Development of thermal sensitive liposomes for targeted delivery and controlled release of drug

by

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Declaration of Originality

I Xin Zhang declare that this thesis is the work of my own. Works of others that are presented in the thesis have been clearly referenced in the Harvard referencing system format.

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Abstract

One of the clinical challenges in the treatment of diseases with a discrete distribution is the local delivery of active drugs without causing systemic side effects. Doxorubicin, an anti-cancer drug, is an example that may cause damage to patients due to its interaction with normal cells. Tissue plasminogen activator (tPA) is another active agent whose effectiveness is compromised due to its unselective effect on both healthy and pathological blood clots.

Thermal sensitive liposome is a drug carrier that is not only able to entrap drug into it hydrophilic interior but can also release encapsulated agents upon mild hyperthermia. Currently, lysolipid-containing thermal sensitive liposome (LTSL) is a standard formulation due to its rapid release behaviour. However, concerns have been raised regarding the negative effect of lysolipid on LTSL stability that results in undesirable leakage of encapsulated drug *in vivo* at physiological temperature.

Therefore, the first aim of the project was to develop a lysolipid-free thermal sensitive liposome (TSL) formulation. For this purpose, doxorubicin was adopted as a model drug. The amount of doxorubicin molecules encapsulated in TSL (0.0123 mg of doxorubicin per 1 mg of lipid) was 2 times the amount in the existing publications (0.05 mg of doxorubicin per 1 mg of lipid). This was done by optimizing the incubation temperature (36^{o}C for 1 hour) during preparation of TSL. When heated from room temperature to 42 ^{o}C, doxorubicin-encapsulated-TSLs release encapsulated doxorubicin molecules faster than their LTSL counterpart. This may result from a higher membrane-bound doxorubicin concentration in dox-TSLs.

The second objective of the project was to develop a tPA-encapsulated TSL (tPA-TSL) formulation that can release encapsulated tPA molecules under hyperthermia condition. It has been demonstrated that the developed tPA-TSL was able to release encapsulated tPA upon 7 minute heating at 45^{o}C. The release of tPA molecules was expected to result from irreversible destruction of liposome upon heating, as was demonstrated by reduction of particle size.

Table of contents

Title page……1
Declaration of originality ……2
Copyright Declaration……3
Acknowledgment……4
Abstract……5
List of tables……11
List of figures……12
Chapter 1. Introduction……14
1.1.Historical context and the emergence of modern medicine……14
1.2.Current stage of magic bullet for tumour treatment……16
1.3.Current stage of magic bullet for blood clot lysis……28
1.4.Emergence of external energy field-triggered drug release system……18
1.5.Aim of the project……20
1.6.Outline of thesis……23
Chapter 2. Literature review……24
2.1. Clinical application of doxorubicin in anticancer chemotherapy and development of doxorubicin-drug delivery system……24
2.2. Development of EPR-based doxorubicin-drug delivery system……26
2.2.1. Doxorubicin-polymer conjugate nanoparticles……26
2.2.1.1.Drug-polymer conjugates and their application to doxorubicin……26
2.2.1.2.Doxorubicin-polymer conjugates nanoparticle……28
2.2.2. Doxorubicin-encapsulated nanoparticle using liposome as carrier……29
2.3.Thermal sensitive nanoparticles for doxorubicin……32
2.3.1.Thermal sensitive liposome……33
2.3.1.1.Traditional thermal sensitive liposome for tumour treatment……33
2.3.1.2.Lysolipid-containing thermal sensitive liposome (LTSL) for tumour treatment……34
2.3.1.3. Failed clinical trial of LTSL vesicle for tumour treatment……36
2.3.2. Polymer-based thermal sensitive nanoparticle……38
2.4. Tissue plasminogen activator-encapsulated TSL for thrombolysis……39
2.4.1. Development of tPA-covalently conjugated carrier……39
2.4.2. Ultrasound sensitive tPA-liposome complex……40

2.4.3. Magnetic guided ultrasound responsive polymer tPA DDS……..…41
2.4.4. Shear-activated tPA-conjugated nano-therapeutics……...41
2.5.Summary……42
Chapter 3. Development of simple and stable thermal sensitive liposomes for the treatment of solid tumour ……..44
3.1. Introduction……....44
3.2. Selection of preparation method for doxorubicin-encapsulated vesicle ……..…46
3.2.1. Preparation of multilamilar and large unilamilar liposome and optimization of preparation process…….45
3.2.2. pH gradient active loading of doxorubicin……..……47
3.2.2.1. Early development of pH gradient method……..…….… 48
3.2.2.2. Selection of acidic buffer for DPPC-based vesicle system for optimal D/L ratio of doxorubicin……50
3.2.2.3. Selection of counterion and its effect on the morphology of liposomal doxorubicin……50
3.2.2.4. Final determination of interior buffer……52
3.3. Selection of method to determine encapsulation efficiency of liposome ……....52
3.4. Preparation and analytical process of doxorubicin-TSL…….52
3.4.1. Preparation of doxorubicin-encapsulate liposome…….53
3.4.2. Determination of D/L ratio of liposome……...….53
3.5. Results and discussion……..54
3.5.1. Influence of incubation temperature on the D/L ratio of dox-TSL……54
3.5.2. Potential mechanism of selective permeability change of TSL membrane with Temperature……56
3.5.2.1. Unsynchronized movement of phospholipid in ripple phase…… 58
3.5.2.2. Relation between the potential occurrence of liquidation at ripple phase in nanosized vesicle and the permeability change……..60
3.5.3. Potential instability of doxorubicin-TSL as a result of ripple phase……61
3.6. Summary……62
Chapter 4. Characterization of dox-TSLs and determination of their thermal sensitivity……...….63
4.1. Introduction……63
4.2. Selection of characterization method for doxorubicin encapsulate vesicle…….65
4.2.1. Determination of particle size of liposome……..65

4.2.2. Determination of phase transition temperature of liposome ……..65
4.3. Selection of assay to study thermal sensitivity of doxorubicin-encapsulated liposome……66
4.3.1. Incompatibility of dissolution test with thermal sensitive liposome…….66
4.3.2. Selection of self-quenching based doxorubicin quantification method……..67
4.3.3. Selection of parameter to determine the thermal sensitivity of doxorubicin-encapsulated liposome……..70
4.3.4. Selection of heating method to determine the release behavior…….72
4.3.4.1. Progressive heating method……73
4.3.4.2. Direct heating method……..73
4.4. Analytical process of doxorubicin-TSL…….74
4.4.1. Determination of particle size of liposome…….74
4.4.2. Determination of phase transition temperature of TSL membrane……74
4.4.3. Determination of release behaviour of doxorubicin-TSL……74
4.4.3.1. Progressive heating assay…….....74
4.4.3.2. Direct heating assay……...75
4.5 Results and discussion……75
4.5.1 Characterization of dox-TSL and dox-LTSL……..75
4.5.2. The determination of dox-TSL release behaviour……..78
4.5.2.1. Release behavior of dox-TSL and dox-LTSL using progressive heating Method…….…..78
4.5.2.2. Reported superior thermal sensitivity of TSL in comparison with LTSL..80
4.5.2.3. Existing reports on interaction of doxorubicin and lysolipid with DPPC Membrane……..…82
4.5.2.4. Potential mechanism behind the superior thermal sensitivity of dox-TSL……84
4.5.2.5. Percentage release of doxorubicin by direct heating method……..86
4.5.2.6. Current theory that explains thermal sensitivity of liposome and a potential new explanation……87
4.6. Conclusion……89
5. Development of tissue plasminogen activator-encapsulated TSL for thrombolysis……..90
5.1. Introduction……..90
5.2. Development of TSL for thrombolytic agents ……....91

