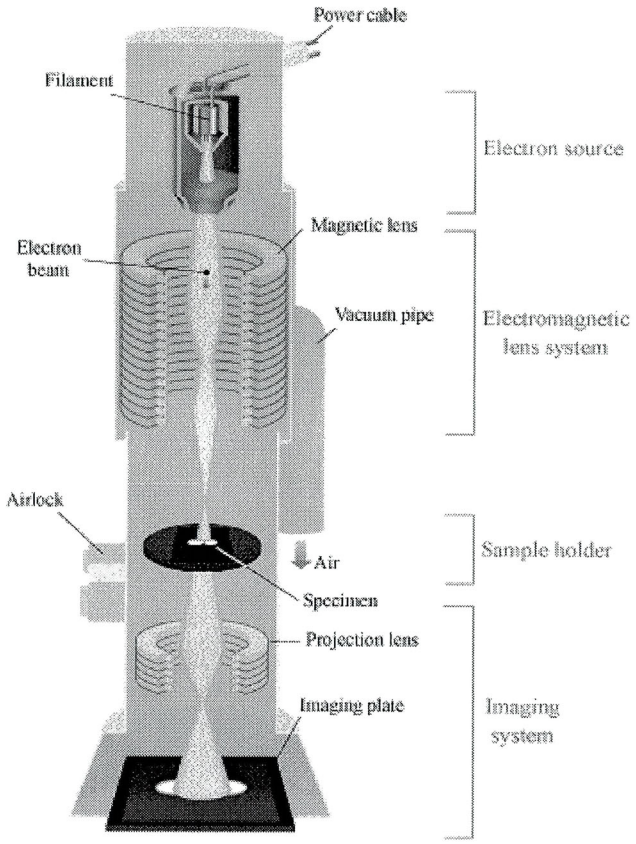


Fig. 2.10 Schematic structure of a TEM
Cited from: http//www.hk-phy.org/atomic_world/tem/tem02_e.html

Transmission Electron Microscopy (TEM) is patterned after Transmission Light Microscopes and will yield similar information. TEM works the same way except that they shine a beam of electrons (like the light) through the specimen (like the slide) and electromagnetic fields works as the glass lens of the light microscopy. Fig. 2.10 shows the Schematic structure of a TEM

which consists of an electron source, an electromagnetic lens system, a sample holder, an imaging system and a vaccum system.

2.5.2 Specimen preparation

Preparation of specimen is extremely important before it is ready to be studied in the TEM. In this section, the preparation procedure of specimen will be introduced.

Formvar membrane coating

The TEM copper grids used in this study are 3 mm in diameter with 400 meshes and they are bare. They need to be coated with a thin electron transparent film, i.e., formvar membrane and carbon membrane, to hold the specimen in place while in the objective lens of the TEM, as shown in Fig. 2.11.

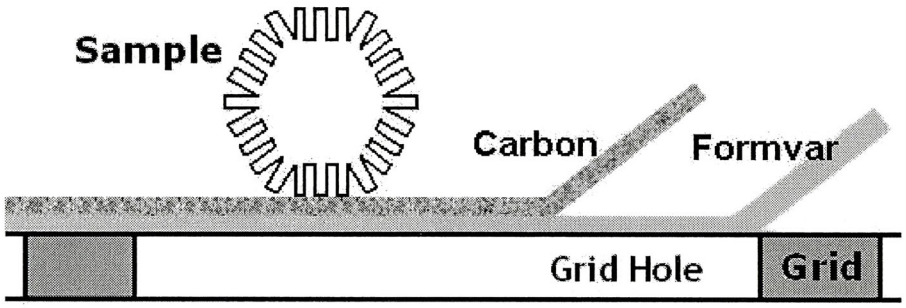


Fig. 2.11 Schematic of liposome specimen for TEM
Reproduced from http://www.grid-tech.com

Formvar is a registered trade name for polyvinyl formal, which is a family of polymers formed from polyvinyl alcohol and formaldehyde as copolymers with polyvinyl acetate; another description is "modified polyvinyl acetal resins" (http://www.2spi.com/). The formvar membrane were prepared according to the following steps,

- Clean film casting device and grid with acetone.
- Clean the water tray with soap and then acetone. Then fill it with de-ionized water).
- Pour formvar solution (1 % (w/w) in chloroform) into the casting device.
- Clean a slide by breathing on it and wiping it with unwoven cloth.
- Put the slide into the casting device and pump the solution to cover the slide.
- Let the slide stay in the solution a few seconds (t1) and drain the solution.
- Wait a few seconds (t2) before taking it out. t1, t2, and the concentration of solution determine the thickness of coat. Longer times and higher concentrations give thicker coatings.
- Let the coated slide dry in air for several seconds and cut it around the edge of slide with a sharp knife.
- Hold the bottom of the slide and put it into water tipsily. The film will float on the surface of the de-ionized water.
- Place grids on the floating film: rough side of the grid facing film.
- Take the grid out of water by placing parallel plastic film over it and let it dry.

Carbon membrane coating

A formvar membrane is relatively weak to the electron beam. It is better to coat a thin carbon film on to the formvar membrane to obtain stable support. The carbon membrane is coated in a vacuum evaporator (JEF-4X, JEOL, Japan) under the condition of vacuum pressure at 1×10^{-5}pa and discharged at 20mA direct current for 1min.

Plasma or glow discharge

PEGylated liposomes are hydrophilic. To get the liposomes well absorbed on the formvar-carbon coated TEM grid surface. The grids were hydrophilized by a Magnetron

Sputtering Equipment (JUC-5000, JEOL, Japan) when the vacuum pressure reached to 6Pa. It was discharged at 2mA for 1min.

Negative staining

The main purpose of negative-staining is to surround or embed the biological object in a suitable electron dense material which provides high contrast and good preservation (M. A. Hayat, 2000). Negative staining is a very useful technique because of its ease and rapidity, and also because it requires no specialized equipment other than that found in a regular EM laboratory.

A 2% solution of ammonium molybdate is particularly useful for staining osmotically sensitive organelles. While this negative stain seems to give the best results for many types of specimen, it does produce a lower electron density than other stains.

The specimen is embedded in a negative stain that is a metal such as ammonium molybdate. On drying, the electron-dense metal atoms envelop the specimen. The difference between the specimen and the surrounding heavy metal atoms with respect to their density produces the necessary contrast. The specimen appears to be light surrounded by a dark background of dried stain (Fig. 2.12). The electron beam passes through the low electron density of the specimen, but not through the metallic background. The specimen substructure is revealed because of the penetration of the stain into its holes and crevices. In other words, the structure is inferred from the distribution that the specimen imposes on the stain. The clarity of the specimen detail depends on the degree to which the stain remains amorphous as it dries as well as on the thickness of the dried negative stain envelope.

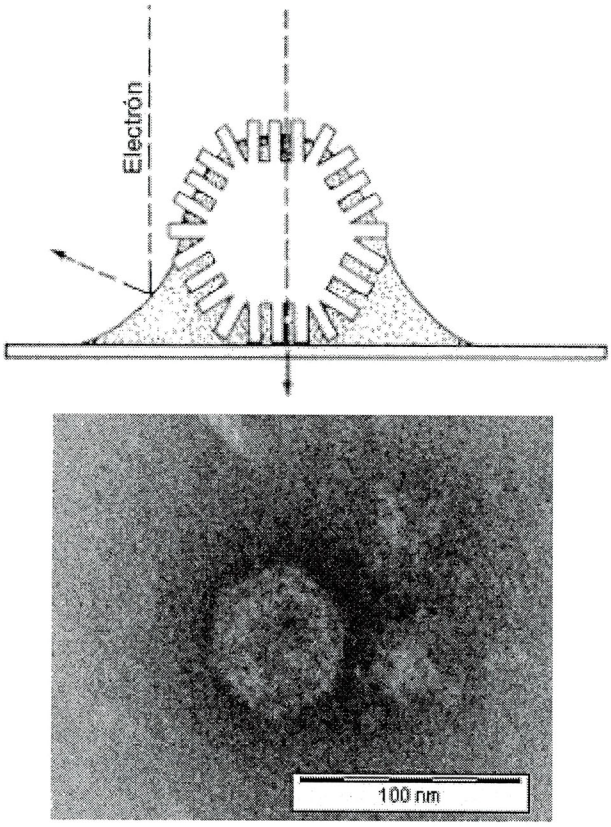


Fig. 2.12 Schematic representation of a specimen particle (e.g., liposome) in TEM observation.

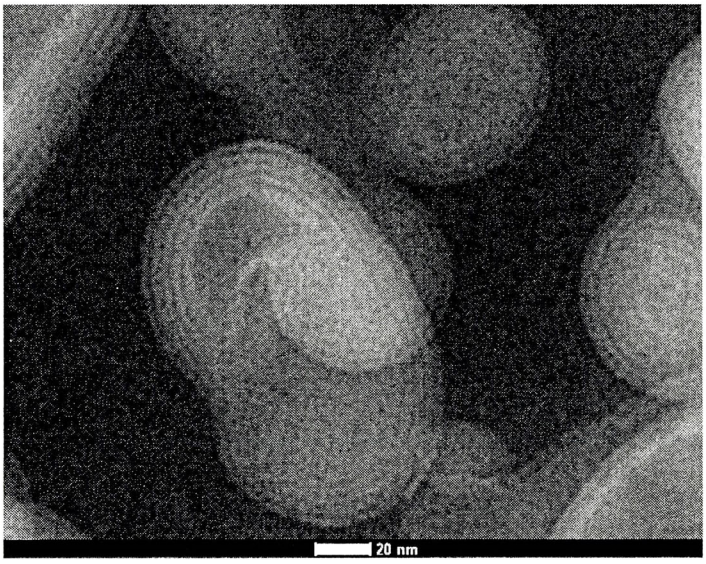
In our study, the negative staining procedure follows the next steps.

- First, a drop of liposome sample was added onto a carbon/formvar-coated copper grid (400 meshes, 3mm in diameter, Nisshin EM Co., Ltd. Tokyo, Japan) for 30s.
- Second, the excess solution was removed by touching a filter paper to the edge of the grid.
- Third, the sample was negative stained by supplying a droplet of ammonium molybdate solution of 2% for another 30s, and the excess solution was blotted off with filter paper.
- Fourth, the specimens were dried in air for 10min before it was set into the equipment for observation.

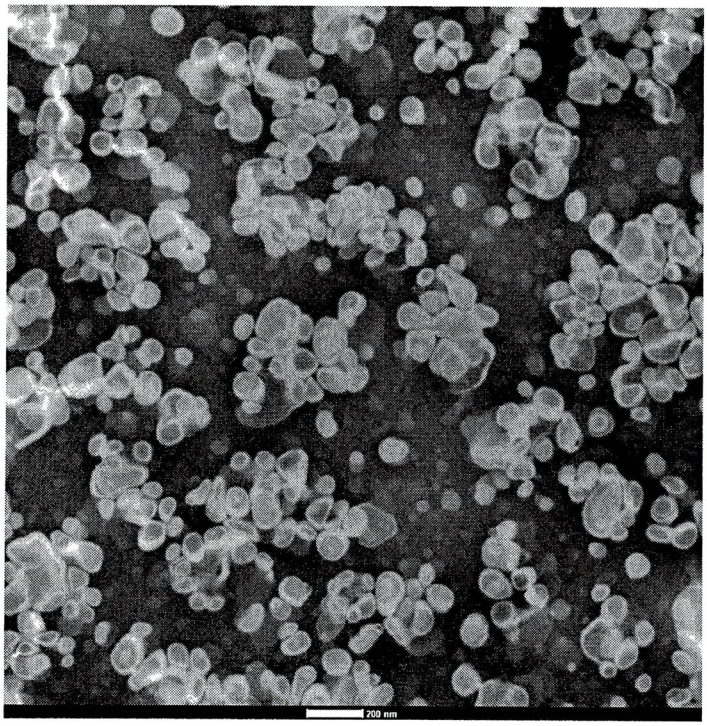
2.5.3 Results of TEM Observation

The observation of the liposomes morphology was carried out by Transmission electrode microscopy (Tecnai G2 Spirit BioTWIN, FEI, USA) at 120kV.

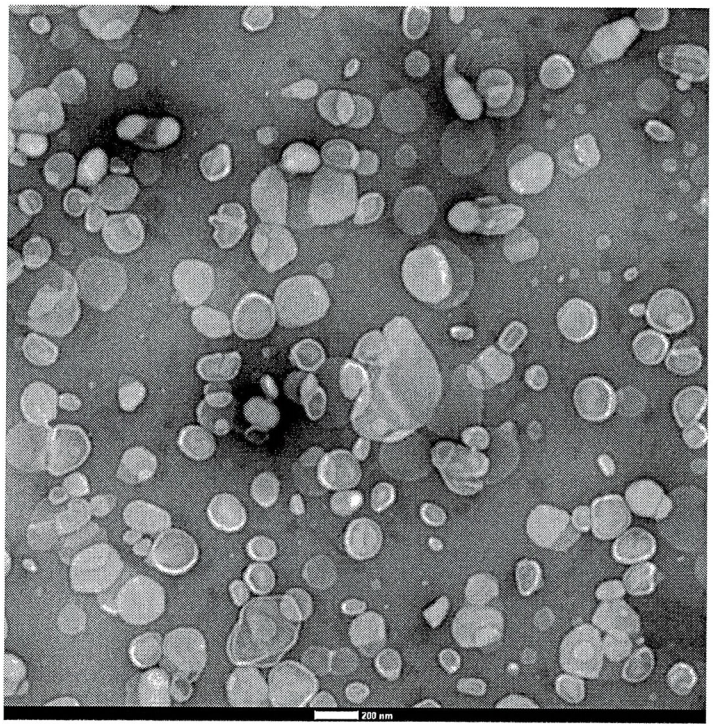
Fig. 2.13 Shows the TEM photos of liposomes after elution through Sephadex 4B column, sample concentration of Fig. a, b, and c were 1mM, 1mM and 0.3mM, respectively. In Fig. 2.13-a, the white enclosed lines represent the liposome membranes. It is obviously that the liposomes are of multilamellar structure and the membrane thickness could be measured to be around 4nm according to the bar given at the bottom of the Fig. 2.13-a. In Fig. 2.13-b, it could be seen that the size of liposomes are around 100nm and there are a slight agglutination of liposomes. It is probably the concentration of the sample for TEM observation was too high (lipid concentration of 1mM). When the sample was diluted to be 0.3mM, a dispersed liposome photo could be obtained, as shown in Fig. 2.13-c.



a)



b)



c)

Fig.2.13 TEM photo of liposome after elution through Sephadex 4B column, sample concentration of Fig. a ,b, and c were 1mM, 1mM and 0.3mM, respectively.

2.6 Summary

In this chapter, the formation principle of liposomes and the preparation methods of liposomes are introduced. The preparation details of liposomes used in our studies are also presented. Further, the morphology of liposomes was studied by transmission electron microscopy (TEM). The result showed that the liposomes were multilamellar structured sphere whose size was around 100nm. It indicates that the liposomes were prepared successfully.

3

FUNDEMENTAL STUDY OF LIPOSOMES

Ultrasound is non-invasive and able to penetrate into the interior of the body, and ultrasound can be focused on targeted sites. Due to these special properties, ultrasound has been put in the spotlight at release control application. However, the mechanism of ultrasound induced release is far from clear. In this chapter, fundamental experiments are carried out to study how the size distribution and zeta-potential of calcein encapsulated liposomes will be influenced by ultrasound irradiation which are important parameters of liposomes. Further, the release properties of calcein encapsulated liposomes are to be evaluated when the liposomes were exposed to different frequency ultrasounds. And the release properties of different amount of PEG modified liposomes are also presented.

3.1 Size distribution measurement by Dynamic Light Scattering (DLS)

Liposome size is a vital parameter of many quantitative biophysical studies, of liposomal drug delivery studies, and of many other applications in both medicine and biology (Goyal, P. et al., 2005; Litzinger D. C. et al., 1994; Uhumwangho M. U. and R. S. Okor. 2005; Woodbury D. J. and C. Miller, 1990; Allen TM, et al., 1993; Oussoren C, et al., 1997). In this study, the size

distribution was investigated by dynamic light scattering (DLS) technology before and after ultrasound treatment.

3.1.1 Principle Behind Dynamic Light Scattering (http://www.malvern.com/.../dynamic_light_scattering.htm)

Dynamic light scattering (DLS), sometimes referred to as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS), is a non-invasive, well-established technique for measuring the size of molecules and particles typically in the submicron region, and with the latest technology lower than 1 nanometer.

Particles, emulsions and molecules in suspension undergo Brownian motion. This is the motion induced by the bombardment by solvent molecules that themselves are moving due to their thermal energy.

If the particles or molecules are illuminated with a laser, the intensity of the scattered light fluctuates at a rate that is dependent upon the size of the particles as smaller particles are "kicked" further by the solvent molecules and move more rapidly. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship, as following,

$$d=kT/3\pi\eta D \qquad (3.1)$$

where, d is the diameter of liposomes, k is the boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, and D is the diffusion constant .

The diameter that is measured in Dynamic Light Scattering is called the hydrodynamic diameter, see Fig.3.1, and refers to how a particle diffuses within a fluid. The diameter obtained by this technique is that of a sphere that has the same translational diffusion coefficient as the particle

being measured.

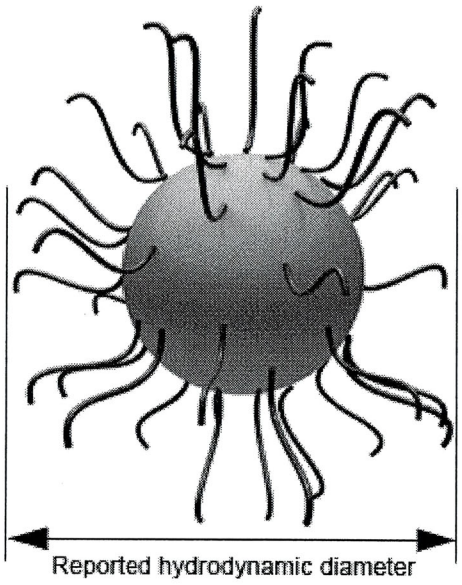


Fig. 3.1 Hydrodynamic diameter of liposomes
Cited from http://www.malvern.com/

The translational diffusion coefficient will depend not only on the size of the particle "core", but also on any surface structure, as well as the concentration and type of ions in the medium. This means that the size can be larger than measured by electron microscopy, for example, where the particle is removed from its native environment.

3.1.2 Ultrasound exposure

Ultrasonic disrupter (UR-20P, Tomy Ceiko, Japan) was used to enhance the release of calcein. The ultrasound probe tip is vertically immerged into the liposome suspension (3 ml) in a rockered test tube, and the run-in part is 4mm beneath the sample surface, as shown in Fig. 3.2. The output power of the ultrasound probe was set at 18W (about 366.7W/cm2). In order to suppress the influence of temperature during the ultrasound treatment and the fluctuation in the electrical impedance measurement, the experiments were carried out in a program incubator (IN602W, Yamato Scientific, Japan) in which the temperature was set at a constant of 25◦C.

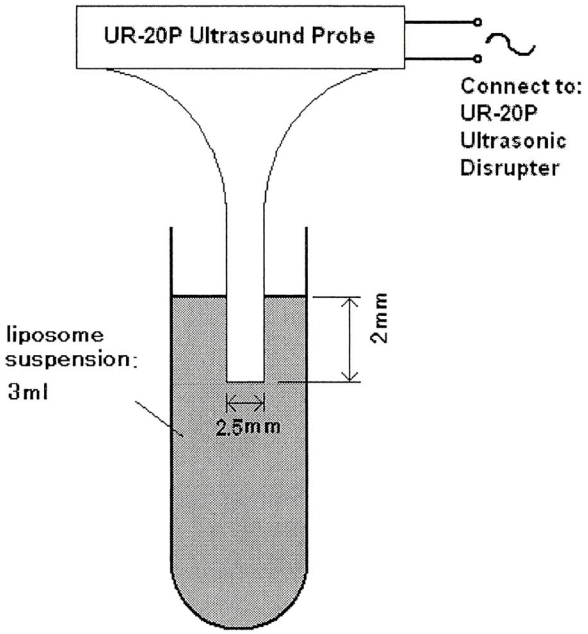


Fig. 3.2 Schematic diagram of ultrasonic irradiation

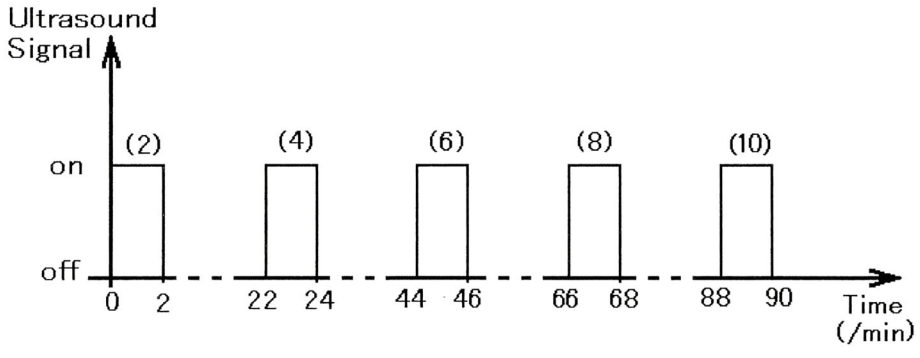


Fig.3.3 Pulsed power mode of ultrasonic irradiation

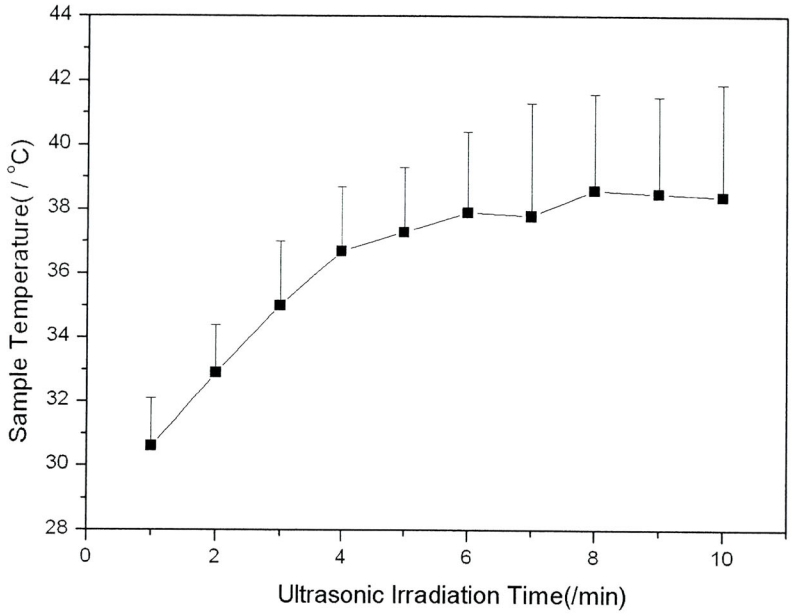


Fig. 3.4 Relationship of sample temperature and ultrasonic irradiation time

The ultrasound was irradiated in a pulsed power mode as shown in Fig. 3.3, i.e., continuously for 2min and then stopped for 20 min. As a result, the temperature of the sample was controlled under 45 ◦C (Fig. 3.4), which was measured by a laser thermometer (HiTESTER 3445, Hioki, Japan). The measurements of the impedance and fluorescence were carried out during the stopping 20 min after the solution temperature was returned to 25 ◦C.

3.1.3 Results of Dynamic Light Scattering (DLS)

In this study, the size distributions were obtained by a zeta potential & particle size analyzer (ELSZ-2, Photal, Otsuka electronics, Co., Ltd., Japan). The results were shown in Fig. 3.5 where the horizontal axis is the diameter and the vertical axis is the intensity of distribution. According to Fig. 3.5, the diameter of liposomes ranges in the region of 80-285nm before

ultrasound treatment, where the peak value is 165nm. When exposed to ultrasound, the diameter range changes to the region of 40-285nm, where the peak value is decreased to be 121nm. It is obvious that ultrasound irradiation destroyed the large liposomes to reform smaller ones. The average size was obtained by cumulant analysis method which decreased from 150nm to 102nm. Further, the size distribution of liposomes did not change after dilution.

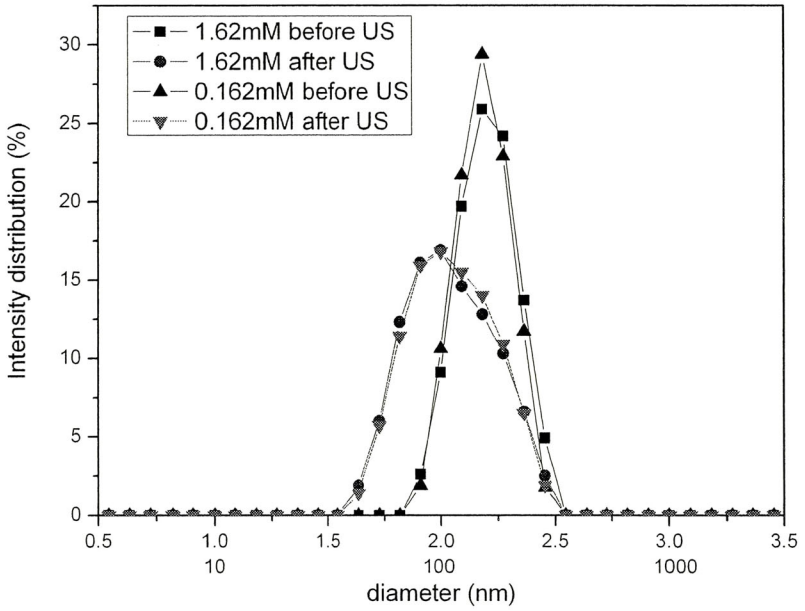


Fig.3.5 Size distribution of liposomes, before and after ultrasound treatment.

3.2 Zeta-potential measurement

The membrane surface potential plays an important role in the rate of aggregation and fusion of vesicles and hence in the physical stability of liposomes. Zeta potential is a critical parameter to characterize the surface charges. In this section, the basic conception of zeta potential will be introduced as well as the zeta potential of liposomes before and after ultrasound treatment where the sample used were the same as that used in section 3.4.

3.2.1 Definition of zeta-potential

(http://www.malvern.com/.../zeta_potential_LDE.htm)

Most particles dispersed in an aqueous system will acquire a surface charge, principally either by ionization of surface groups, or adsorption of charged species. These surface charges modify the distribution of the surrounding ions, resulting in a layer around the particle that is different to the bulk solution. As shown in Fig.3.6, if the particle moves, under Brownian motion for example, this layer moves as part of the particle. The zeta potential is the potential at the point in this layer where it moves past the bulk solution. This is usually called the slipping plane. The charge at this plane will be very sensitive to the concentration and type of ions in solution.

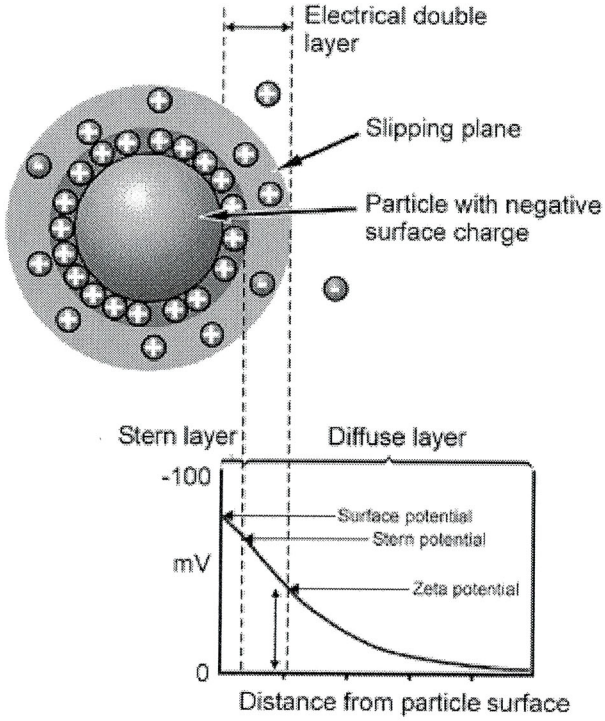


Fig. 3.6 Schematic representation of zeta potential

Cited from http://www.malvern.com/

3.2.2 Principle of zeta potential measurement (http://www.malvern.com/.../zeta_potential_LDE.htm)

Zeta potential is measured by applying an electric field across the dispersion. Particles within the dispersion with a zeta potential will migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. This velocity is measured using the technique of laser Doppler anemometry. The frequency shift or phase shift of an incident laser beam caused by these moving particles is measured as the particle mobility, and this mobility is converted to the zeta potential by inputting the dispersant viscosity, and the application of the Smoluchowski or Huckel theories. These theories are approximations useful for most applications. More recent models are available which can give a more exact conversion, but require more knowledge of the chemistry of the dispersion.

Zeta potential was determined by smoluchowsik relationship, as following,

$$\xi=4\pi\ \eta\ U/\varepsilon \qquad (3.2)$$

where, ξ is the zeta potential, η is the viscosity of the solvent, U is the electrophoretic mobility, ε is the dielectric constant of the solvent.

3.2.3 Results of zeta-potential measurement

Figure 3.7 shows the zeta potential results of calcein-liposomes in de-ionized water, before and after ultrasound irradiation (the irradiation condition is introduced in section 3.1.2). The zeta potential is negative which indicates that the phosphate ester in the dipole group of POPC lies on the outside of the membrane and the choline group towards to the inside of the membrane. It induces negative charges on the neutral POPC membrane (Tong Hua and Yao Songnian, 1998).

Because the liposomes were eluted with de-ionized water, the surroundings of high lipid concentration liposomes (1.62mM) and that of 10 times diluted were basically of the same. Thus the zeta potentials of the two samples were of the same level. While liposomes of 1.62mM were exposed to ultrasound, because of the release of inner-calcein, the ion concentration increased sharply which leaded to the decrease of zeta potential (absolute value). This phenomenon was considered to be induced by the increase of ion concentration which leaded the compression of the diffusion layer of the electric double layer (Tong Hua and Yao Songnian, 1998). When the diluted liposomes were exposed to ultrasound, the zeta potential did not changed much which indicated that the ion concentration was too low to influence the diffusion layer as well as the zeta potential, even the encapsulated calcein were released. The changes of zeta potential indicate that ultrasound irradiation could enhance the release the calcein encapsulated in liposomes.

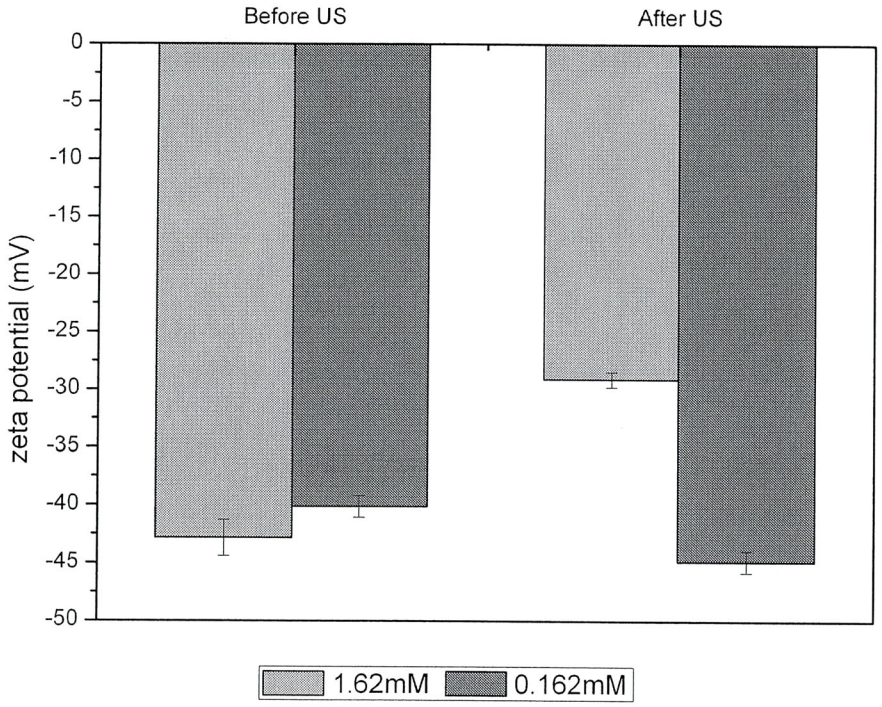


Fig. 3.7 Zeta potential of calcein-liposomes in de-ionized water, before and after ultrasound irradiation

3.3 Fluorescence intensity evaluation method

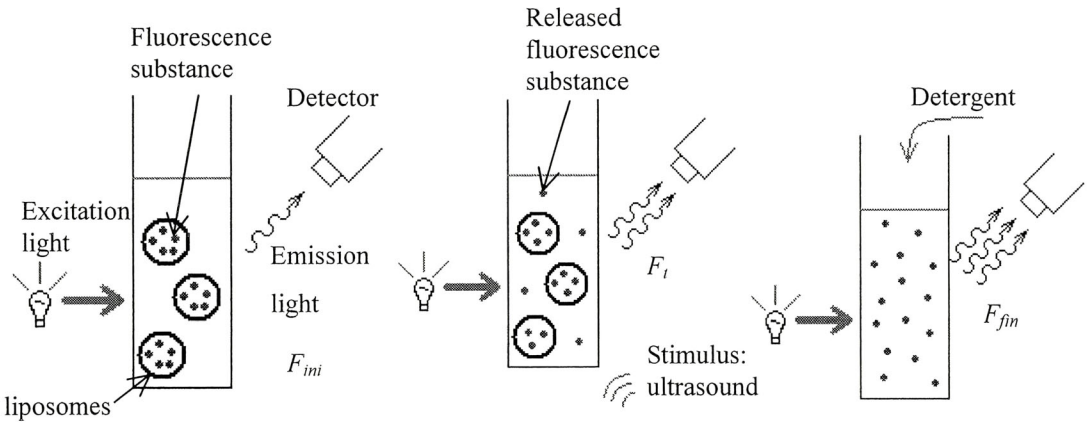


Fig. 3.8 Schematic diagram of fluorescence method for evaluation of drug encapsulated liposomes. a) high concentration calcein encapsulated liposomes gives weak fluorescence emission which could be ignored. The fluorescence intensity is F_{ini}; b) Stronger fluorescence intensity could be obtained when the calcein is released out of the liposomes into the extra-liposomal medium. The fluorescence intensity is F_t; c) Maximum fluorescence intensity F_{fin} is after excess detergent is added.