5.2.1. Selection of streptokinase as a pilot thrombolytic agent……91
5.2.2. Quantification method for thrombolytic agent……...92
5.2.3. Vesicle preparation method for thrombolytic agent-TSL……93
5.2.3.1. Ideal physical characteristics of vesicle for high encapsulation capacity…..93
5.2.3.2. Available preparation methods for large vesicles with low lamilarity…….94
5.2.3.3.The role of solute-engulfing mechanism in encapsulation capacity of vesicles…….. 96
5.2.3.4. REV method for the preparation of thrombolytic agent-TSL……96
5.3. Preparation procedure of thrombolytic agent-encapsulated TSL or LTSL.....98
5.3.1. Preparation method for thrombolytic agent using reverse phase evaporation method……..98
5.3.2. Quantification of SK and determination of D/L ratio of SK-encapsulated liposome……98
5.3.3. Quantification of tPA concentration and determination of D/L ratio of tPA-encapsulated liposome……..99
5.3.4. Thermal sensitivity test of SK or tPA-encapsulated vesicles……99
5.3.5. Determination of phase transition temperature and particle size of tPA-TS100
5.4. Results and discussion……100
5.4.1. Feasibility of SK-encapsulated LTSL……100
5.4.2. Feasibility of tPA-encapsulated TSL……102
5.4.3. Potential release mechanism of tPA from TSL upon heating……..103
5.4.4. Hypothesis of structure of tPA-TSL……106
5.5. summary……107
6. Conclusions……108
6.1. Main conclusions……108
6.1.1. Development of lysolipid-free doxorubicin-TSL……...108
6.1.2. Development of lysolipid-free tPA-TSL……..109
6.2. Limitations of the analytical methods employed ……110
6.2.1. Limitations of doxorubicin and calcein quantification method……111
6.2.2. Limitations of heating method for measurement of thermal sensitivity…….112
6.2.3. Limitations of s-2251^{TM}-based assay for thrombolytic agent quantification….112
6.3. Future work……...113
6.3.1. Determination of PEG functionality and fluidity of membrane……....113
6.3.2. Determination of release behaviour of TSL at temperatures above T_m……113

6.3.3. Optimization of tPA-TSL for stability enhancement……...113
Reference……..115
Achievement……..130
Appendix.....131
Appendix I- List of chemicals used and their abbreviations……..……132
Appendix II- Information on analytical equipment used in the project……133
Appendix III-Calibration curve for lipid quantification……134
Appendix IV-Calibration curves for doxorubicin quantification……135
Appendix V-Calibration curve for SK quantification encapsulated in TSL……136
Appendix VIII-Calibration curve for tPA quantification ……137
Appendix VII- Heating DSC Thermogram of TSL membrane……..138
Appendix VIII- Heating DSC Thermogram of pure DPPC membrane……....139
Appendix IX- Heating DSC Thermogram of pure DPPC membrane……..140

List of tables

Table 1.1. Summary of reported doxorubicin release behaviour of DPPC:DSPE.2000 formulation in the literature……21
Table 3.1. Summary of reported D/L ratio of liposomal doxorubicin in the literature……45
Table 4.1. Summaries of property determination during novel thermal sensitive liposome development in existing publications……..……..6 4
Table 4.2. Summary of prior studies where the thermal sensitivity of doxorubicin-encapsulated thermal sensitive liposomes was determine based on the self-quenching nature of doxorubicin……...…69
Table 4.3. Characterization of liposomal doxorubicin prepared in the present study and comparison with publications in the literature……77
Table.4.4 summary of prior studies that demonstrate lysolipid-free thermal sensitive liposome having faster release rate than LTSL at various temperature points……81
Table 4.5. List of existing publications that reported thermal sensitivity of liposome being uncompromised at temperatures above T_m of membrane. ……88
Table 5.1. Characterization of tPA-TSLs and blank TSLs prepared by REV method……103

List of figures

Figure 2.1.The structure of doxorubicin and its ancestor molecule daunorubicin…….25
Figure 2.2. Molecular structure of Polyethyleneglycol, PEG, the first polymer for therapeutic conjugation……..…27
Figure 2.3. Molecular structure of hydroxypropylmethacrylamide, HPMA, and the first doxorubicin-polymer conjugate……..…28
Figure 2.4. Demonstration of doxil®, the first doxorubicin-encapsulated nanoparticle….31
Figure 2.5. Schematic that demonstrates the main function of ThermaDox®……35
Figure 2.6. Molecular structure of PIPAAM, the first thermal sensitive material. ……..38
Figure 2.7. Schematic that demonstrates the concept of tPA/RBC……..40
Figure 2.8. Schematic that demonstrates the concept of shear-activated tPA-Ns aggregates……42
Figure 3.1. Schematic of pH gradient method for doxorubicin encapsulation……..…48
Figure 3.2. Brief description of ammonium gradient method for doxorubicin encapsulation……50
Figure 3.4. Influence of incubation temperature on the D/L ratio of dox-TSL (*n=3*). Inubation process lasted for 1 hour……..……55
Figure 3.4. Permeability changes of DPPC membrane to unprotonated and acetic acid molecules within temperature range of 25 to 35 °C……57
Figure3.5.Demonstration of various stages of lipid membrane upon temperature rise ……..……58
Figure 3.6. Demonstration of C=O (carbon and oxygen double bond) in the DPPC molecules……59
Figure 4.1. Influence of D/L ratio on stability of liposomal doxorubicin using % release (A) or amount of released doxorubicin/lipid (w/w) (B)……72
Figure 4.2. Physical appearance of intact dox-TSLs (A), dox-TSLs after the addition of triton *X-100* (B) and dox-TSLs after 2 minutes of progressive heating assay……...76
Figure 4.3. Amount of doxorubicin released /lipid (w/w) from dox-TSL *(n=3)* and dox-LTSL *(n=5)*……79
Figure4.4.Potential interaction site of doxorubicin and MSPC with phosphotadylcholine……..……83
Figure 4.5. Proposed theory that explains why thermal sensitivity of dox-TSLs is higher than that of dox-LTSLs……...85

Figure 4.6. % of released doxorubicin from TSL and LTSL using direct heating assay (*n=3*)……..……...86
Figure 5.1. A schematic of the preparation process of classical REV using water-immiscible phase……95
Figure 5.2. D/L ratio (IU/mg) of SK-encapsulated LTSL (*n=3*)……...101
Figure 5.3. The extent of SK release from LTSL at 42 and 45^{o}C (*n=3*)……..102
Figure 5.4. Changes of tPA-TSLs and blank TSLs particle size prior to and after heating at 45 ^{o}C for 7 minutes (A) (*n=3*). Change of tPA-TSLs particle size prior to and after the addition of triton X-100 (B) (*n=3*)……104

Chapter 1. Introduction

1.1. Historical context and the emergence of modern medicine

Drug, a general term that describes the substance humans consume for curing disease, has evolved with the development of human civilization since antiquity. In the past century, the world level life expectancy increased from 47 years in 1950-1955 to 68 years in 2005-2010 (Zhao. 2011) and advances in medicine is a crucial contributor to the increase of well state of human being. In the world we are living today, the magic bullet concept (to be detailed below) is the predominant thinking behind the development of modern medicine. This is especially the case when a detectable pathogen is the cause of a disease and pathogen-specific toxin is administered to patients in order to destroy the pathogen without harming the host. While this may seem obvious to people living today, the magic bullet concept was not established until the beginning of the 20th century as a cross-field product that benefited from advances in organic chemistry, synthetic chemistry, pharmacology and parasitology (Bosch. 2008).