Fluorescence method is a traditional evaluation method for controlled release of liposomes. Calcein shows weak fluorescence at high concentration above 70mM because of self-quenching. The fluorescence of calcein entrapped in liposomes at high concentration could be ignored. While the fluorescence will increase greatly if the calcein encapsulated inside the liposome is leaked out of the liposome into the extra-liposomal medium.

The release rates of calcein-liposomes were also evaluated by fluorescence percentage which is calculated by

$$R^{"}(t) = \left(F_t - F_{ini}\right) / \left(F_{fin} - F_{ini}\right) \times 100\% \qquad (3.3)$$

where $R(t)$ is the fluorescence release percentage, F_t is the fluorescence intensity observed at time t, F_{ini} is the initial intensity, and F_{fin} is the final fluorescence intensity when calcein-liposome is completely destroyed by adding excess detergent as shown in Fig. 3.8. In this thesis, the fluorescence intensities were measured with a spectrofluorometer (FP-6300, Jasco Corporation, Japan) in the conditions that the wavelength of excitation light was set at 492nm and emission light at 517nm. In order to get comparable data, all the data were measured right after the excitation and emission conditions were set up.

The final fluorescence intensity in liposomes was an important parameter which is employed to determine the release rate of liposome encapsulated calcein. Because there are quite amount of calcein removed by gel filtration, the total calein concentration of the calcein-liposomes is unknown, though the concentration of calcein encapsulated in liposomes remains to be the initial concentration as 100mM. It is necessary to re-determine the final concentration, or the final fluorescence intensity, of the calcein-liposomes.

The final fluorescence intensity was measured in the condition of completely decomposing the liposomes by adding detergent solution. As reviewed by Memoli (Adriana Memoli et al., 1999), when it is at low concentration, the surfactant monomers are incorporated within the lipid bilayer according to a partition equilibrium between aqueous and lipid phase; When the surfactant concentration exceeds a saturation value, phospholipids are gradually solubilized into mixed micelles that coexist with surfactant-saturated vesicles; and complete solubilization of vesicles occurs and only mixed micelles are present in solution, i.e., the entrapped calcein is released thoroughly. Thus, the proper amount of surfactant is necessary to be determined when it is used to destroy liposomes. Fundament experiment has been carried out for this information.

Sodium Cholate

Sodium cholate (3,7,12-Trihydroxy-5β-cholan-24-oic acid, monosodium salt) is water soluble ionic detergent/bile salt commonly used in applications ranging from cell lysis, liposome preparation, isolation of membrane proteins and lipids, preventing nonspecific binding in affinity chromatography and as a cell culture media supplement. In this study, it was used to destroy the liposomes.

Fig.3.9 shows the fluorescence intensities of calcein-liposome when sodium cholate solutions of different concentrations were mixed into them. According to Fig.3.9, the maximum fluorescence intensities of 20μM, 50μM and 100μM calcein-liposome were about 640, 925 and 784 respectively. And the corresponding sodium cholate concentration was about 56mM, which was over 500 to 2500 times of the liposome concentration. Thus, it is proper to have the concentration of sodium cholate 1000 times as higher as that of calcein-liposomes.

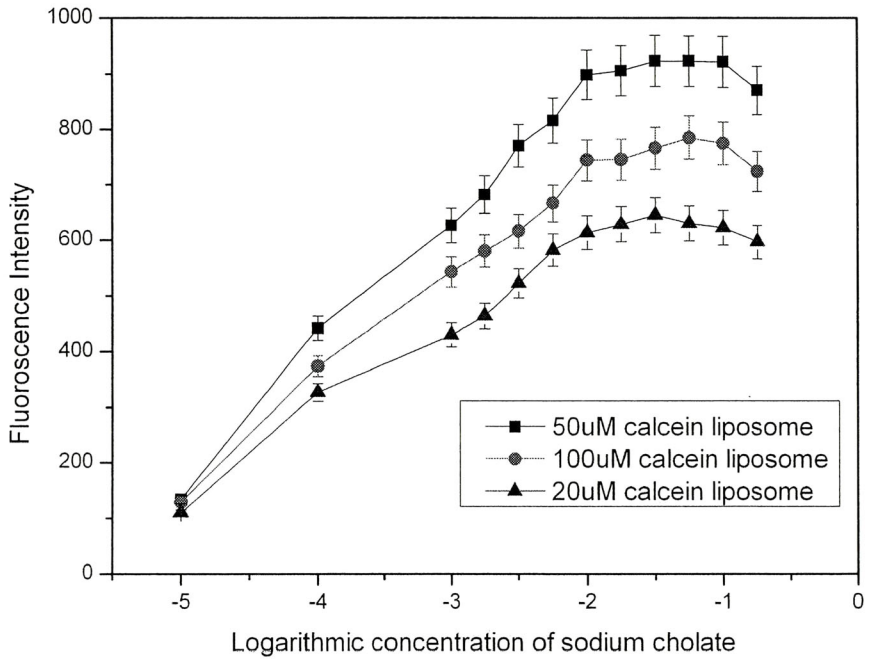


Fig.3.9 Fluorescence intensities of calcein-liposome when sodium cholate solutions of different concentrations were added, the unit of sodium cholate concentration is mole/liter

Triton X-100

Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) is a kind of non-ionic detergent which works the same as sodium cholate. To avoid the influence of additional ions for impedance measurements, Triton X-100 was employed to destroy the liposomes. The proper amount of Trition X-100 was also determined beforehand. The relation of fluorescence intensities of calcein-liposomes *vs.* the logarithmic concentration of Triton X-100 solutions is given in Fig. 3.10. According to Fig.3.10, the fluorescence intensities of calcein liposomes arrived to the maximum value after the concentration of Triton X-100 reached 0.05%. In this study, the Triton X-100 added to destroy calcein liposomes were 1%.

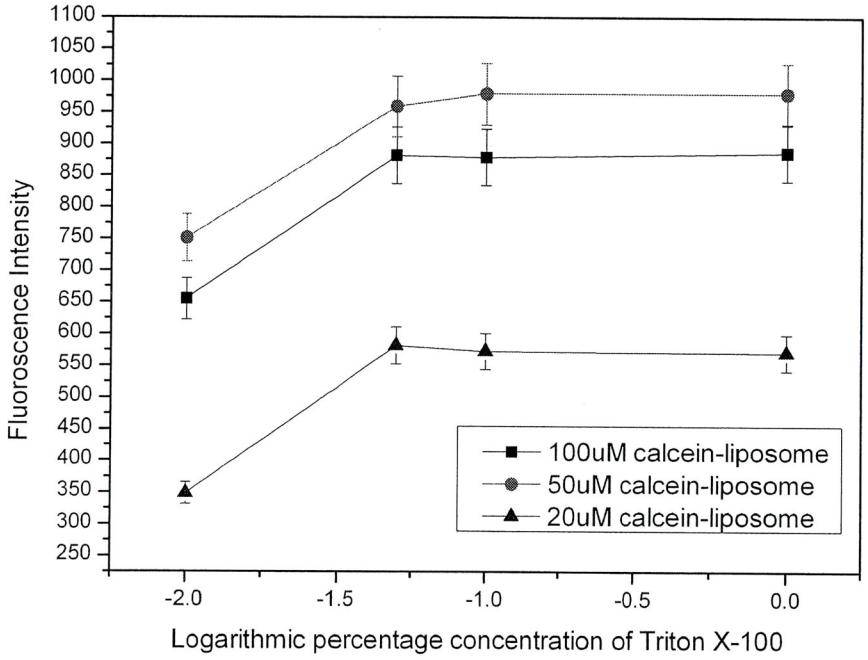


Fig. 3.10 Fluorescence intensities of calcein-liposome when Triton X-100 solutions of different concentrations were added, the unit of triton X-100 is w/v percentage.

3.4 Temperature induced release

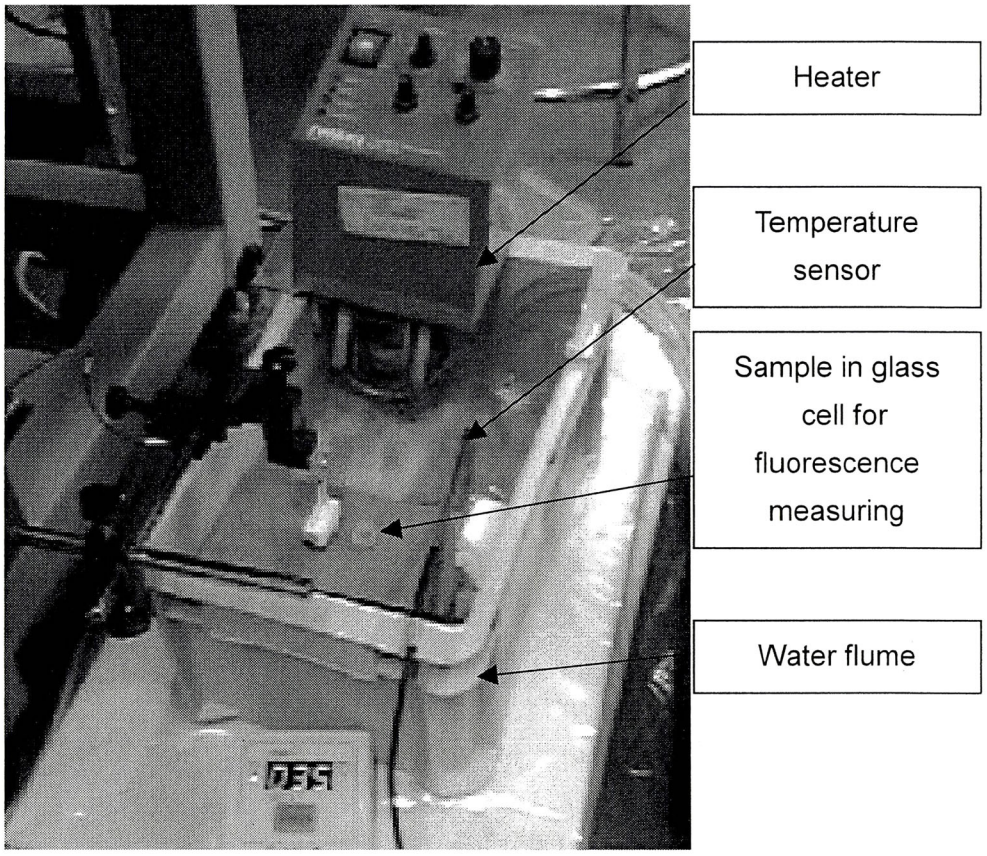


Fig. 3.11 Temperature induced release experiment setup

Temperature is an important parameter which would possibly induce the release of drug encapsulated liposome. The release property of calcein-encapsulated liposome to temperature was studied. In this experiment, liposome sample was prepared by freezing-thawing method and the lipid concentration of the sample was 50 μ M with 100mM calcein/NaOH solution encapsulated in the inner of liposomes. The experiment setup was shown in Fig. 3.11. The sample (3ml) was put in a quartz cell which was kept in a water flume to control the temperature. The fluorescence intensities at 25℃, 35℃, 45℃, 55℃ was measured after the sample was kept

in the flume for 20min. The release rate of calcein-liposomes was evaluated with the fluorescence percentage method introduced in section 3.3. Fig. 3.12 shows the release rate changes induced by temperature. According to Fig. 3.12, the max release rate was appeared at 55℃ and it was less than 10%. It indicates that the sample is insensitive to temperature.

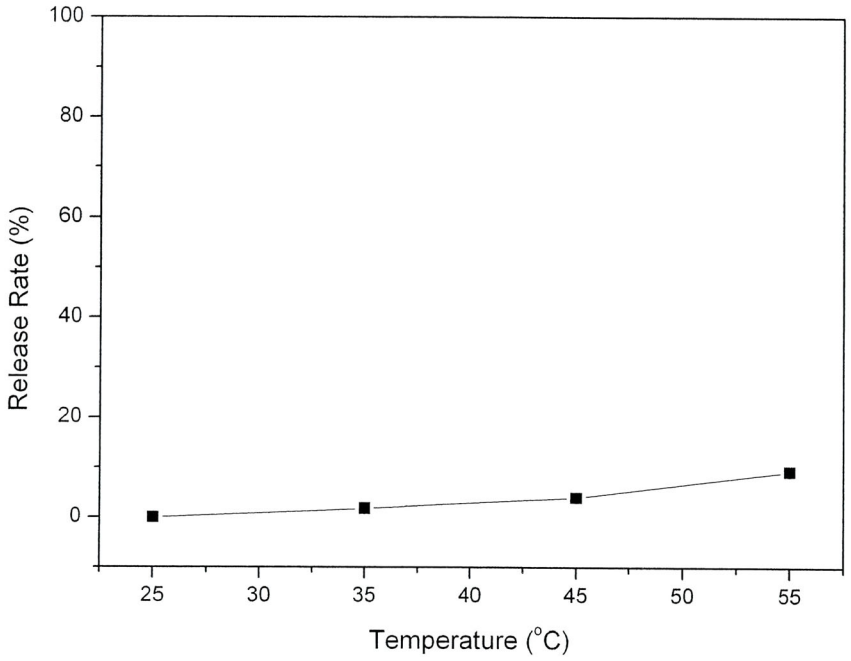


Fig. 3.12 Release rate changes induced by temperature.

3.5 Ultrasound induced release

The calcein-liposome was also treated with ultrasound to study its release property. An ultrasound cleaner (iuchi-III) with 3 kinds of frequencies (28kHz, 45kHz, and 100kHz) was used to induce the calcein release of liposomes.

The sample used in this experiment was prepared with reverse phase method in Tris-buffer (pH=7.4±0.1, [NaCl] =0.1M) whose lipid composition is POPC:DPPE-PEG2000: Cholesterol=57:7:36 at molar ratio and the lipid concentration of the sample was 50 μ M with 100mM calcein/NaOH solution encapsulated in the inner of liposomes. The experiment setup

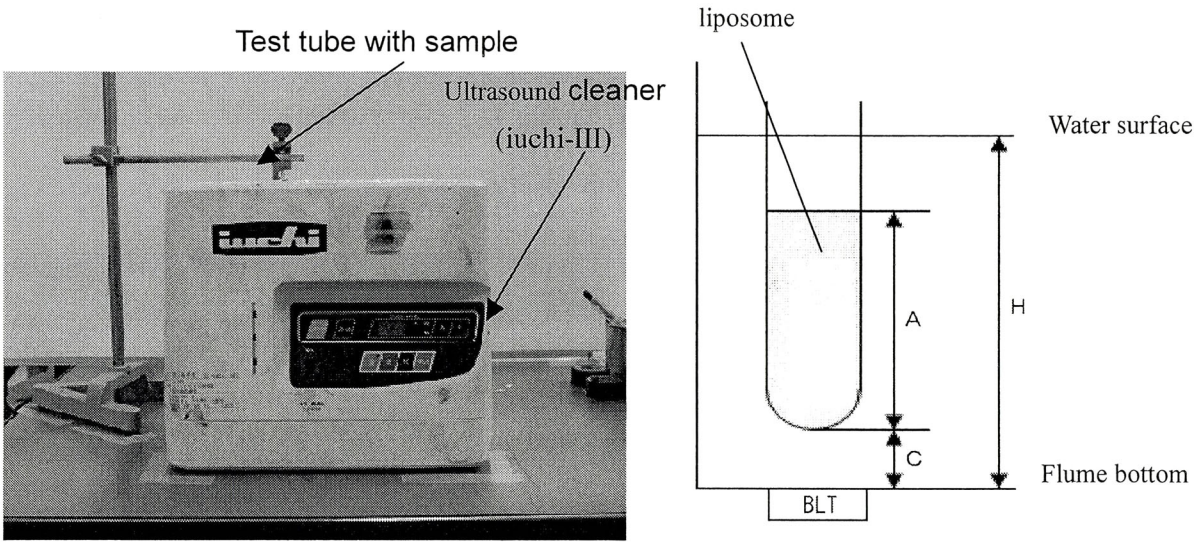


Fig. 3.13 Ultrasound induced release experiment setup and the dimension of the sample in the water flume where A =35mm, H=60mm, C=1mm.

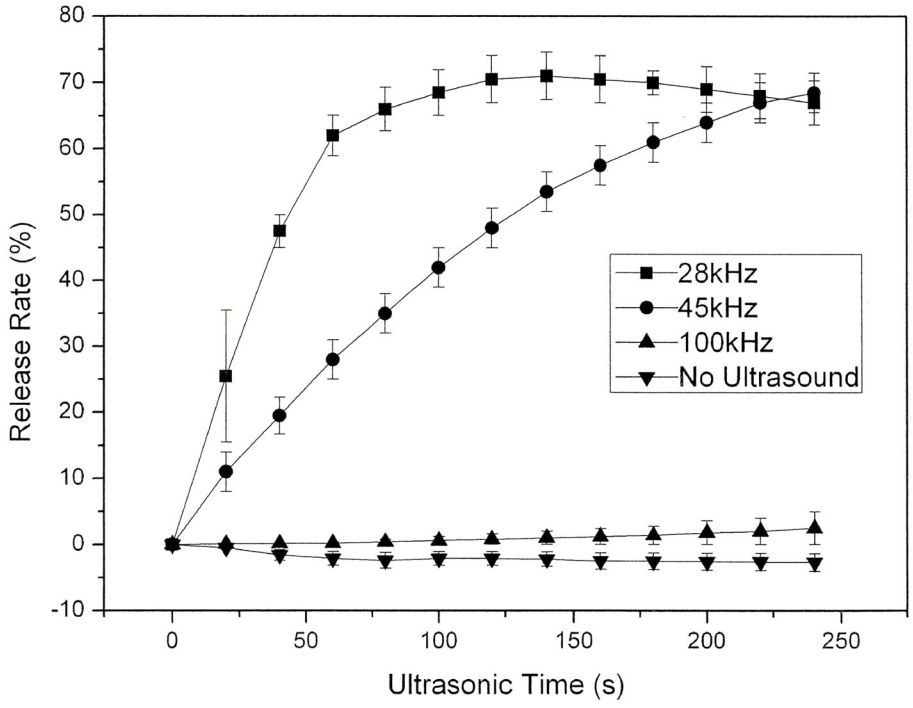


Fig. 3.14 Release rates of calcein liposomes induced by different frequencies ultrasound with an ultrasound cleaner compared by that without ultrasound treated sample.Mean ± S.D. n=4.

was shown in Fig. 3.13. The sample was put in a round bottom test tube which was vertically kept in a water bath in the cleaner. As shown in Fig.3.13(b), the depth of the water in the flume is 60mm, the distance between test tube and flume bottom is around 1mm and the depth of the sample in the test tube is 35mm. The results are shown in Fig. 3.14. According to Fig. 3.14, no calcein released if there is no ultrasound exposure. And the release rate at 28kHz increases sharply and has the biggest values. The release rate at 45kHz increases much slower than that at 28kHz while no obvious changes at 100kHz. It indicates that the calcein liposomes are sensitive to low frequency ultrasound.

It is reported that PEG enhances ultrasound-induced leakage in a dose dependent fashion until approximately 5% (Mona Pong et al., 2006). But they did not discuss the influence of cholesterol. In this study, four kind of liposomes composes of POPC, DPPE-PEG2000 and cholesterol in different molar ratios, i.e., POPC:DPPE-PEG2000:Cholesterol =60:0:40, 58:3:39, 70:5:25 and 40:10:50, which were named as 0%sample, 3%sample, 5%sample and 10%sample respectively hereinafter. The samples were prepared with reverse phase method in Tris-buffer and the lipid concentration of the sample was 50 μ M with 100mM calcein/NaOH solution encapsulated in the inner of liposomes. Samples were exposed to 45kHz ultrasound by the ultrasound cleaner (iuchi-III). The dimension of sample in the flume is shown in Fig. 3.15. As shown in Fig.3.15, the depth of the water in the flume is 50mm, the distance between test tube and flume bottom is around 1mm and the depth of the sample in the test tube is 28mm. The results are shown in Fig. 3.16. According to Fig. 3.16, the release increased while the ultrasonic time prolonged. The 0%PEG sample has the smallest release rate while 3%PEG, 5%PEG and 10%PEG have much faster release speeds and the higher the PEG amount is the faster the

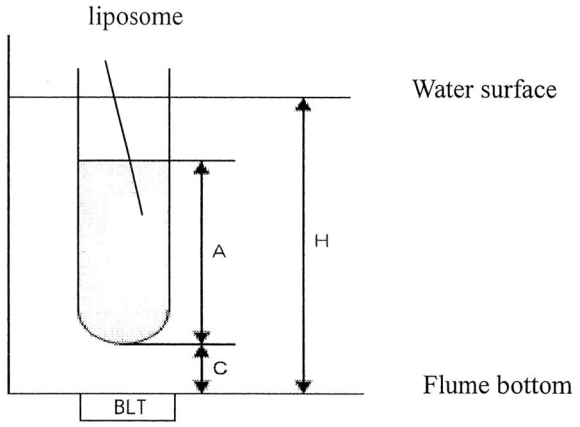


Fig. 3.15 Dimension of the sample in the water flume where A =28mm, H=50mm, C=1mm.

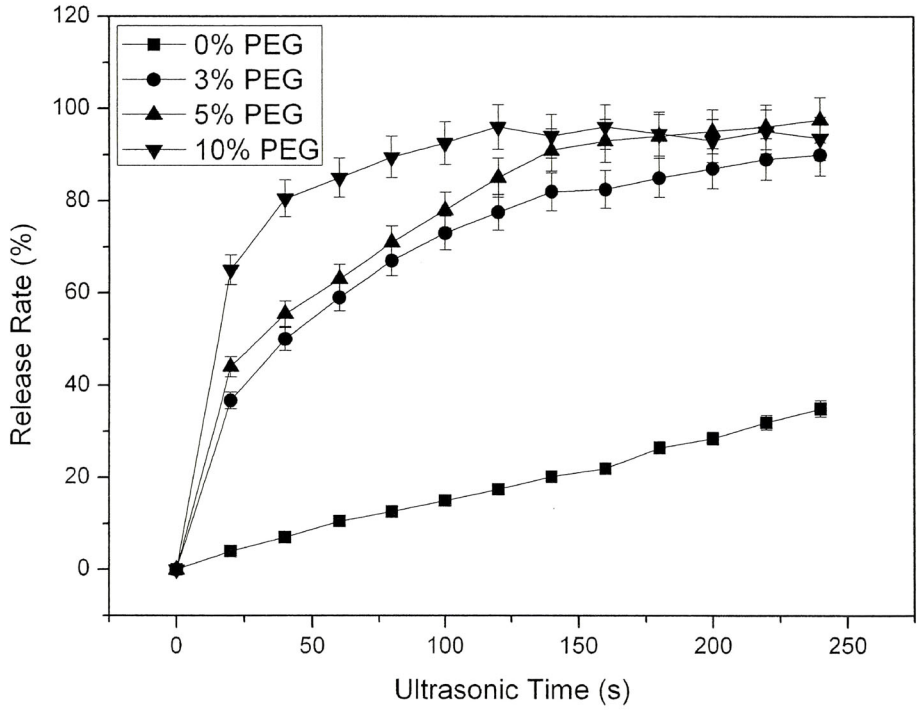


Fig. 3.16 Release rates of calcein liposomes of different PEG amount induced by 45kHz ultrasound with an ultrasound cleaner. Mean±S.D. n=4.

release will be. It indicates that PEG enhances ultrasound-induced leakage in a dose dependent fashion up to 10% PEG.

3.6 Summary

In this chapter, the size distributions and zeta potentials of calcein liposomes before and after ultrasound irradiation were studied. The results showed that ultrasound irradiation could destroy the liposomes and form smaller liposomes. Moreover, the results of zeta potential indicated that ultrasound could enhance the release of inner substances of liposomes. Further, the release property of Pegylated calcein-liposomes were studied when kept in different temperature water bath and exposed to temperature low frequency ultrasound with an ultrasound cleaner. Results showed that the liposomes prepared in our study were insensitive to temperature but very sensitive to low frequency ultrasound in a mode of dose dependent fashion up to 10% PEG.

In the other hand, we found that the frequency and power of commercial ultrasound equipments could not be adjusted easily. It is inconvenient to study the relation of frequency and release property accurately because of the shift of resonant frequency. Further, the tradition fluorescence evaluation method is only fit for fluorescent substances and low concentration samples. Also it is only used in off-line measurement. A new controlled release method and an evaluation method will be discussed in chapter 4 and 5, respectively.

4

RELEASE CONTROL OF DRUG-ENCAPSULATED LIPOSOMES BY ULTRASOUND IRRADIATION

In the study of ultrasound induced release of liposomes, researchers usually use commercial ultrasound equipments which have fixed frequency or power. While the property of an ultrasound probe (resonant frequency) would change if it is was acted on different mediums. This would probably influence the stability of controlled release. This chapter introduces the basic concept of ultrasound in drug delivery and concerns with the study on how to control the release rate of the inner-substances from liposomes by the ultrasonic irradiation. Two kinds of ultrasonic control methods are tested for investigation of the efficiency of the release property of the liposomes. One control method is to increase the input power by fixing its frequency at a frequency near the probe resonant frequencies. Another method is to adjust the input frequency around the probe resonant frequencies but fixing the input power. In the experiments, three kinds of liposomes with different lipid ratio were prepared and calcein as an indicator was enclosed into the liposomes. The release effect was evaluated from the fluorescence intensity changes which were measured by a spectrofluorometer. The results showed that the efficient release property could be achieved both by control of the input power and the input frequency, but adjusting the input frequency around the probe resonant frequency can get higher efficient

release performance than that controlled by the input power.

4.1 Introduction

Up to now, many strategies for controlling drugs release from liposomes have been proposed, including chemical approaches （X. Guo and F.C. Szoka Jr, 2003）, biological approaches (P. Meers, 2001), and methods based on physical external stimulations such as electric fields (S. Murdan, 2003), magnetic fields (M. Babincova et al., 2002), temperature (K. Kono et al., 1999), visible light (V.C. Anderson and D.H. Thompson, 1992; A. Mueller et al., 2000), pH (D.C. Drummond et al., 2000), and ultrasound (R.J. Price et al., 1998; D. Cohen-Levi et al., 2000; A. Marin et al., 2002). Among these methods, ultrasound is non-invasive and able to penetrate into the interior of the body. In addition, ultrasound can be focused on targeted sites. Due to these special properties, ultrasound has been put in the spotlight at release control application.

Ultrasound is an acoustic mechanical wave which having a frequency above 20 kHz. Most modern ultrasound devices are based on the piezoelectric effect that change an applied voltage into mechanical displacement of a surface (the face of the transducer) that is in contact with water, gel, or some other media that can efficiently transmit ultrasonic waves. Ultrasound is well established as a medical technology for a wide range of applications: imaging, flow analysis, physiotherapy, tumor and fibroid ablation, kidney-stone shattering, and others (Avi Schroeder et al., 2009). In recent years, however, research interests in biomedical ultrasound have shifted gradually from pure diagnostic imaging towards the therapeutic application of ultrasound energy (Myhr G, 2007). Starting with the pioneer works of Kost and Langer on sonopheresis (J. Kost and R. Langer, 1992), efforts of a number of research groups have been focused on using ultraound as an effective drug delivery modality.

Ultrasound in drug delivery has been extensively reviewed by many reviewers (Ilana Lavon and Joseph Kost, 2004; Ghaleb A. Husseini and William G. Pitt, 2008; Junru Wu and Wesley L. Nyborgm 2008), and the basic aspects are to be introduced here.

Ultrasound could be divided into three distinct sets based on frequency range and applications:

- High-frequency or diagnostic ultrasound in clinical imaging (3–10 MHz).
- Medium-frequency or therapeutic ultrasound in physical therapy (0.7–3.0 MHz).
- Low-frequency or power ultrasound for lithotripsy, cataract emulsification, liposuction, cancer therapy, dental descaling and ultrasonic scalpels (20–100 kHz).

There are many significant advantages that render ultrasound useful in drug delivery. Ultrasound can penetrate fairly deeply into the body, depending upon the wavelength and the tissue type. Water and gel have very little absorption and scattering (collectively called attenuation), but muscle has fairly high attenuation, and bone and lung tissue have very high attenuation. Though ultrasound does not penetrate easily into or through bone or lung tissue, it can penetrate into tissues well. In general, the amount of attenuation increases as the frequency of the ultrasound increases, so low frequency ultrasound can penetrate more deeply into tissues.

Ultrasonic waves can be focused, reflected, and refracted through a medium, especially the living tissues, just like optical and audio waves do. Thus ultrasound can be carefully controlled and focused onto the tumor site or a particular tissue volume in the body via a number of parameters including frequency, power density, duty cycles, and time of application (Zhong-gao gao, et al., 2005). Such site-specific treatment is beneficial in drug delivery to localize the drug interactions to the target tissue only, thus sparing the body from deleterious side effects. The application of ultrasound has obvious advantages because no surgery or other invasive

procedures are needed, hence eliminating pain and minimizing patient aversion compared to traditional therapies.

Moreover, Tachibana and coworkers reported that there is a synergistic effect between the pharmacological activity of some drugs and ultrasound (K. Tachibana et al., 2000). The absorption of ultrasound can be used to create local tissue hyperthermia, which is often employed by itself or as an adjuvant to chemotherapy in treating some tumors (C.-W. Cho et al., 2002). Ultrasound has also been reported that it can increase the permeability of blood–tissue barriers and cell membranes (Shao-Ling Huang, et al., 2004)

This paper concerns with a study on effective control method to release the drugs encapsulated in liposomes. It has been reported that ultrasound of low frequency (20 kHz-100 kHz) were more efficient than the therapeutic ultrasound (1MHz-10MHz) (Shao-Ling Huang and Robert C. MacDonald, 2004; D. Cohen-Levi et al., 2000; Mona Pong et al., 2006). In this study, a piezoelectric ceramic ultrasonic probe was employed to produce ultrasound to the liposome solution for investigation of the release property of the encapsulated calcein. The lipid liposomes as capsules are prepared from POPC, DPPE-PEG2000 and cholesterol in different molar ratios in order to make different strength of liposomes. Instead of drugs, the calcein is used as a water-soluble indicator and was encapsulated into the inner side of liposomes, so that the release effect can be easily evaluated by the fluorescence intensity changes which is easily measured by a spectrofluorometer. A new control method to release the inner-substances of calcein-encapsulated liposomes was investigated. The practical method to select a suitable input frequency based on piezoelectric impedance measuring technique is proposed. Further, the release property of calcein-liposomes is tested for validation of the proposed method.