Prior to the acceptance of the magic bullet concept, drug was predominately guided by our cultural understanding of the nature of the cosmos, disease and human body. In Europe, since the Black Death that killed nearly one-third of the European population, theories other than superficial explanations (e.g. witchcraft, divine judgment) had been proposed to explain why the plague occurred with an attempt to cure diseases. In the late 15th century (100 years after the Black Death), syphilis (also known as “sexual plague”) became the major killers in Europe since renaissance time till the early 20th century (Kelly. 2009) and is still portrayed in some culture as a divine judgment from God due to its sexual transfer nature (Bosch. 2008).

Prescribed in the canon of medicine (the most influential medical textbook in medieval time), mercury was the standard treatment for syphilis before the 18th century, which gave rise to the famous saying “*a night in the arm of venus leads to a lifetime on mercury*”, demonstrating both the false shame brought to the infected people and the length of the treatment (Semaan et al. 2006). Sadly, oral intake of mercury caused severe side effects to the treated patients (*e.g*. tremors, impaired cognitive skills); the

cure rate was extremely low and syphilis still claimed peoples' life (e.g. Vladimir Lenin, based on official report) as late as the early 20th century (Service . 2000). In the modern era, patented arsenic containing solutions (historically called tonic for their wide range of "curable" diseases) became an alternative remedy of syphilis other than the more toxic mercy (Jolliffe. 1993). When looking back into the history, the two major medicines used to treat syphilis share the same problem: using medicine without acknowledging the disease-causing target.

In the early 20th century, the major development in parasitology not only allowed diagnosis of the true cause of some of the well-known "disease" (e.g. African sleep sickness is caused by the infection of Trypanosoma brucei (Goddard 2013), syphilis is caused by Treponema pallidum), but also opened the door for medical research by enabling the direct effect of drug to be assessed. In 1909, Paul Enrich, the founding father of therapeutic pharmacology, successfully synthesised Arsphenamine (marketed as Salvarsan), a Treponema pallidum-targeting chemicals with two well defined characteristics: parasitotropic but not organotropic (i.e. drug that harms the parasitic invader without harming the host) (Witkop. 1999.). In 1910, he published the rationale behind the development of an ideal version of parasite—targeted medicine:

*"These antibodies are exclusively '**parasitotrop'** and **not 'organotrop'**, and hence it can be no surprise that they find their target as a kind of **magic bullet**. In this way, I also explain some of the marvellous cures of that [therapeutic] direction. It is therefore eo ipso self-evident, that the serum method must be ceteris paribus superior to every other type of therapy precisely through the **pure parasitotropy of these medical substances**"* - stated by Paul Ehrlich in Boyer. 2013

Since its introduction to the medical world, the concept of magic bullet had revolutionized the strategy people used to develop medicine: from finding the chemicals that can relieve the symptom of the disease to **finding the magic target and relevant magic target.** In the field of antibacterial drug development, thanks to the distinctive cell structure between mammalian eukaryotic and prokaryotic bacteria, the antibiotic (including penicillin) developed under the magic bullet strategy had saved numerous human life from once life-threatening infectious diseases.

However, finding the magic target is the pre-condition of rational design of the magical bullet. Without it, magic bullet will lose its “not organotropic” features and compromise its efficacy at the disease-causing site. As a result, while drug may still be able to kill the target effectively, it could also damage normal cells and result in undesirable side effects or render the treatment completely ineffective.

Cancer and life-threatening blood clots are the two main areas where the development of magic bullets is compromised by the lack of ideal magic sites. In this project, both areas will be explored.

1.2. Current stage of magic bullet for tumour treatment

Tumour is the end product of abnormal growth or division of cells and malignant tumour (or cancer) is now the major cause of death of elderly people (50-74 years old) in the United Kingdom (Cancer mortality by age, available from http://www.cancerresearchuk.org). Since tumour cells are originated from normal cells, no magic targets that are distinctively present in tumour cells have been found. Consequently, current strategy of tumour treatment is mainly focused on improving the chances of drug deposition to tumour rather than normal cells by making use of the abnormal up-regulation of various receptors (most notability epidermal growth factor receptor, EGFR) (Normanno et al, 2006) or the hyper vascular permeability of tumour.

In 1985, Mike Sporn and Anita Robert discovered that various types of carcinoma tumour (e.g. gastric, breast, colorectal, ovarian, cervical, bladder and oesophageal cancer) have up-regulation levels of EGFR (a cell-surface receptor that regulates cell growth and proliferation) (Sporn and Robert. 1985). This discovery spurred the development of EGFR receptor-targeted drugs. Since the first EGFR inhibitor was approved to treat chronic myeloid leukaemia (CML) in 2001, imatinib®, has received positive reception and was regarded as a “miracle drug”. In one of the most important clinical trials carried out by Brian and his colleagues, 53 out of 54 patients had their white blood cell counts restored to the normal range within 1 month of treatment, with nearly 90% of patients survived 5 years after treatment (Pray. 2008). For their discovery, Drucker and his colleagues (the team behind the invention of imatinib®) received the

Lasker-Debaker clinical medical research award for “converting a fatal cancer into the manageable condition” and “Japan prize” for **development of “therapeutic drug targeting cancer-specific molecules**”.

However, as the drug was designed to target EGFR, successful therapeutic outcome of imatinib® treatments depends on the quality of its target (*i.e.* the extent of up-regulation of EGFR and level of its mutation). For instance, while most CML patients have positive up-regulated EGFR levels, only 15% of patients with invasive breast cancer have up-regulated level of EGFR (DiGiovanna et al. 2005) and 30% of patients with non-small-cell-lung cancer (NSCLC) have the required level of EGFR expression (Pirker. 2012). In one of the latest phase III clinical trials, NSCLC patients who had low levels of EGFR expression did not show survival benefit from cetuximab®, a recently designed EGFR inhibitor (Pirker. 2012).

A serious drawback of imatinib® is its negative influence on immunoregulatory system: through the same mechanism of action as it inhibits tumour proliferation, imatinib® substantially reduces the proliferation of activated T cells (responsible for cell-mediated immunity) (Seggewiss et al 2005). As a result, for patients who are treated with imatnib®, even vaccine may become the source of infection.

Another major discovery of target site for tumour is made on the tissue, rather than molecular level. In 1984, Hiroshi Maeda of Kumamoto University discovered that nano-sized particles could accumulate in tumour (Hoffman. 2008). This tumour specific phenomenon is known as the enhanced permeability and retention effect (*i.e.* EPR effect) which can be utilized to develop specialized particles for tumour treatment. In parallel, long circulating nanoparticle developed in 1987 has significantly increased the circulation time of nanoparticles, fulfilling the condition required for satisfactory tumour accumulation (24-hour circulation time is needed to achieve maximum nanoparticle accumulation in tumour) (Working et al. 1994). These two discoveries initiated the development of EPR-guided nanoparticles which can incorporate molecules ranging from toxin (chemotherapy, to be detailed below) to the latest tumour-specific gene therapeutics for tumour treatment. Similar to EGFR targeted drugs, the effectiveness of EPR-guided nanoparticles depends on the quality of the target (the leakiness of tumour blood vessel), a condition that till recently has been proved to be highly heterogeneous

among patients and is considered to be the main reason for the diverse clinical results of EPR-guided nanoparticles (Prabhakar. 2013).