4.2 Materials and methods

4.2.1 Liposome preparation

Three kinds of calcein-liposomes were prepared by reverse phase evaporation method as introduced in chapter 2. Mona Pong et al. reported that PEG (up to 8 mol%) enhanced ultrasound-induced leakage in a dose dependent fashion until approximately 5mol% (Mona Pong et al., 2006). But they did not discuss the influence of cholesterol. In this study, the lipid of liposomes composes of POPC, DPPE-PEG2000 and cholesterol in different molar ratios, i.e., POPC:DPPE-PEG2000:Cholesterol = 58:3:39, 70:5:25 and 40:10:50, which were named as 3%sample, 5%sample and 10%sample respectively hereinafter. 100mM Calcein/NaOH solution was encapsulated in the liposomes and the liposomes size was regulated by extrusion through a polycarbonate membrane (porous sizes of 100nm). Non-encapsulated calcein was eliminated by eluted through a Sephadex-4B column with tris-buffer (pH=7.4±0.1, [Nacl] =0.1M). And the final lipid concentration was determined with by choline oxidase · DOAS method. All samples were diluted with tris-buffer.

4.2.2 Ultrasound irradiation

The ultrasonic probe of nominal resonant frequency of 28 kHz was used to control the release of calcein from liposomes in experiment, which is modified from commercially produced ultrasonic disrupter (UR-20P, Tomy Ceiko, Japan). The experiment setup is shown in Fig. 4.1. A function generator (WF 1966, NF, Japan) was used to generate the input voltage signal in different frequency and amplitude. The input signal is magnified by a power amplifier (4010,

NF, Japan) and then applied to the ultrasonic probe. In the ultrasonic irradiation experiment, the liposome suspension of 2ml is filled in a quartz cell and the tip of the ultrasound probe is immerged into the suspension just 3mm beneath the solution surface. Two input control methods for driving the ultrasound probe were tested to investigate the release efficiency on liposomes, i.e., increasing the input power at a fixed the input frequency and varying the input frequency around its resonant frequency at a fixed input power. In the ultrasonic irradiation experiment, the ultrasonic input was applied to the liposome solution continuously for every 30 seconds.

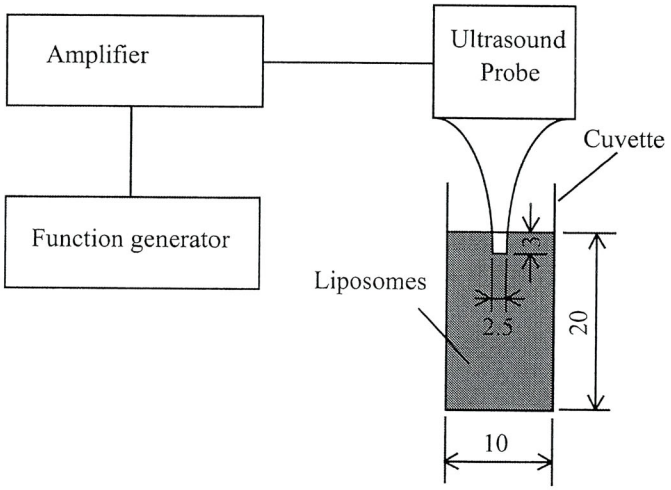


Fig.4.1. Ultrasonic irradiation experiment setup.

4.3 Results and discussion

In order to investigate the ultrasonic effect more detail, the resonant property of the ultrasonic probe was first measured experimentally. The generative displacement at the probe tip was measured by a laser displacement meter (SONY, VL10), where the sinusoidal input at the constant voltage amplitude of Vp-p=10V with varying the frequency was applied to the probe.

The obtained result is plotted in Fig. 4.2. The nominal resonant frequency of this probe is 28 kHz, but the experimental results shown that there are tow peak frequencies appeared around 28.28 kHz and 38.06 kHz. This phenomenon might come from the nonlinear property of piezoelectric ceramic cells. Further, the displacement amplitudes of the probe changed sharply near the peaks, which hints that the output of the probe could be adjusted effectively by changing the input frequency.

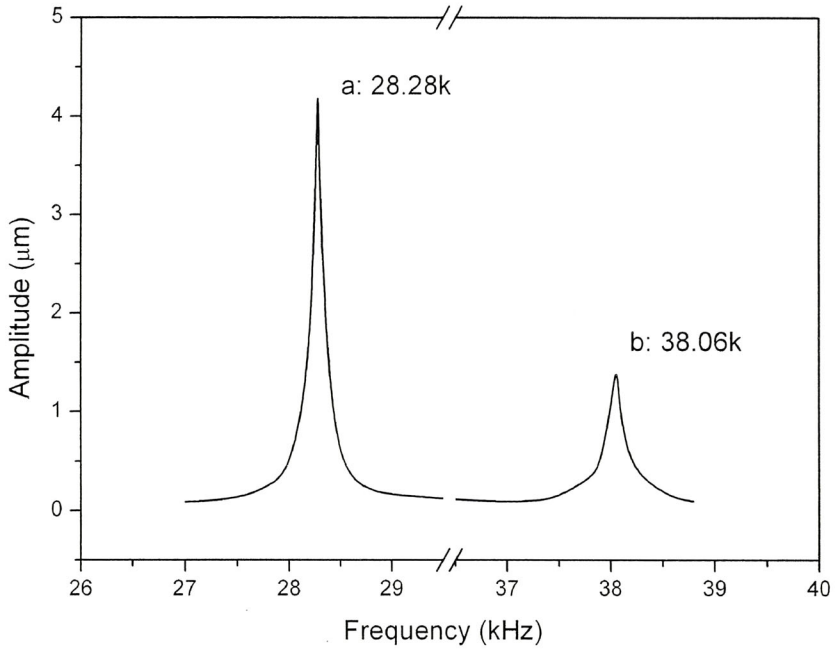
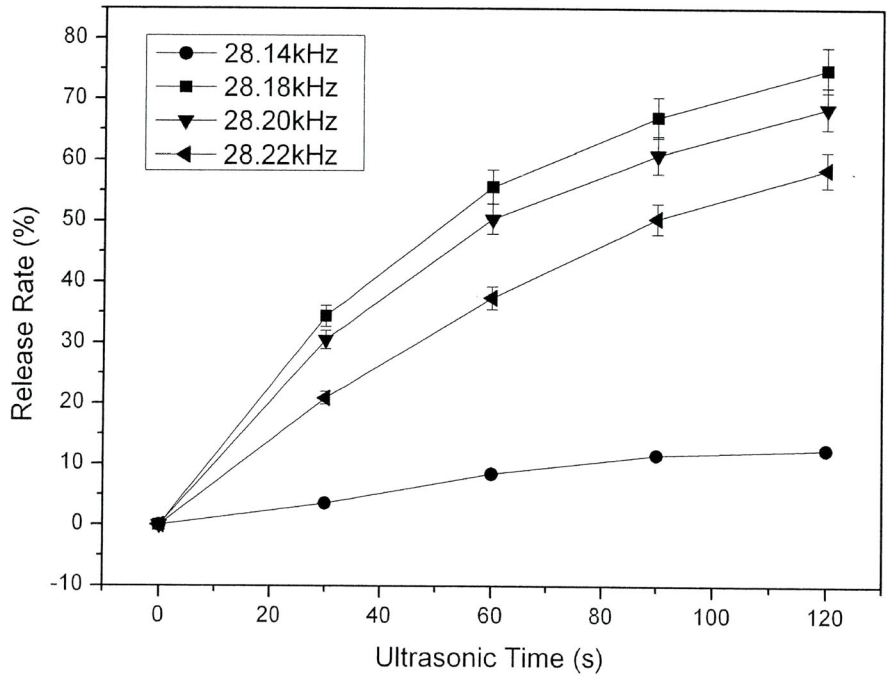


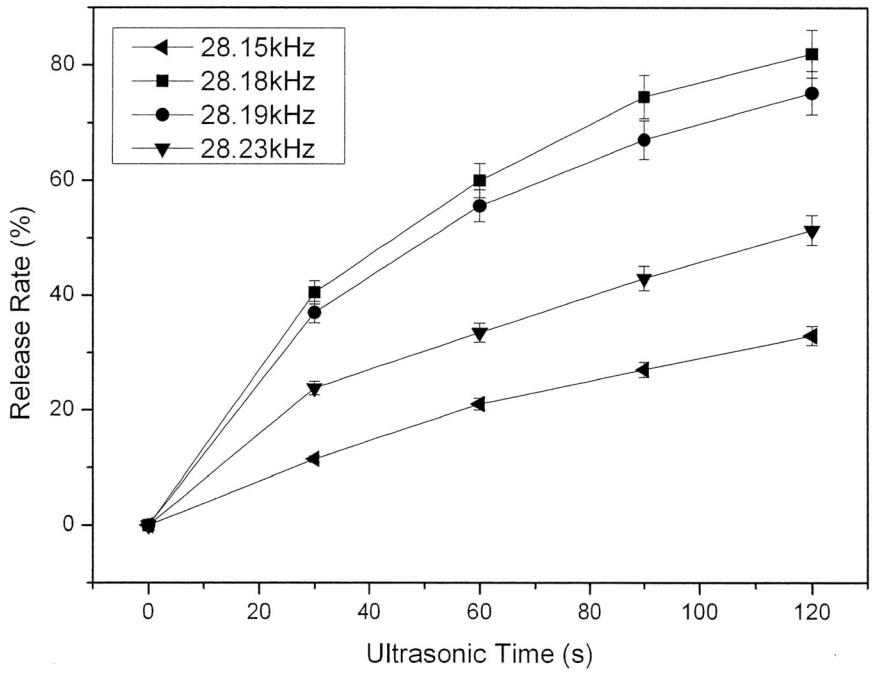
Fig.4.2. Frequency response of the tip displacement of probe at a constant input voltage 10Vp-p.

In the first experiment, the ultrasonic probe was driven by adjusting the input frequency around its peak frequencies and the input power was fixed at 1.4W. Figure 4.3 shows the results of the release rate due to ultrasonic irradiation obtained by adjusting the input frequency around the first peak frequency 28.28 kHz. Figure 4.3(a) shows the results of 5%sample. It has the maximum calcein release rate at 28.18 kHz and the effect was reduced significantly with a small

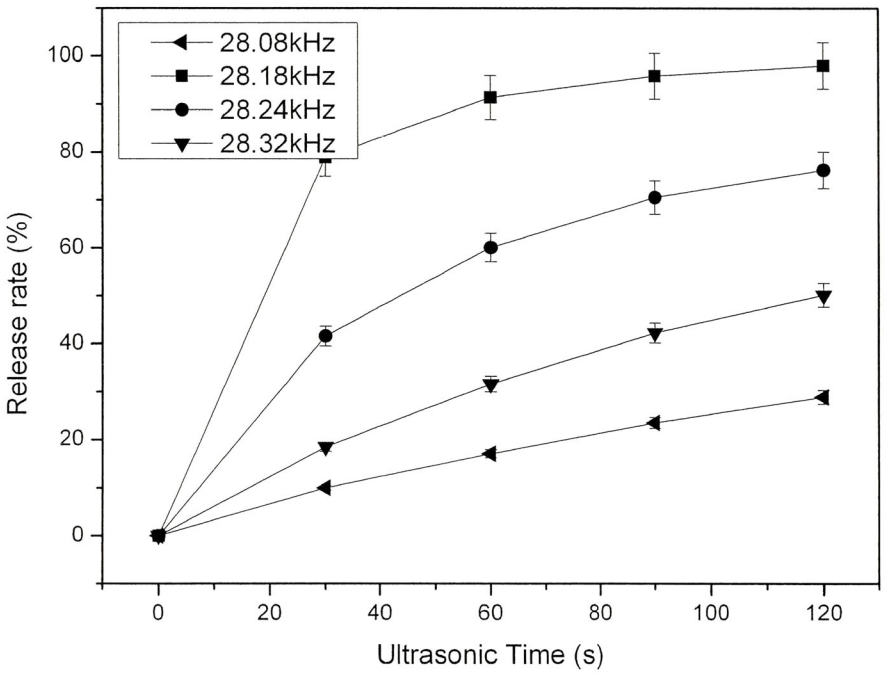
change from 28.18 kHz. Further, it shows that few calcein was released around the boundary frequencies before 28.14 kHz and after 28.23 kHz. Comparing with the results in Fig. 4.2, it is evidence that the efficient frequency is lower than the peak frequency of the probe. The reason is that the resonant frequency goes lower after the probe is inserted into the water or attached to the object compared with the probe in free condition. Figure 4.3(b) is the results of 3%sample. It shows similar to the results of 5%sample. Figure 4.3(c) is the results when the PEG increased to 10%, in which condition the liposome becomes much weak and easy broken. The release curves of 10%sample show a rapid start-up and the ultrasonic excitation still works well even the input frequency is around the boundary frequencies of 28.14 kHz and after 28.23 kHz.



a)



b)

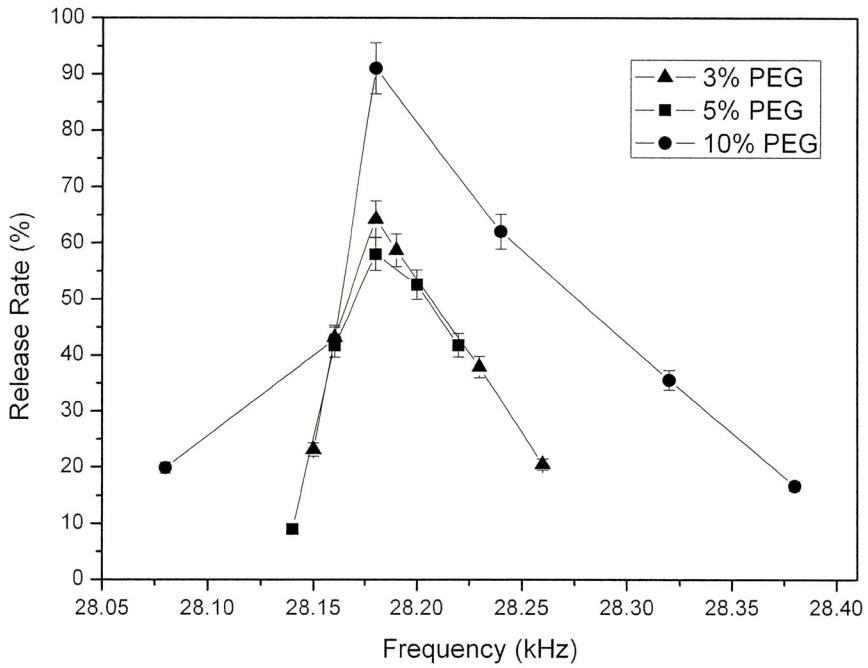


c)

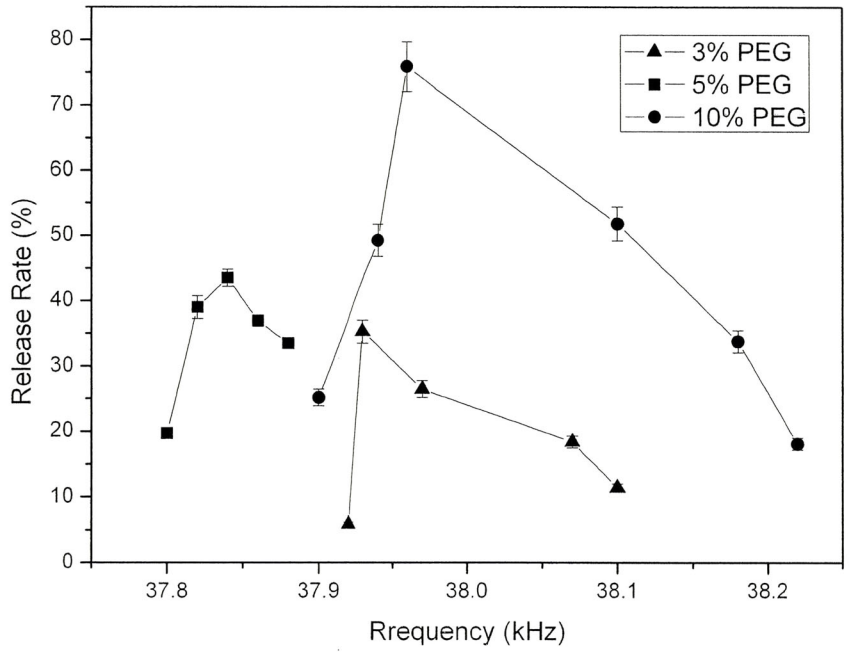
Fig. 4.3 Release effect of calcein-liposome by input frequency control. a) 5%sample (5% PEG); b) 3%sample (3% PEG); c) 10%sample (10% PEG.), input power 1.4 W. Mean S.D. n=4.