1.3. Current stage of magic bullet for blood clot lysis

Blood clot formation (*i.e.* thrombogenesis) is an essential function in human body that enables the rapid repair of injured blood vessels. As the end product of a complicated cascade reaction (*i.e.* coagulation process), a polymerized-fibrin blood clot is eventually formed at the damaged vessel, the formation of which prevents the loss of blood upon vascular injury and serves as a provisional matrix in subsequent tissue repair (Yakovlev et al. 2000). The damaged part of the vessel is then recovered after the dissolution of initially formed blood clot through fibrinolysis process (blood clot lysis): Plasmin and tissue plasminogen activators (tPA) are two types of proteins that account for the fibrinolysis activity in the vessel. Through activation, plasminogen (*a* precursor enzyme of plasmin) is converted into plasmin, which dissolves the fibrin network (the building block of blood clot) and restores blood flow in the vessel (Yakovlev et al. 2000). TPA, produced by endothelial cells, is responsible for the conversion from plasminogen to plasmin. If the balance between fibrinogenesis and fibrinolysis is tilted towards the former, excessive blood clots may be formed in the vessel resulting in reduction of blood flow. Stroke and myocardial infarction (MI) are two of the most severe consequences of pathophysiological blood clot formation in a large blood vessel (Francis et al. 1987, Meunier. et al. 2007). Clinically, administration of tPA is the only accepted method to treat acute ischemic stroke and myocardial infarction in the U.S.A and U.K.

On the other hand, as tPA can also dissolve hemostatic blood clots that are present in the vessel, administration of tPA may result in internal bleeding of the affected area or areas remote from the clot (Ganguly et al. 2005, Ganguly et al. 2006).

1.4. Emergence of external energy field-triggered drug delivery system

External energy field-triggered drug delivery system (DDS) is defined as a drug delivery strategy that is able to selectively release drug at the desirable site when exposed to an external energy field. It has become a branch of clinically-applicable technique in the

past 30 years. The concept of drug delivery strategy was firstly introduced by Yatvin et al (Yatvin et al. 1978): inspired by the early work of Papahadjopoulos et al (Papahadjopoulos et al. 1973), Yavin and his colleagues demonstrated for the first time the ability of liposome (a type of nanoparticle composed of phospholipids with a hydrophilic interior that can be used to entrap drug molecules) to selectively release encapsulated model drug (*i.e.* neomycin) in response to temperature rise from 37 to 42^0C, which led to a 100 time increase in local drug concentration (Yatvin et al. 1978). This type of nanoparticle was defined by Yatvin as thermal sensitive liposome (Yatvin et al. 1978). Apart from suggesting tumour and local infection being the possible applications of thermal sensitive nanoparticles, Yatvin also described the core concept and benefit of thermal sensitive liposome compared with conventional drug treatment (Yatvin et al. 1978):

"*On passing through the heated area in the circulation, the liposome would be expected to release their contents at a greater rate than elsewhere and thus to develop higher local concentrations.* "- from Yatvin et al. 1978

In other words, by increasing the temperature of desirable target in the body, drug can be locally released in response to temperature rise. This will greatly benefit patients who cannot be effectively treated by the magic bullet strategy due to the lack of a magic target, which is the case for blood clot-suffering patients or tumour patients who lack sufficient up-regulation of EPR and EGFR. For these patients, external energy field-triggered drug delivery systems offer the advantage of local release of drug without the need for a magic target.

In parallel with the emergence of thermal sensitive liposome, high intensity focused ultrasound (HIFU) was developed as a non-invasive surgical technique and became clinically available in 1990 (Hynynen et al. 1990): Generated by a transducer, multiple ultrasound waves can provide localised heating to a specifically targeted area as a result of added intensity of multiple wave interactions, while the rest of the body is left unaffected due to the relatively low wave intensity of individual waves (Haar et al. 2007). HIFU has been applied as an external energy source to trigger the local release of drug from thermal sensitive liposomes (de Smet et al. 2011, de Smet et al. 2013, Dromi et al. 2007, Grull et al. 2012).

Radio frequency ablation (RFA) is another clinically available heating method that has been used as an external energy source to trigger local release of drug from thermal sensitive liposomes (Hong et al. 2013, Poon and Borys. 2009, Poon and borys. 2011). Unlike HIFU, RF heats up the body tissue by directly contacting the target area with a heating probe, delivering high frequency current and increasing the temperature of the targeted area (Haemmerich et al. 2002). Aside from its invasive nature, RFA does not have the ability to accurately control the temperature of heated area at the precision of HIFU. As a result, when RFA is used as a heating source, the affected area experiences a wider range of temperatures (Gasselhuber et al . 2010) when compared with the narrow range generated by HIFU (Dromi et al. 2007).

Although other DDS exists that can be triggered by different external sources (*e.g.* ultrasound, electric field, magnetic field, *etc*), thermal sensitive liposome is the only external energy field-triggered drug delivery strategy that has reached the clinical stage so far (Landon et al. 2011).

1.5. Aim of the project

Currently being considered as a crucial component in most thermal sensitive liposome formulations, lysolipid has been reported to be a drug release accelerator that can influence both the rate and extent of drug release (Anyarambhatla, and Needham. 1999. Mills and Needham. 2005). As a result, lysolipid-containing thermal sensitive liposome (*i.e.* LTSL) is currently the gold-standard formulation (Needham et al. 2013. Poon and Borys. 2009, Tagami et al. 2011). However, concerns have been raised regarding the negative effect of lysolipid on the stability of LTSL that has been found to result in undesirable leakage of encapsulated drugs *in vivo* at physiological temperature (Al-Ahmady et al. 2012, Li et al. 2013, Hossann et al. 2012). Furthermore, such undesirable leakage was predominantly reported in doxorubicin-encapsulated LTSL, a well-studied formulation with strong stability due to the formation of doxorubicin crystal within the liposome (to be detailed in section.3.2.2.3). Hence, due to the fact that majority of small molecules lacks the ability to form organized and stable crystals, the extension of LTSL to other therapeutic small molecules is limited.

On the other hand, the lysolipid free formulation (e.g. DPPC:DSPE.2000) has been found to lack the ability to rapidly release encapsulated doxorubicin. The retarded doxorubicin release behaviour reported in the literature is summarized in Table 1.1.

Table 1.1. Summary of reported doxorubicin release behaviour of DPPC:DSPE.2000 formulation in the literature

Formulation	Extent of doxorubicin release in 10 min (%)	Reference
DPPC:DSPE.2000	~20	Chiu et al. 2005
	~25	Needham et al. 2013
	~22	Banno et al 2010

More recently, however, this view has been challenged by the demonstration that lysolipid free thermal sensitive liposomes can rapidly release 70% of encapsulated cisplatin (another anticancer agent) at a rate similar to its LTSL counterpart (Woo et al. 2008). Moreover, owing to the absence of lysolipid, cisplatin-encapsulated DPPC:DSPE.2000 liposomes are more stable in the circulation compared to its LTSL counterpart (Woo et al. 2008). However, as cisplatin was encapsulated as free drug molecules (cisplatin cannot be encapsulated through pH gradient and does not form crystal), it might be reasonable to speculate that doxorubicin-encapsulated DPPC:DSPE.2000 would not be able to release encapsulated doxorubicin as rapidly as its cisplatin-encapsulated counterpart due to the formation of doxorubicin crystals. **Hence, the first aim of the project is to confirm the release pattern of doxorubicin-encapsulated DPPC:DSPE.2000 liposome**. By doing so, the application of DPPC:DSPE.2000 liposome may be expanded to other small therapeutic molecules that can form a stable complex within the liposome by increasing the stability of membrane at physiological temperature. Doxorubicin is selected as a model drug in the project due to the extensive literature on its release behaviour from DPPC:DSPE.2000 liposome. On the other hand, as DPPC:DSPE.2000 liposome often have a low doxorubicin encapsulation efficiency compared with its non-thermal sensitive counterparts (Chiu et al. 2005), **effort has also been made to increase the doxorubicin loading capacity of DPPC:DSPE.2000 formulation**: As long as the presence of doxorubicin molecules

does not compromise the integrity of liposome membrane, a higher amount of encapsulated doxorubicin per lipid (drug to lipid ratio D/L) can help reduce the undesired leakage of encapsulated doxorubicin at physiological temperature (Jonhnston et al. 2008). Hence a higher D/L ratio is a desirable property of doxorubicin-encapsulated DPPC:DSPE.2000 liposomes .