The averages of release rate after 120 seconds irradiation in Fig. 4.3 as a function of input frequency are taken for each samples and summarized in Fig. 4.4(a). The release rates of 5%sample and 3%sample are almost same for all the testing frequencies. However, 10%sample has much high release rate over all the testing frequencies. Furthermore, all the samples have the most effective frequency at 28.18 kHz. It indicates that the membrane of 10%PEG lipsomes is most easy to be destroyed under ultrasonic irradiation and have wide control frequency region.

The same experiment was carried out also for the testing frequency around the second peak frequency 38 kHz (Fig. 4.2) and their results are summarized in Fig. 4.4(b). Comparing to the results in Fig. 4.4(a) the 10%sample has higher release rate and wide control frequency region than other samples. Furthermore, the release rates are higher for the ultrasonic input frequency around 28kHz than that around 38kHz for each sample. However, the most effective frequency and the control frequency region are different from each sample. For example, the most effective frequency for 5%smaple is 37.84 kHz, for 3%smaple and 10%sample are 37.93 kHz and 37.96 kHz. To explain this phenomenon, let consider the wave length in the liposome suspension, which is defined by $\lambda=c/f$ where f is the input frequency of ultrasonic wave and c is the velocity of the ultrasound in solution. Suppose the velocity in distilled water at 23 ℃-27℃ is 1500 m/s, the wave length at frequency 38 kHz will be 39.5 mm and at 28 kHz is 53.6 mm. In our ultrasonic irradiation experiment, the liposome suspension 2ml was filled into the quartz cell which made the depth d of suspension as 20mm. It is clear that the testing suspension depth is just equal to the half length of the wave length in distilled water (i.e., $d=\lambda/2$), which means the self-induced oscillation in suspension will strongly influence the release rate. Based on the formula $\Delta\lambda / \lambda = \Delta f / f$ or $\Delta d / d = \Delta f / f$, if Δd is controlled under 0.1mm or 0.01ml, the shift of



a)



b)

Fig. 4.4 Average release rates of each sample after 120 seconds irradiation as function of the input frequency. (a) input frequency around 28kHz and (b) around 38kHz, where input power fixed at 1.4 W.

frequency will be $\Delta f = 0.19$kHz. However, in our present experiment it is difficult to control the liposomes suspension at accuracy less than 0.005ml. On the hand, the frequency at 28 kHz is not on the self-induced oscillation frequency, which might be the reason why input frequency at 28 kHz is more stable than that at 38 kHz in control of ultrasonic irradiation. The results also indicate that the release of inner-substances from liposomes can be realized by the ultrasonic treatment and can be controlled easily and suitably by adjusting the input frequency.

From the above results, the most effective input frequencies to ultrasonic probe become lower than the probe resonant frequency measured in free condition as shown in Fig. 4.2. It is well known that the resonant frequency of the probe will be easily influenced by the condition where is to be used. Now the problem comes how to measure the resonant frequency of the probe in its practical use condition and how to determine the effective frequency. Based on the piezoelectric impedance measurement technique (Tetsuya Morisaki and Zhongwei Jiang, 2005; Zhongwei Jiang et al., 2006; Fenlan LI and Zhongwei JIANG, 2007), it can be simply done in the following way. First attach the probe to the object to be irradiated, and then connect the input wires from the probe to an impedance analyzer and measure its admittance (the inverse of impedance) response at the expected frequency region. As an example, the admittance frequency response of the ultrasonic probe when it is in free condition, attached on the finger tip and nail surface, was measured by an impedance analyzer (Agilent 4292A, Agilent Co., JAPAN). The absolute admittance responses at frequency region 27.5 kHz to 29.5 kHz are shown in Fig. 4.5. From the results the peak value of admittance is at 28.322 kHz when the probe tip is in the state of free, and it is reduced to 28.207 kHz as the probe tip was touched by finger tip. After the curve of admittance response and the peak frequency related to the object are obtained, the effective input frequency should be selected a little lower than the peak frequency. Based on

authors' experience, the piezoceramic actuator usually has strong nonlinear properties at its resonant frequency and the piezoceramic actuator will cause high temperature when it is driven at its resonant frequency. Based on the results in Fig. 4.5 and considering to release the drug in human body, the input frequency to the probe is better to be selected lower than but much near 28.2 kHz.

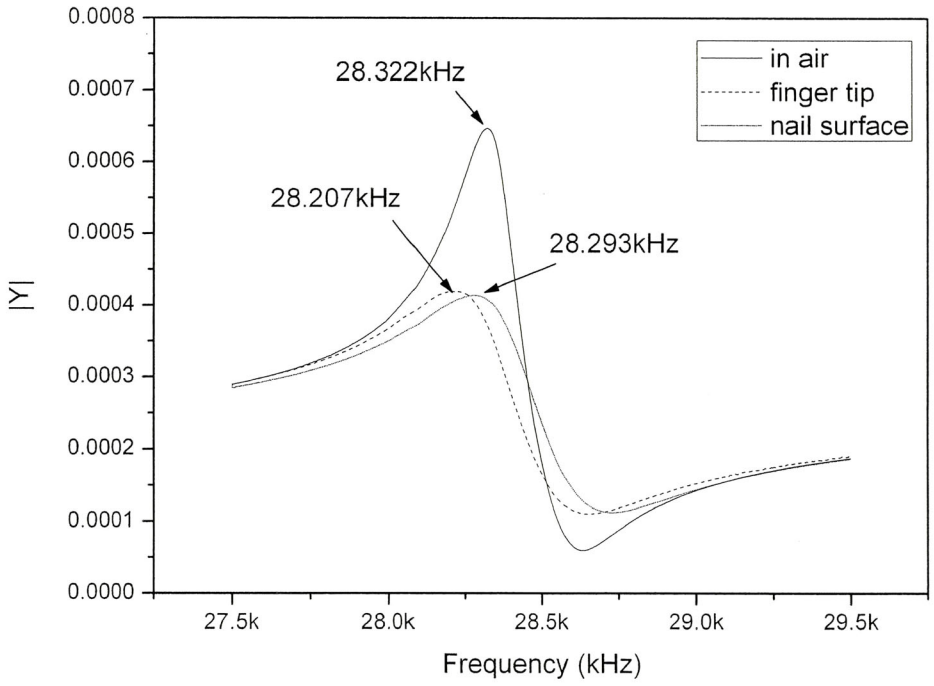


Fig. 4.5 Admittance frequency responses obtained when the ultrasonic probe attached to different objects.

Based on above discussions, the release rate can be controlled in such a way that first determine the input frequency based on the piezoelectric impedance measurement, and then adjust the input power. To validate this simple control method, in the second experiment the release property of calcein-liposomes was tested by adjusting the input power at a fixed input frequency of 28.18 kHz. Figure 4.6 shows the average release rates of each sample after 120 seconds irradiation at input frequency 28.18 kHz when the input powers were set at 0.7W and 1.4W. It

is evident that the release rate was increased with the increase of the input power for each sample. Further, the release rate of 10%sample was higher than the other samples. It indicates the lipid membranes have different stiffness due to the ratio of composed materials, for example, the lipid membrane at a mole ratio of POPC:DPPE-PEG2000:Choresterol=40:10:50 (10%sample) was most easily to be destroyed under the ultrasonic irradiation, while 5%sample (at ratio of 75:5:25) was relative strong. Mona Pong et al. reported that PEG enhanced ultrasound-induced leakage, but according to the results shown in Fig. 4.6, at low PEG concentration (<5 mol%), the leakage of water soluble substances decreased while increase the ratio of cholesterol in lipid membrane. And at high PEG concentration (10 mol%) the leakage was mainly determined by PEG even the ratio of cholesterol reached to 50 mol%. The results also hint that the calcein-liposomes release control method proposed in this study could be a power tool for evaluating the lipsome strength in biomaterial science.

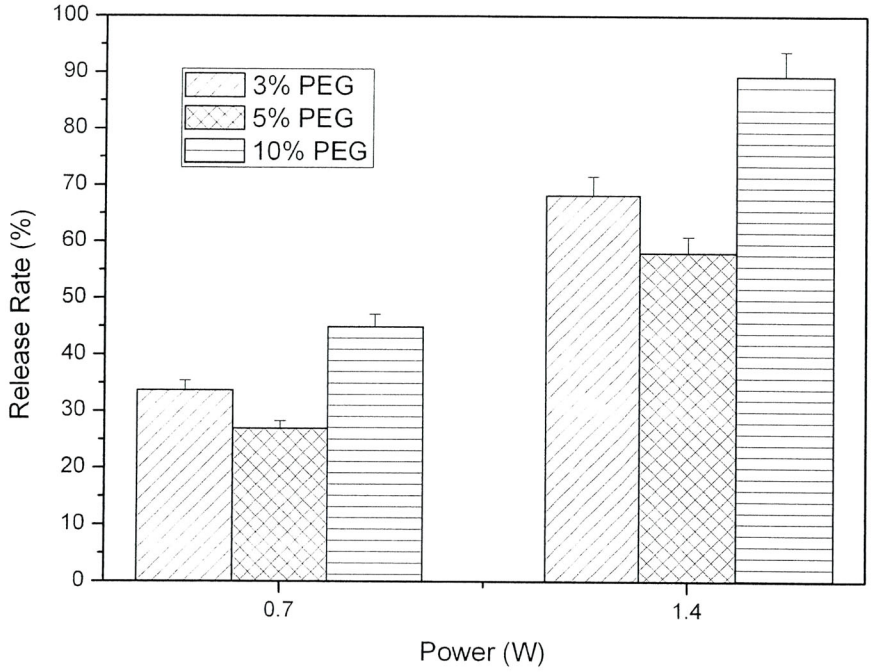


Fig. 4.6. Average release rates of each sample after 120 seconds irradiation at 28.18 kHz with adjusting the input power. Mean S.D. n=4

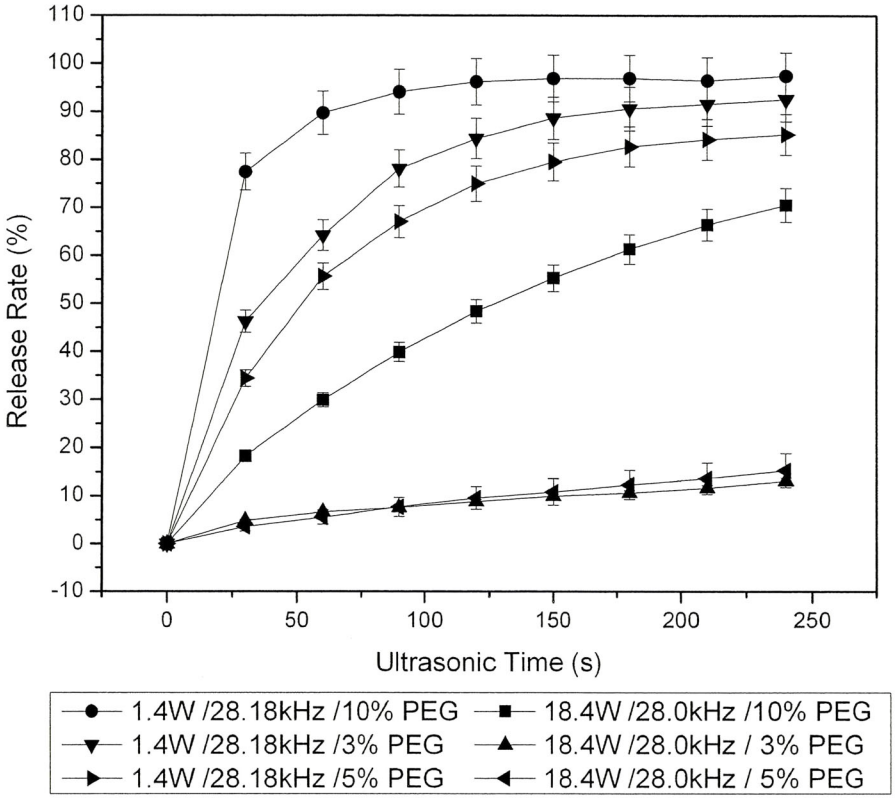


Fig. 4.7. Release effect comparison of the input at 28 kHz with 18.4W to 28.18 kHz with 1.4W.

Finally, Figure 4.7 shows the influence of the selected input frequency on the release effect. Two inputs, 28.0kHz with 18.4W and 28.14kHz with 1.4W, were tested in this experiment. It shows that the release of calcein in 5%sample and 3% sample almost have not achieved at the input frequency of 28 kHz even a very high input power was applied. However, the release speed becomes very high if the control input is set at a suitable frequency 28.18 kHz and even with very low input power. This experiment result indicates that the release property of liposomes could be controlled effectively by selecting a suitable input frequency based on the admittance response curve and then adjusting the input power to achieve the expected release result.

4.4 Summary

The release effect of calcein-liposomes controlled by the ultrasonic irradiation was studied experimentally. A new control method to release the inner-substances of calcein-encapsulated liposomes was investigate. The method how to select a suitable input frequency based on piezoelectric impedance measuring technique is proposed. The experiment results showed that release property of calcein-liposomes could be controlled more effectively by selecting the input frequency based on the admittance response curve of the probe in the practical use condition. Further, increase of the input power also leads to an increase of the release performance, but it is usually associated with high power which needs high current and easily causes a raise in temperature. As a conclusion, the release property of liposomes could be controlled efficiently by an ultrasonic probe in the way that the input frequency should be selected a little lower than its resonant frequency, so that the release speed of drugs from the encapsulated liposomes can be adjusted suitably by the input power.

5

IMPEDANCE EVALUTAION METHOD

In the study on ultrasound induced release of liposomes, it was found that the fluorescence intensity measurement and ultrasound treatment were separated into two steps. The release property could not be evaluated while ultrasound is irradiating. Also the fluorescence method is not fit for non-fluorescent drugs. Further, the fluorescence method does not fit for high concentration samples because of self-quenching. To fulfill these problems, this chapter is concerned with the study on development of a novel method for evaluation of the liposomes release property by measuring the electric impedance changes of liposome suspensions. Calcein/NaOH encapsulated liposomes (calcein- liposomes) were prepared with deionized water and were treated with ultrasonic irradiation in order to investigate the release property of the liposomes. To validate the proposed impedance measuring method, the calcein release rates were evaluated both by the impedance changes and the fluorescence intensity changes in calcein-liposome suspensions. With the comparison of these results obtained by the two methods, it is shown that the impedance method has much wider detecting concentration range than the fluorescence one. Furthermore, the impedance method can be efficiently used for evaluation of the release property on various ionic substances encapsulated within liposomes.

5.1 Introduction

Development of Drug Delivery System (DDS) is present interesting to researchers because it gives the hope of delivering drugs to the diseased part in the body and releasing them under control. Since polyethylene glycol (PEG) was first suggested for preparing liposomes in the 1990s (A.L. Klibanov, et al., 1990), some of the liposomes have been improved endurable for clinical application. Based on these technologies, researchers have focused their interests on the areas of drug encapsulation, targeting and release control. However, the properties, especially the release property of liposomes under external excitation, have not been clarified yet. Furthermore, how to evaluate the release property simply is one of the key techniques to be developed first.

Since fluorescent compounds like carboxyfluorescein (M.C. Sandström et al., 2005; D. Volodkin et al., 2007)/calcein (X. Liu et al., 2005; D. Yi et al., 2007) or fluorescence-labeled agents are widely used for fundamental research instead of real encapsulated drugs, the spectrophotometer and fluorophotometer are often used to study the release property of fluorescer-encapsulated liposomes, and the release property of liposomes is usually evaluated by fluorescence percentage (M. Kokkona et al., 2000; M. Pong et al., 2006; V.P. Torchilin and V. Weissig, 2003a). Due to the self-quenching property of fluorescence agents, the measurement precision will be greatly influenced by the concentration of fluorescence agents. Moreover, this method does not fit for evaluating the encapsulated drugs which are non-fluorescent. Otherwise, the non-fluorescent molecules should be grafted with fluorescent dyes, which will increase the experiment cost.