The other aim of the project is **to expand the catalog of feasible drug for thermal sensitive liposome to high molecular weight protein-based therapeutics**. In the thesis, tPA is used as a model drug not only because of its high molecular weight, but also because tPA is a thrombolytic agent which is effective at treating blood clots in myocardial infarction and acute ischemic stroke. As such, it is a good example of cases where the development of magic bullet is extremely hard as a result of non-differentiable structure between pathological and hemostatic blood clots. Prior to the application of expansive tPA, however, streptokinase (another widely used thrombolytic agent) is selected as a model drug due to its low cost and yet similar mechanism of action.

In summary, the objectives of the thesis are:

- Development of lysolipid-free TSL that is able to encapsulate doxorubicin
- Improve the amount of doxorubicin encapsulated in DPPC:DSPE.2000 liposome
- Determine the thermal sensitivity of doxorubicin-encapsulated DPPC:DSPE.2000 liposome

- Feasibility study of thrombolytic agent-encapsulated thermal sensitive liposome
- Determine the feasibility of thermal sensitive liposome to encapsulate and release streptokinase
- Determine the feasibility of thermal sensitive liposome to encapsulate and release tPA

1.6. Outline of the thesis

Chapter 2. Literature review: this chapter introduces the drug delivery system developed for the treatment of tumour and blood clots.

Chapter 3. Preparation of simple and stable dox-TSL with high D/L ratio for the treatment of solid tumour: In this chapter, work on increasing the encapsulation of doxorubicin in DPPC: DSPE.PEG.2000 liposomes are described in detail. One of the properties of liposomes, Doxorubicin/lipid ratio was determined and compared with existing publications.

Chapter 4. Preparation of simple and stable dox-TSL with high D/L ratio for the treatment of solid tumour: In this chapter, hydrodynamic diameter and phase transition temperature of DPPC: DSPE.PEG.2000 liposomes that encapsulated with doxorubicin molecules have been determined. The release rate of encapsulated doxorubicin both from lysolipid-free thermal sensitive liposomes and their lysolipid-containing counterparts was determined using two heating methods.

Chapter 5. Development of tissue plasminogen activator-encapsulated DPPC: DSPE.PEG.2000 liposome (tPA-TSL) for thrombolysis: This chapter describes the development of tPA-TSLs. TPA to lipid ratio, hydrodynamic diameter and phase transition temperature of tPA-TSLs have been determined. The ability of tPA-TSLs to thermally release encapsulated tPA molecules was determined at 45 ^{o}C

Chapter 6. Conclusion: In this chapter, the main findings and limitation of the work presented in this thesis are summarized. This is followed by a discussion of the limitations associated with the analytical methods used in the project. At the end of the chapter, future works are proposed for further optimization of the formulations and testing of the proposed hypothesis.

Chapter 2. Literature review

2.1. Clinical application of doxorubicin in anticancer chemotherapy and development of doxorubicin-drug delivery system

The use of chemotherapy in cancer treatment can be traced back to the middle of the last century: tested by Louis Goodman and his colleagues, nitrogen mustards, a derivative of the dangerous mustard gas (used as chemical weapon in the first world war) was first tested clinically for treatment of various cancers in the 1940s (Joensuu. 2008). In the 1950s, inspired by the discovery that antibodies could be produced from bacteria, collaborative studies were carried out by two groups of researchers from France and Italy in order to find an anticancer compound that could be produced by bacteria (Weiss 1992). Derived from *Streptomyces* (a soil-based microbe), **daunorubicin** (shown in Figure 2.1), the first anticancer chemotherapy compound of the anthracycline family was discovered. Later, the more powerful compound, **doxorubicin** (also shown in Figure 2.1), was produced by a mutant strain of *Streptomyces* by the same research group (Lomovskaya et al. 1999). The main mechanism of action of doxorubicin and other anthracycline chemotherapy is the inhabitation of cell growth through intercalating the base pairs of DNA/RNA strands (Rubin and Hait. 2000). However, as doxorubicin cannot distinguish tumour cells from normal cells with a fast growth span, the administration of doxorubicin also hinders the production of fast growing normal cells. This includes the production of red and white cells, leading to the occurrence of anaemia and neutropenia (Schwartz et al. 2005). Cells composed of skin, hair, and the gastrointestinal tracts are also the likely target of doxorubicin due to their rapid growth rate (Schwartz et al. 2005). Furthermore, the main serious side effect of doxorubicin is its cardiotoxicity which may cause congestive heart failure (Minotti et al. 1999). While the actual mechanism of its cardiotoxicity is still unknown, two hypotheses have been proposed to explain the route through which doxorubicin causes heart damage:

- Production of free radicals: Catalyzed by various reductase enzymes, doxorubicin (and other anthracycline) is transformed into a semiquinine-free radical that eventually produces $^{\cdot}OH$, a potent free radical that can damage heart cells (i.e. cardiomyocytes) through oxidation (Xu et al. 2005). In addition, the

presence of free radicals is also responsible for the disruption of cardiac gene expression program and reduces the production of several important proteins and enzymes (Akimoto et al. 1993).

- Formation of harmful metabolite: Doxol is a hydroxyl metabolite formed by cytosolic enzyme. It was detected within the cardiomyocytes of postmortem tissue from patients who had previous undergone anthracycline treatment (Minotti et al. 1999). Such a metabolite is able to impair cardiac contraction and relaxation through affecting the myocardial energy metabolism and ionic gradient movement (Minotti et al. 1999).

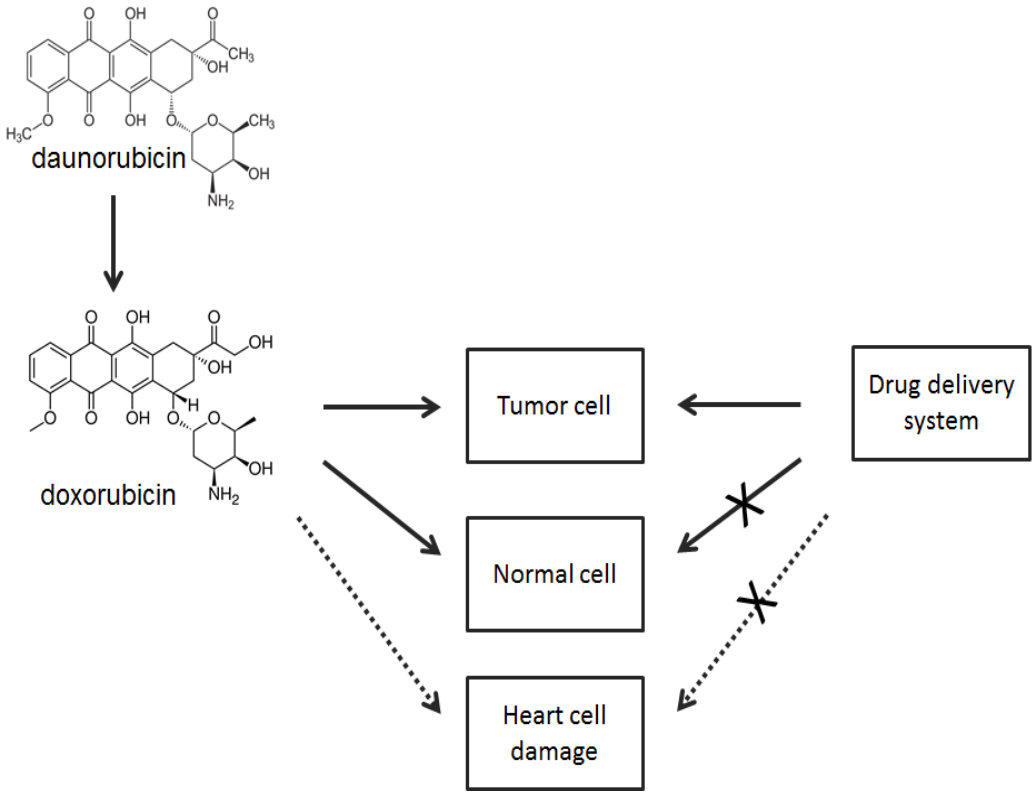


Figure 2.1.The structure of doxorubicin and its ancestor molecule daunorubicin. The desirable and undesirable targets of doxorubicin are also demonstrated: doxorubicin damages the tumour and normal fast growing cells through DNA/RNA binding. Doxorubicin causes heart cell damage mainly through the production of free radicals and hydroxyl metabolite.

2.2. Development of EPR-based doxorubicin-drug delivery system

Irrespective of its undesirable victims (i.e. normal fast growing cells or cardiomyocytes), it is apparent that DNA/RNA binding of normal cells, production of free radicals and the metallization of doxorubicin require the direct contact between doxorubicin and specific enzyme or target. Hence, by shielding doxorubicin from its potential victims or interacting enzymes, the harmful side effect of doxorubicin can be avoided. In 1984, a group led by Maeda discovered that the high permeability of solid tumour *(i.e.* EPR effect) could be exploited for drug accumulation (Iwai,.1984). However, in order to make accumulation of the drug possible through EPR effect, a longer circulation time of around 24 hours (Working et al.1994) and an ideal particle size (~100 nm) are needed (Nagayasu et al. 1999). Based on these criteria, two drug delivery systems (DDS) have been developed with the aim to maximize the availability of doxorubicin to tumour cells whilst reducing its toxicity by avoiding interaction between doxorubicin and healthy cells:

- Doxorubicin-polymer conjugate nanoparticles (Masayuki et al. 1990)
- Doxorubicin-encapsulated nanoparticle using liposome as a carrier (Barenholz. 2012)

The effectiveness of these doxorubicin DDSs depends on two factors:

- The extent of doxorubicin-DDS deposition in the tumour
- Availability of free doxorubicin molecules at tumour site.

2.2.1. Doxorubicin-polymer conjugate nanoparticles

2.2.1.1. Drug-polymer conjugates and their application to doxorubicin

Drug-polymer conjugates are a type of DDS that aims to increase the effectiveness of drug by directly attaching polymer molecules onto drug molecules and is the first type of DDS that has been applied to toxic chemotherapy (Kopecek and Kopeckova. 2010). Polyethyleneglycol (PEG, shown in Figure 2.2) was the first polymer used to conjugate therapeutic molecules and was initially used by Davis et al (Davis. 2002) to reduce the adverse immunological response of proteins of non-human origin (e.g. insulin). It was proposed by Davis that, by conjugating protein with a hydrophilic polymer, the undesirable interaction between the immune system of human and therapeutic proteins can be prevented (Davis. 2002). Hydrophilic PEG was chosen both as a result of their

non-immune nature and the presence of terminal hydroxyl group that is available for linking proteins (Davis. 2002). Surprisingly, while fulfilling their main purpose, PEGlyation of the protein also increased the circulation time of protein (Davis. 2002). Unfortunately, these pioneering techniques were never used to conjugate doxorubicin.

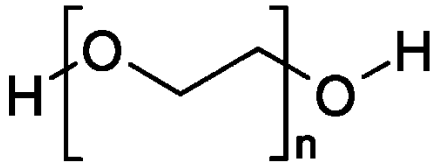


Figure 2.2. Molecular structure of Polyethyleneglycol, PEG, the first polymer for therapeutic conjugation. The terminal group is available for drug conjugation.

Independently, hydroxypropylmethacrylamide, (HPMA, see Figure 2.3) a novel synthetic polymer with a methylene backbone and a stable amide side chain, was first synthesized in the 1970s (Kopecek and Bažilová et al. 1973). Compared with drug decorated by PEG, HPMA was intentionally developed as a cell selective drug delivery system with the required multifunction:

- Selectively targeting cells with a surface attachment of cell targets (e.g. galactose, hepatocytes targeting, etc.): A relatively high MW of HPMA makes endocytosis of the cell the only pathway for drug uptake, rather than by diffusion (as is the case for small molecules). With the help of cell-targeting molecules, the drug has an increased chance of uptake by tumour cells.

- Ability to free drug from polymer conjugate: Using enzyme degradable oligopeptides as a link between the polymer backbone and drug molecules, the drug can be “released” from its polymer-conjugate form upon interaction with specific enzymes.

In 1983, a specially designed oligopeptide which is degradable only by lysosome was synthesized (Kopecek and Kopeckova. 2010). This enabled conjugate drug molecules to remain in an inaccessible state before they reached the cytosolic structure of cells (Rejmanová P et al. 1985). In 80s, the first doxorubicin-polymer conjugate (doxorubicin-HPMA, see Figure 2.3) was patented (Rihova et al. 1985). With its ability to add cell-targeting group, doxorubicin-HPMA has become a good candidate for an effective tumour-targeting doxorubicin delivery system.

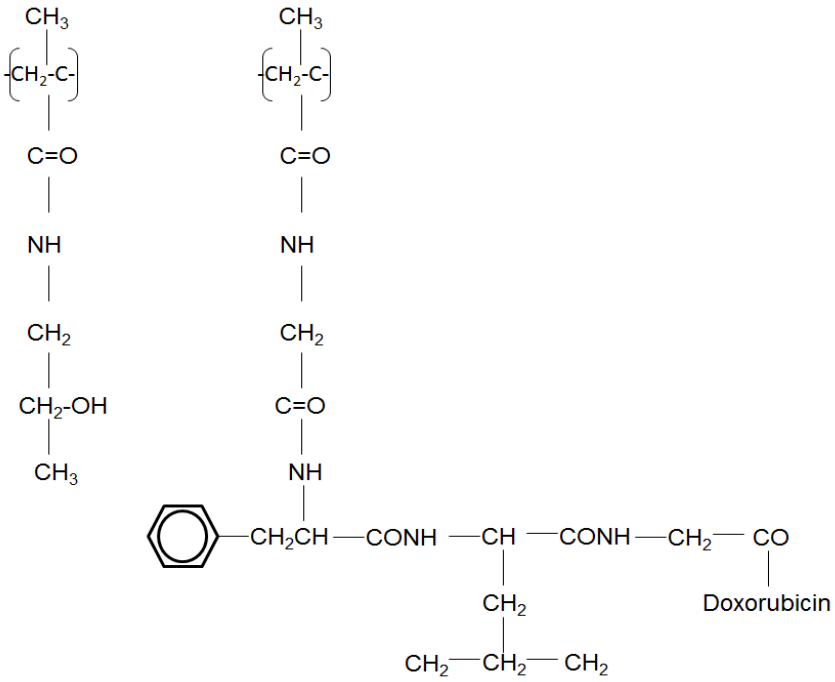


Figure 2.3. Molecular structure of hydroxypropylmethacrylamide, HPMA, and the first doxorubicin-polymer conjugate (adapted from Kopecek and Kopeckova. 2010). The link between HPMA backbone and doxorubicin was specially designed to be broken only by lysosome.