It is well known that the existence of free ions in the aqueous phase induces the conductivity of

the solution. The electrical property of liposome suspension will be changed when the encapsulated ionic materials are released out of liposome population. By monitoring the impedance or conductivity changes of the solution, the release property of drugs might be evaluated. But there are a few reports on evaluation of the liposome release property with the electrical impedance method. Bordi, et al., have monitored the electrical conductivity of NaCl-trapped liposome suspensions before and after ultrasonic treatment (F. Bordi et al., 2006). They aimed to find out the conducto-metric evidence for intact polyion-induced liposome clusters. The purpose of this study is to establish an impedance-based method for evaluation of the release property of drug-encapsulated liposomes.

In this paper, calcein/NaOH solution of high concentration was encapsulated into PEGylated liposomes, and 20kHz ultrasound was employed to enhance the release of encapsulated calcein because low frequency (tens of kilohertz) ultrasound exposure is more effective for introducing permeability change of the liposome membrane (M. Pong et al., 2006). The impedance changes of liposome suspensions before and after ultrasound treatment were measured with an impedance analyzer at the frequency of 1kHz which is often adopted in solution conductivity testing. Based on the analysis of the experiment results, the method for evaluating the release property of liposomes was proposed. Further, this proposed impedance method was validated by comparing the results obtained from the traditional fluorescence method.

5.2 Methods

5.2.1 Sample preparation

The sample used in this study was prepared by freezing-thawing method as was introduced in chapter 2. The lipid was composed of POPC, DPPE-PEG-2000 and cholesterol at molar ratio of

9:1:10. The samples were prepared with deionized water and the inner substance encapsulated in liposomes were 100mM calcein/NaOH.

5.2.2 Ultrasound exposure

Ultrasonic disrupter (UR-20P, Tomy Ceiko, Japan) was used to enhance the release of calcein. And the experiment setup and experiment conditions were shown in chapter 3 section 3.1.2. In brief, the output power of the ultrasound probe was set at 18W (about 366.7W/cm2), and was driven in a pulsed power mode (2min ON; 20min OFF) to control the temperature.

5.2.3 Impedance measurement

Basic principle

The electrochemical double layer impedance Z_{bl} of the electrode/solution interface could be modeled as a parallel connection of faraday impedance Z_f and double layer capacity $C_d(M)$. Further, the solution impedance $Z_s(M)$ could be characterized as a parallel connection of inner resistance $R_s(M)$ and capacitance $C_s(M)$. So the equivalent electric model for solution impedance measurement could be expressed by a series connection of $Z_s(M)$ and Z_{bl} as shown in Fig. 5.1, where $R_s(M)$, $C_s(M)$, $C_e(M)$ and $C_d(M)$ are functions of calcein ion concentration M.

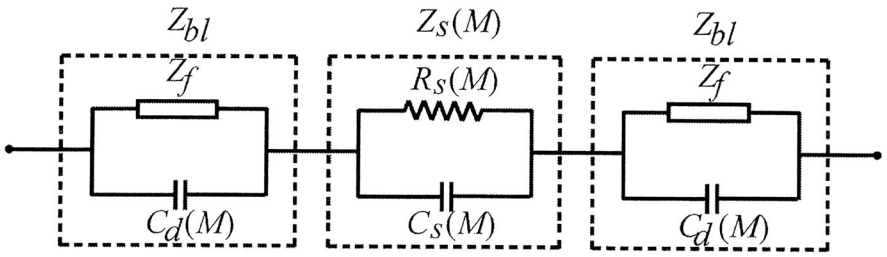


Fig. 5.1 Equivalent electrical model of solution impedance measurement

Since there is no electrical active material in the calcein-liposome solution and the applied voltage is low enough, there will be no electrode reaction occurring at the electrode/solution interfaces. Thus, the faraday impedance Z_f could be viewed as infinite ($Z_f = \infty$) (R. Tamamushi, 1991). In this case, figure 5.4 can be simplified as a series connection of solution impedance $Z_s(M)$ and double layer capacity $C_e(M)$ ($=C_d(M)/2$). The total impedance Z_t is then expressed as

$$Z_t = Z_s(M) + \frac{1}{j\omega C_e(M)}$$

$$= \frac{R_s(M)}{1+\omega^2 R_s^2(M) C_s^2(M)} - j\left[\frac{\omega R_s^2(M) C_s(M)}{1+\omega^2 R_s^2(M) C_s^2(M)} + \frac{1}{\omega C_e(M)}\right] \qquad (5.1).$$

When the encapsulated substances in the liposomes are released, both the solution impedance $Z_s(M)$ and double layer capacity $C_e(M)$ will be changed. It is clear from Eq. (5.1) that the real part of the impedance acquired by impedance analyzer only corresponds to the solution impedance $Z_s(M)$ and it is not influenced by the double layer capacitive $C_e(M)$. In the subsequent sections, only the real parts of the impedances recorded by impedance analyzer were used for evaluating the release property of calcein-liposomes.

Impedance measurement

The setup of impedance measurement system is shown in Fig. 5.2. The solution impedance was measured with two stainless steel electrodes (CUY560, NEPA GENE, JAPAN) which were connected to an impedance analyzer (Agilent 4292A, Agilent Co., JAPAN). The measurement frequency of the impedance analyzer was set at 1kHz and the voltage was 0.5Vp-p. Since the arrangement of the electrodes influence the impedance measurement, the distance between the two electrodes was set at 5mm in constant. All other parameters were also fixed in all

experiments as was shown in Fig. 5.2. Since the solution impedance is susceptible to temperature, all the measurements were carried out in a program incubator after the sample temperature reached to 25℃.

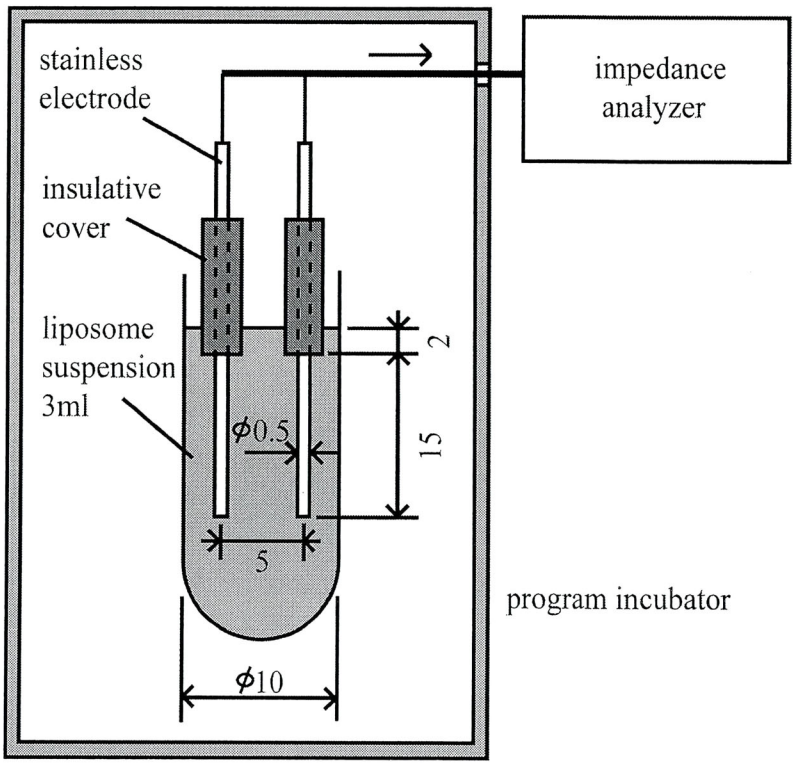


Fig. 5.2 Impedance measurement system setup

Calibration

The release rate of calcein-liposomes can be evaluated by the concentration percentage as follows

$$R'(t) = \left(M_t - M_{ini}\right) / \left(M_{fin} - M_{ini}\right) \times 100\% \tag{5.2}$$

where, $R'(t)$ is the concentration release percentage, M_t is the calcein/NaOH concentration at time t, M_{ini} is the initial concentration (i.e., the concentration at time 0), and M_{fin} is the final concentration when the liposomes were completely destroyed by Triton X-100.

To determine the concentration of released calcein M_t, a calibration between the calcein concentration and the impedance Z_s should be made beforehand by experiment. In calibration experiments, the Calcein/NaOH solution samples were diluted with deionized water in a logarithmical variation, and the impedance of each Calcein/NaOH sample was measured carefully at condition shown in Fig. 5.2. The results of the impedance Z_s as a function of the calcein concentration M are plotted in Fig. 5.3.

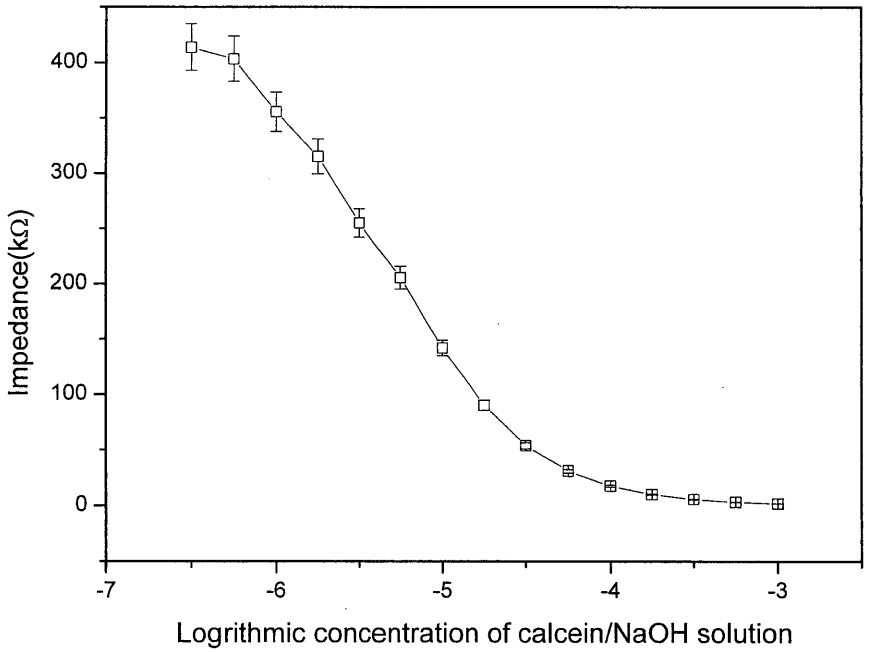


Fig. 5.3 Relationship between impedance (real part) and Calcein/NaOH solution concentration.

Mean±S.D. n=4

It is found from the figure that the impedance is not linear to the logarithmical concentration of calcein. It can be divided into three parts in order to get high accuracy calibration equations, the first part at the high concentration region from -3 to -3.75 in logarithmical concentration, or from $2x10^{-3}$ to $2x10^{-4}$M in molar concentration, the second part at the middle concentration from -3.75 to -5,or from $2x10^{-4}$M to $1x10^{-5}$M, and the third part at the low concentration from -5 to

-6.75, or from $1x10^{-5}$ M to $5x10^{-7}$ M. By applying the least-square regression method to the experiment data in these three regions receptively, three calibration equations were obtained as following,

$$M_t = 1.93 Z_s^{-1.03} \times 10^{-3} \quad at\ 2\,k\Omega \le Z_s \le 10\,k\Omega \tag{5.3}$$

$$M_t = 2.59 Z_s^{-1.13} \times 10^{-3} \quad at\ 10\,k\Omega \le Z_s \le 200\,k\Omega \tag{5.4}$$

$$M_t = 0.0618 \exp(-0.0116 Z_s) \times 10^{-3} \quad at\ 200\,k\Omega \le Z_s \le 500\,k\Omega \tag{5.5}$$

Because of the high electric insulation of lipid membranes, one can supposes that the calcein/NaOH solution encapsulated in the inner cores does not influence the solution impedance. Further, the influence of liposome population on solution impedance could be ignored if the liposome solution does not contain ionic material. Therefore, the calcein concentration M_{ini} and M_t can be determined by calibrating the measured impedances at time 0 and t according to calibration equations (5.3)-(5.5). Further, considering that Triton X-100 is non-ionic detergent which is used to destroy the liposome population to release the calcein completely, the final concentration M_{fin} were obtained by adding 1% triton X-100 (30μl) to the 3ml liposome sample and measuring its impedance.

5.2.4 Fluorescence measurement

For comparison, the release rates of calcein-liposomes were also evaluated by traditional fluorescence method which was introduced in chapter 3. In brief, the calcein release rate is calculated by

$$R''(t) = (F_t - F_{ini}) / (F_{fin} - F_{ini}) \times 100\% \tag{5.6}$$

where $R''(t)$ is the fluorescence release percentage, F_t is the fluorescence intensity observed at time t, F_{ini} is the initial intensity, and F_{fin} is the final intensity when calcein-liposome is completely destroyed by adding triton X-100. The fluorescence intensities were measured with a spectrofluorometer in the conditions that the wavelength of excitation light was set at 492nm and emission light at 517nm. In order to get comparable data, all the data were measured right after the excitation and emission conditions were set up. The samples prepared in section 5.2 were also measured by the spectrofluorometer after their impedances were recorded. Figure 5.4 shows the results of the fluorescence intensity versus the calcein concentration. The intensity goes up as the calcein concentration is increased and it reached to the highest value around the concentration of -5 in logarithmic concentration or 1.0×10^{-5}M in molar concentration. After the peak the intensity decreased with the increase of the concentration. This is well known as self-quenching phenomena. According to the description of Torchilin and Weissig (V.P.

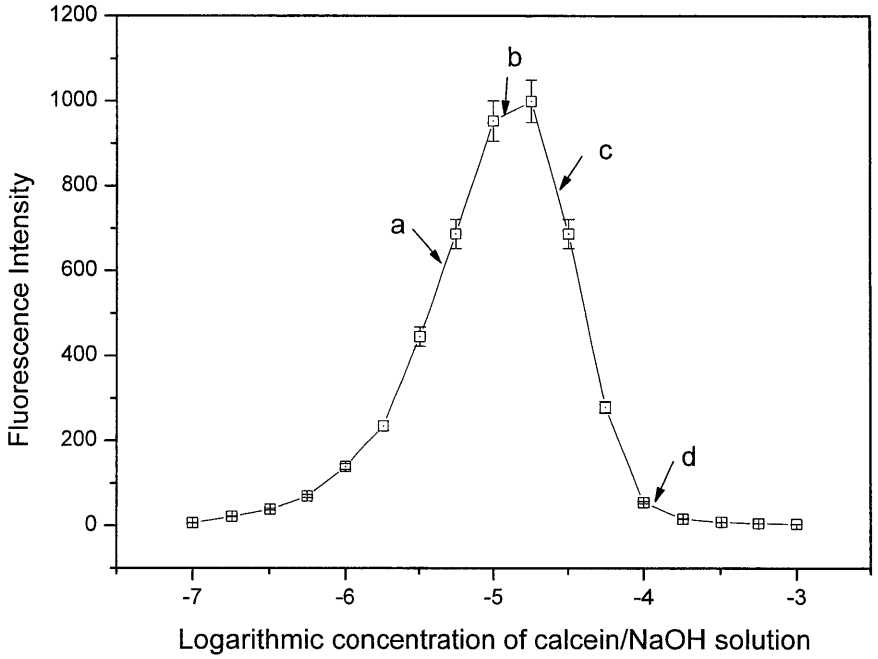


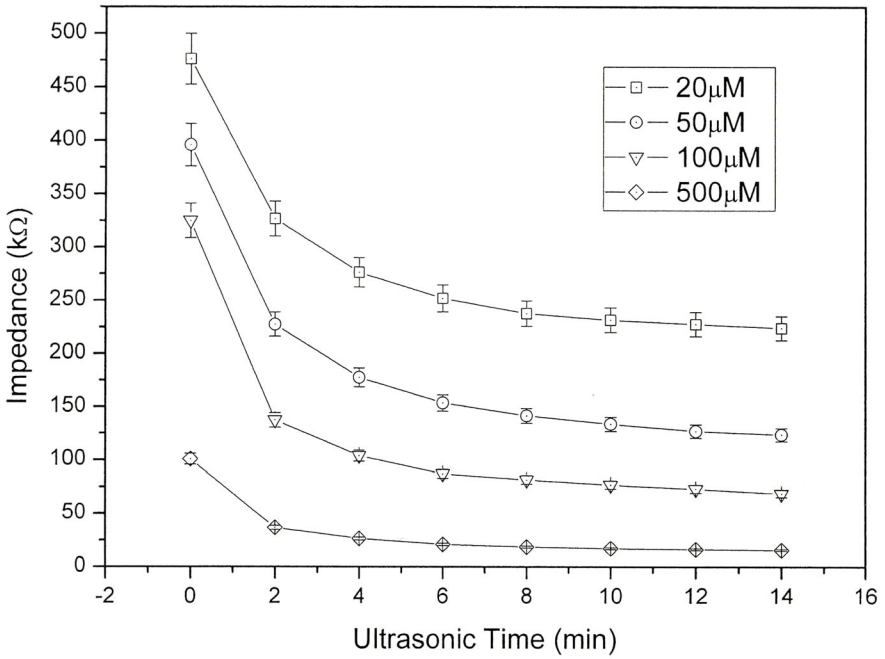
Fig. 5.4 Relation between calcein concentration and fluorescence intensity. Point *a*,*b*,*c* and *d* represents the maximum fluorescence intensities of 20μM, 50μM, 100μM and 500μM liposomes in the relation curve, respectively. Mean±S.D. n=4

Torchilin and V. Weissig, 2003b), only when the sample is diluted enough, i.e., no self-quenching occurs, the accurate fluorescence intensity measurement can be carried out. From this result it is evident that the accurate fluorescence intensity measurement is just available for the calcein concentration lower than $1.0x10^{-5}$M.