2.2.1.2. Doxorubicin-polymer conjugate nanoparticle

Irrespective of the detailed structure of the drug-conjugate, a short half-life ($h_{1/2}$) was a major drawback in the early stages of drug-conjugate development as increasing $h_{1/2}$ of the drug was not the initially-intended purpose of designing a drug-polymer conjugate. For instance, HPMA-doxorubicin has a $h_{1/2}$ of only 1.8 hours (Vasey et al. 1999). This is largely due to the low MW (25KDa) of the HPMA-doxorubicin (Loadman et al. 1999) which is readily filtered out of the circulation by renal excretion (cut off range <60 KDa). Hence, drug-conjugates can hardly benefit from the discovery of EPR effect as a result of their small size and short circulation time. With an aim to overcome the short half-life of drug-polymer conjugates and their small particle size, the first doxorubicin–

incorporated polymeric nanoparticle was invented in the 90s. The main component of the nanoparticle is the newly synthesized poly(ethylene glycol)-poly (aspartic acid) block co-polymer, an amphiphilic polymer with PEG as hydrophilic group and poly aspartic acid as hydrophobic group that can conjugate with doxorubicin (Masayuki et al. 1990). Due to their amphiphilic nature, the polymers form micelles in the aqueous system and shield poly aspartic acid along with doxorubicin from exposure to the circulation (Masayuki et al. 1990). Owing to its large particle size, the half-life of such doxorubicin-conjugate was significantly increased compared with its drug-conjugate counterpart (Masayuki et al. 1990). The actual release mechanism and detachment of doxorubicin from polymer, however, are not fully understood. More recently, a nanoparticle composed of HMPA-doxorubicin has been developed and the resultant formulation not only offers a long half-life but also has the ability to specifically release doxorubicin upon reaching the cytosol of cells (Etrych et al. 2011).

2.2.2. Doxorubicin-encapsulated nanoparticle using liposome as carrier

Liposomes are artificial bilayer vesicles composed mainly of a mixture of phospholipids, which are essentially an amphiphilic substance with a hydrophilic polar phosphate head and two hydrophobic hydrocarbon tails (Drummond et al. 1999). After following an appropriate preparation procedure, vesicles with a hydrophilic interior that is isolated from the exterior water environment by a bilayer composed of phospholipid molecules, can be produced (Barenholz 2001). Using this bilayer as a barrier, it has already been shown in the early 60s that hydrophilic molecules can be encapsulated into the hydrophilic interior of the liposome (*i.e.* liposomal doxorubicin) to prevent undesirable doxorubicin exposure (Bangham et al. 1965). In stark contrast to the fast moving pace of drug-polymer conjugate research in the 70s, however, development of liposome-based drug delivery systems has been slow since the early finding in the 50s. It was not until the 1980s that major discoveries had been made which catalysed the development of nanoparticle as an ideal doxorubicin carrier:

- Invention of extrusion method for the preparation of liposomes in 1979: A liposome reduction technique that can produce liposome with very homogenously distributed particle sizes ranging from 100 - 400 nm (Olson et al. 1979).

- Discovery of EPR effect in 1984: The group led by Maeda discovered that the high permeability of solid tumour can be exploited for drug accumulation (Iwai et al. 1984). Later on, the ideal size of particles was discovered to be 100 nm, a size that enabled particles to avoid renal secretion and liver uptake, whilst being able to penetrate into the tumour region (Nagayasu et al. 1999).

- Invention of long circulating liposomes in 1987 and 1990: Liposomes incorporated with specially designed lipids (i.e. gangliosides and sphingomyelin) were first developed in 1987 and were found to be able to significantly increase the $h_{1/2}$ of the liposomes in circulation to 24 hours (Allen et al. 1987). In 1990, liposome was, for the first time, surface decorated with a polymer (i.e. PEG) which increased the $h_{1/2}$ of liposome to ~24 hours (Klibanov et al. 1990). This formulation was later referred to as stealth liposome.

- Invention of pH gradient encapsulation method for doxorubicin in 1990: This encapsulation method creates a cross-membrane pH gradient that drives doxorubicin to move into the interior of liposome. The amount of encapsulated doxorubicin in doxorubicin-encapsulated liposome is thus 4 times higher than doxorubicin-polymer conjugates (Madden et al. 1990).

On the basis of these inventions and discoveries, Doxil®, the first doxorubicin-encapsulated liposome claimed to be able to "selectively accumulate doxorubicin loaded liposome into the tumour", was developed in the early 90s (Barenholz 2012). The structure of Doxil® is illustrated in Figure 2.4.

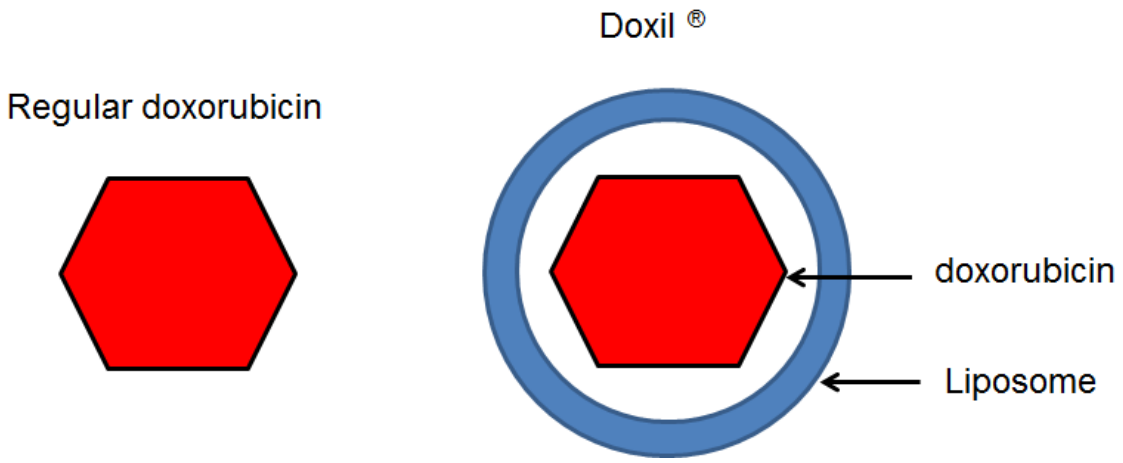


Figure 2.4. Demonstration of doxil®, the first doxorubicin-encapsulated nanoparticle, adapted from http://www.doxil.com. Crystalized doxorubicin through a pH gradient method was encapsulated in the interior of liposome.

As the first doxorubicin loaded nanoparticle, Doxil® has become a commercial success and ranked the second bestselling medical product in the US after launch. When compared with drug-conjugate, its compatibility as a passively accumulative nanoparticle and far superior doxorubicin loading make Doxil® a stronger competitor than doxorubicin-conjugate nanoparticles. The most serious problem with Doxil®, however, is the lack of a suitable approach to release the encapsulated doxorubicin after accumulation. This has hampered the *in vivo* effectiveness of Doxil (Kong et al. 2000).