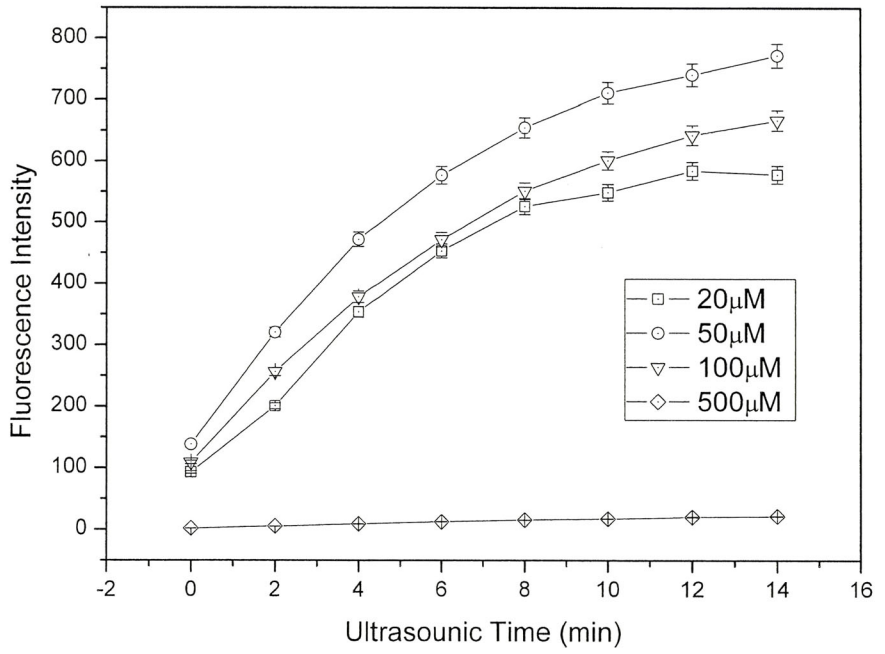
5.3 Results and discussion

In this paper, four different lipid concentrations of calcein-liposomes (20μM, 50μM, 100μM and 500μM) were prepared for validation of the proposed impedance measuring method. Their final fluorescence intensities of 20μM, 50μM, 100μM, and 500μM samples measured by the spectrofluorometer were 644, 923, 785 and 22 respectively. They were corresponding to *a*, *b*, *c* and *d* in Fig. 5.4. As was discussed in the former section, one can easily understand that the sample whose lipid concentration is 20μM could be evaluated accurately by the fluorescence intensity method.

The ultrasonic treatment was set at 20kHz, with the output power of 18W. Further the ultrasonic treatment was applied to the sample for two minutes and stopped 20 minutes for measuring the impedance change and the fluorescence intensity. This process was repeated until the measurement result approached to a constant value. Figure 5.5-a is the plot of the impedance results at each two-minute ultrasonic treatment. The impedance of each sample decreased exponentially with increase of the treatment time, and it approached to a constant value as the ultrasonic treatment is proceeded long enough. This indicates that the calcein ions were released from the liposome population gradually with the increasing of treatment time. Furthermore, the impedance at the low concentration sample has higher impedance values than those of the high concentration sample as was demonstrated in Fig. 5.3.



a)



b)

Fig. 5.5 Impedances (a) and fluorescence intensities (b) results obtained from calein-liposomes at four different lipid concentrations (20μM, 50μM, 100μM and 500μM) when ultrasound was applied. Mean±S.D. n=4

The fluorescence intensity measurement was carried out simultaneously right after its impedance results were obtained. Figure 5.5-b shows the fluorescence intensity changes as function of the ultrasonic treatment time. The fluorescence intensity of each sample is increased gradually with the treatment time. Furthermore, the intensity of the sample at 50μM lipid concentration has the highest value than the others. In comparison, the intensity at 500μM has the lowest value.

As discussed above, the release profile of 20μM liposomes could be evaluated accurately by the fluorescence method. The release rate as a function of the ultrasonic treatment was calculated by Eq.(5.6) from the fluorescence intensity results and the result was plotted in Fig.6 (R", circled curve). Further, the release rate calculated by Eq.(5.2) from the impedance measurement data was over-plotted in Fig. 5.6 (R', squared curve). According to Fig. 5.6, it is evident that the release rates obtained by both methods are agreed well with each other. It indicates that the

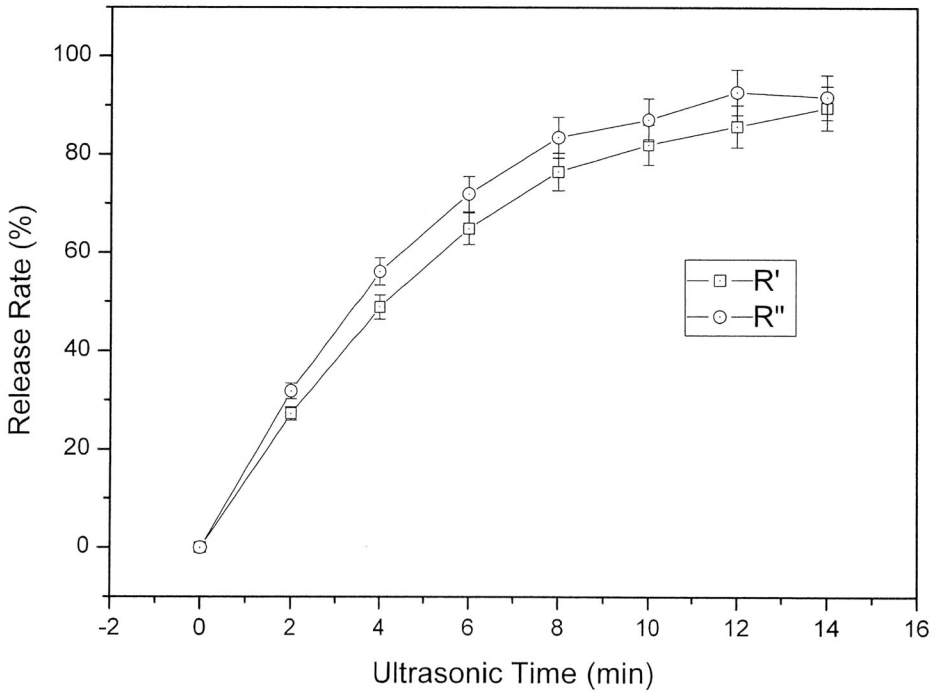


Fig. 5.6 Calcein release rates of 20μM calcein-liposomes by concentration percentage (R') and fluorescence percentage (R"). Mean±S.D. n=4

impedance evaluation method is satisfied for the evaluation of liposome release rate, and has the accuracy as good as, that of fluorescence method which is only available for low calcein concentration.

In the following, more comparisons of the results obtained by these two methods are discussed. Figure 5.7 shows the release rates of the four samples evaluated by fluorescence percentage while Fig. 5.8 shows the corresponding results acquired by impedance measuring method. In Fig. 5.7, the release rates of high concentration liposomes do not agree well with that of 20μM liposomes, which was due to calcein self-quenching as discussed in the previous section. It means that the detecting range of fluorescence method should be limited within the non-quenching concentration region. However, Fig. 5.8 shows a good consistency of each sample obtained by the impedance measurement method. This implies that the impedance measurement method could be used for wide range of the calcein concentration if the target

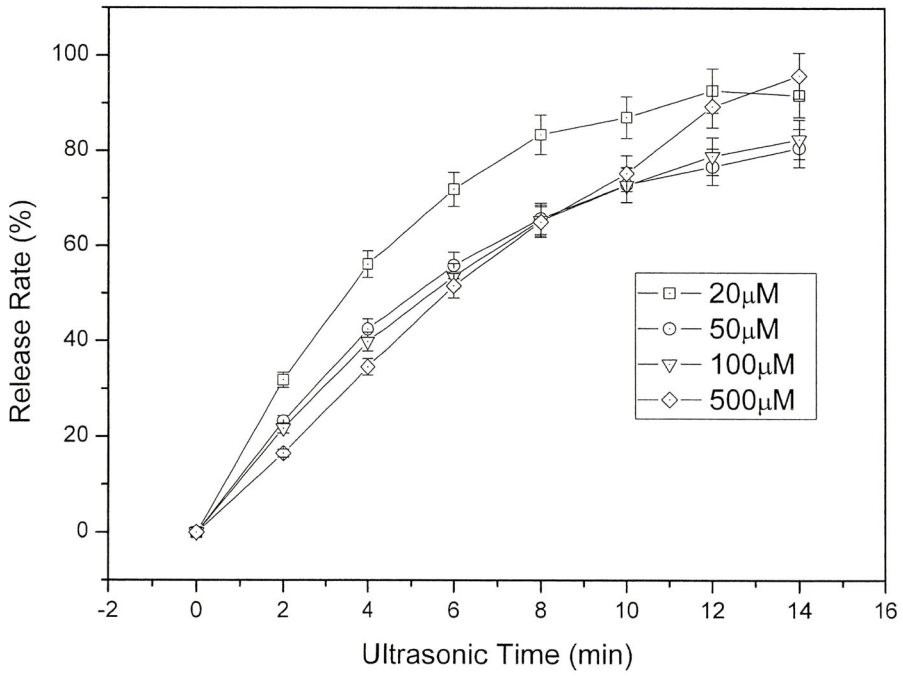


Fig. 5.7 Calcein release rates of 20, 50μM, 100μM and 500μM calcein-liposomes by fluorescence percentage (R"). Mean±S.D. n=4

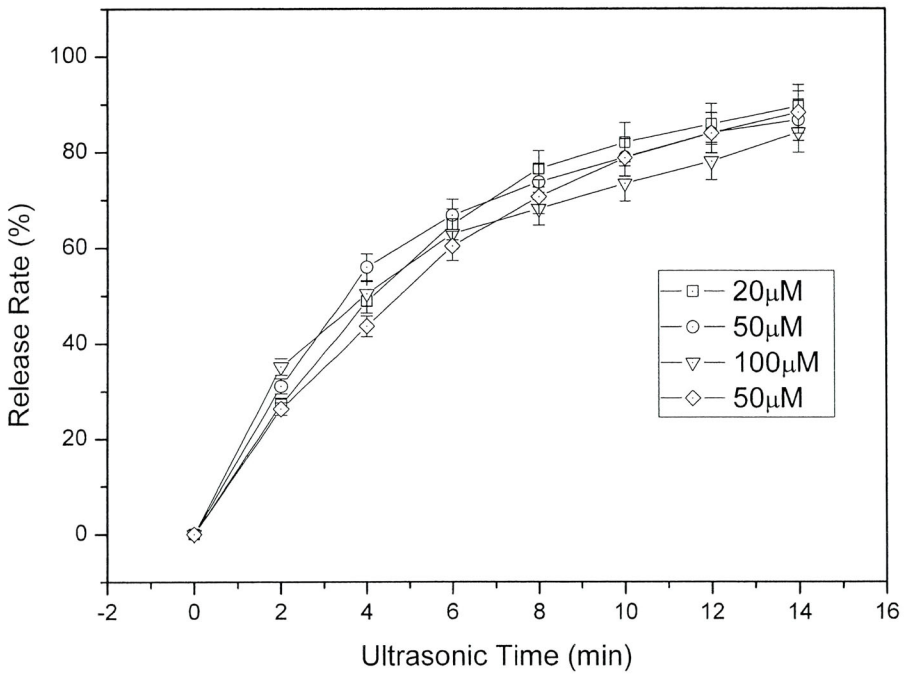


Fig. 5.8 Calcein release rates of 20μM, 50μM, 100μM and 500μM calcein-liposomes by concentration percentage (R'). Mean±S.D. n=4

solution calibration on its impedance to concentration is previously obtained. Further, it is obvious that the impedance method can be also efficiently used for evaluating the release rate of the liposomes encapsulated with various ionic materials without fluorescent dyes.

5.4 Summary

In this paper, ultrasonic treatment at 28kHz was employed as a stimulus to enhance the release speed of calcein-liposomes in deionized water. The release rates of calcein-encapsulated liposomes were evaluated by impedance measuring method and fluorescence intensity method, simultaneously. The results showed that the accuracy of fluorescence method is correlated with the final calcein concentration of the liposomes and it is available only for the samples at low

lipid concentration where the maximum calcein concentration should be lower than quenching concentration. The release rates of the samples at different calcein concentration showed their consistency in the results obtained by impedance measuring method. It indicates that the impedance method is not affected by the calcein self-quenching and has a wider testing range than the fluorescence method. Furthermore, since the impedance method is based on the common conductive property of ionic substances, it has high potential to evaluate the release property of ionic-substances encapsulated liposomes.

6

CONCLUSIONS AND PROSPECTS

Liposomes as drug delivery system have been intensively researched because their potential application in controlled release of drugs. In this dissertation, a study on ultrasound induced release of calcein-liposomes as well as a new evaluation method based on impedance measurement was proposed.

As a first step of this study, PEGylated calcein-liposomes with different lipid components were prepared and their morphologies were investigated by transmission electron microscopy. The release of calcein liposomes induced by temperature and ultrasound was studied, respectively. Results showed that the sample prepared with POPC, DPPE-PEG200 and cholesterol was insensitive to temperature but was very sensitive to low frequency ultrasound. The size distributions and zeta potentials were studied by particle size & zeta-potential meter before and after ultrasound irradiation. Results showed that the liposomes were well prepared and had a mean size around 100nm. The zeta potential of liposomes in de-ionized water is negative. After exposed to ultrasound, the mean size of liposomes decreased slightly and their absolute zeta potential value decreased for high concentration liposomes which indicate the release of encapsulated calcein.

In a second step, the release effect of calcein-liposomes controlled by the ultrasonic irradiation was studied experimentally. A new control method to release the inner-substances of calcein-encapsulated liposomes was investigated. The method on how to select a suitable input frequency based on piezoelectric impedance measuring technique is proposed. The experiment results showed that release property of calcein-liposomes could be controlled more effectively by selecting the input frequency based on the admittance response curve of the probe in the practical use condition. Further, increase of the input power also leads to an increase of the release performance, but it is usually associated with high power which needs high current and easily causes a raise in temperature. As a conclusion, the release property of liposomes could be controlled efficiently by an ultrasonic probe in the way that the input frequency should be selected a little lower than its resonant frequency, so that the release speed of drugs from the encapsulated liposomes can be adjusted suitably by the input power.

In a third step, the release rates of calcein-encapsulated liposomes were evaluated by impedance measuring method and fluorescence intensity method, simultaneously. The results showed that the accuracy of fluorescence method is correlated with the final calcein concentration of the liposomes and it is available only for the samples at low lipid concentration where the maximum calcein concentration should be lower than quenching concentration. The release rates of the samples at different calcein concentration showed their consistency in the results obtained by impedance measuring method. It indicates that the impedance method is not affected by the calcein self-quenching and has a wider testing range than the fluorescence method. Furthermore, since the impedance method is based on the common conductive property of ionic substances, it has high potential to evaluate the release property of ionic-substances encapsulated liposomes.

Although these initial results establish the necessary experimental procedures and resultant accuracy, further measurements are clearly needed to provide more accurate control and measuring methods. In the long run, the release property of drug encapsulated liposomes induced by ultrasound *in vivo* as well as the control method is needed to be established. And the impedance measuring method is also need to be proved in animal testing. The impedance method will provide an online measuring method for ultrasound induced release.

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