In 1980 (about the same time when the extrusion method was developed), a novel liposome based-drug delivery system , pH sensitive liposome was first proposed with an intention to make use of the low pH environment of solid tumour: using a pH sensitive liposome (*i.e.* n-palmitoyl homocysteine), encapsulated model molecules were able to be rapidly released on a low pH environment (Yatvin et al. 1980). In theory, the combination of Doxil® and pH sensitive liposome should be able to resolve the doxorubicin release issue. However, the major hurdle of the utilization of a pH sensitive liposome as a doxorubicin carrier is the lack of a suitable doxorubicin encapsulation method that can achieve satisfactory loading: as pH gradient method and more advanced Mn or Copper doxorubicin-complexation method all require the establishment of a low pH liposome interior as the driving force for doxorubicin encapsulation (Cheung et al. 1998). Thus pH

sensitive liposomes do not currently have a high loading efficiency due to their pH sensitive nature.

2.3. Thermal sensitive nanoparticles for doxorubicin

Both drug delivery methods described above aim to deliver doxorubicin in two synchronized steps.

- Deposition of doxorubicin-carrying nanoparticles through EPR-based tumour accumulation
- Rapid release of encapsulated-doxorubicin upon reaching the target site.

As the efficiency of doxorubicin molecules to kill tumour cells is directly related to their local concentration, most of nanoparticle-based DDSs aim to release the encapsulated/conjugated doxorubicin as soon as the nanoparticles reach the tumour region despite their different release mechanisms (Bertrand et al. 2010). This strategy however, may cause the tumour to be exposed to inadequate doxorubicin concentrations over a long time period and this may increase the likelihood of the tumour developing multi-drug resistance. This, ironically, is the consequence of a combination of slow tumour accumulation of nanoparticles and their rapid release of doxorubicin. For instance, even with an ideal particle size, liposomal doxorubicin is progressively accumulated in the tumour region during its circulation *in vivo* and its concentration in the tumour is estimated to reach a peak 24 hours after administration (Uster et al. 1998). In contrast, most of spontaneously-triggered stealth liposomal doxorubicin vesicles are designed to rapidly release encapsulated doxorubicin upon reaching the tumour region (Bertrand et al. 2010). As a result of such slow accumulation and rapid release combination, the local concentration of doxorubicin should increase in a progressive pattern that is proportional to the liposome concentration. Hence, the tumour is exposed to doxorubicin molecules at a sub-lethal concentration throughout the accumulation duration (*e.g.* 24 hours) and this increases the likelihood of the tumour to developing drug resistance (Sterpetti et al. 2006).

Compared with EPR based-DDS, DDS designed to be triggered by hyperthermia has the advantage of being able to rapidly release most of the encapsulated doxorubicin upon

introduction of the required external force. This helps to maximize the local concentration of doxorubicin and minimize the likelihood of the tumour being subjected to a sub-lethal concentration.

While both liposome and polymeric nanoparticles have been made to be thermal sensitive, currently, thermal sensitive liposomes are the only available DDS that can be used to rapidly release the encapsulated agent upon heating.

2.3.1. Thermal sensitive liposome

With DPPC (1,2-dihexadecanoyl-sn-glycero-3-phosphocholine) as its main lipid component, thermal-sensitive liposome (TSL) is a category of liposomes that, on the one hand, possess the capacity to release encapsulated drug in response to an increase in temperature in a mild-hyperthermia range, whilst on the other hand, is able to prevent premature leakage of encapsulated drug at physiological temperatures (Yatvin et al. 1978). Based on the mechanism of drug release upon heating and the lipid it contains, thermal-sensitive liposome can be further divided into traditional thermal sensitive liposome (TTSL) and lysolipid incorporated thermal-sensitive liposome (LTSL) (Ickenstein et al. 2003).

2.3.1.1. Traditional thermal sensitive liposome for tumour treatment

Compared to the other type of TSL (*i.e*. lysolipid-containing thermal sensitive liposome, to be discussed later), tradition thermal sensitive liposome (TTSL) vesicles solely utilize DPPC as their source of thermal sensitivity, as well as being the main membrane component. In general, the membrane of liposome undergoes solid crystalline, liquid crystalline, and an intermediate co-existence phase that contains both phases, at below, above, and the phase transition temperature of the lipid membrane, respectively. These three membrane states differ from each other in their permeability and other physical and chemical characteristics (Mills and Needham. 2005). For TTSL, at physiological temperature (37^{o}C), a rigid solid-crystalline phase of liposome, characterized by its tight membrane packing, gives the membrane a low permeability, preventing premature leakage of encapsulated material (Mills and Needham. 2005). Upon heating, the

solidified membrane starts to melt from the grain boundary area of the membrane (Ickenstein et al. 2003). As a consequence of molecular mismatch of lipid chains at the boundary, a highly compressible interface begins to form, a phenomenon which is currently believed to account for an increase in permeability (Winter et al. 2010). The total area of the interface reaches its maximum at around 42^{o}C (phase transition temperature of TTSL) which is within the temperature range required for the maximum permeability of the membrane (Mills and Needham. 2006). Upon further heating, the interfacial area disappears gradually until the formation of a pure liquid phase, accompanied by a reduction in permeability (Mills and Needham. 2005).

The first attempt to develop chemotherapy-encapsulated TTSL was reported by Yatvin et al (Yatvin et al. 1978): using DPPC and DSPC as membrane components, more than 50% of encapsulated cisplatin were rapidly released after 3 min heating at 42^{o}C. After further formulation optimization, stealth doxorubicin-encapsulated TTSL composed of DPPC, HSPC (hydrogenated soy phosphatidylcholine), cholesterol and PEG.lipid was developed (Gaber et al. 1995). The resultant vesicles were reported to be able to release 50% of encapsulated doxorubicin after 30 min heating at 42^{o}C. (Gaber et al. 1995). Unfortunately, although the group that developed this TTSL system did not carry out further *in vivo* testing, its anti-tumour effect was reported to be ineffective compared with a more advanced LTSL system (Needham.et al. 2000). This particular publication may have played a part in the lack of further development of such potentially highly promising TTSL vesicles, a formulation that until very recently proved to be very effective both *in vitro* and *in vivo* (de Smet et al. 2013).

2.3.1.2. Lysolipid-containing thermal sensitive liposome (LTSL) for tumour treatment

Different from TTSL, the thermal-sensitivity of LTSL vesicles has been claimed to stem from the combined effect of DPPC and lysolipid (a phospholipid that has one rather than two lipid chains). Being regarded as impurities, lysolipids are thought to be incorporated at the boundary region of the lipid membrane during the preparation of vesicles (Mills and Needham 2005). Upon heating which initiates the formation of a solid-liquid interface, lysolipids (with a conical molecular shape), at a desirable concentration (a concentration between 5-10% is mostly applied), have been reported to create and stabilize the hydrophilic water pores along the highly compressible liquid–solid

boundaries which significantly enhances the permeability of liposome (Mills and Needham. 2005). Among various anticancer agent-encapsulated LTSL vesicles, ThermoDox® - a doxorubicin-encapsulated LTSL is considered the most widely studied and successful formulation. Consisting of 10% C-18 lysolipid, 5% PEG.DSPE.2000 (PEG lipid with a molecular weight of 2000), and 85% DPPC (Poon and Borys. 2009), ThermoDox® has been reported to release 100% of the encapsulated doxorubicin within 30 s (Mills and Needham. 2005). Accompanied with the first *in vivo* application of ThermoDox®, a novel vesicle delivery strategy via intravascular release has been developed with the aim to maximize the anti-tumour efficiency of LTSL (Needham.et al.2000).

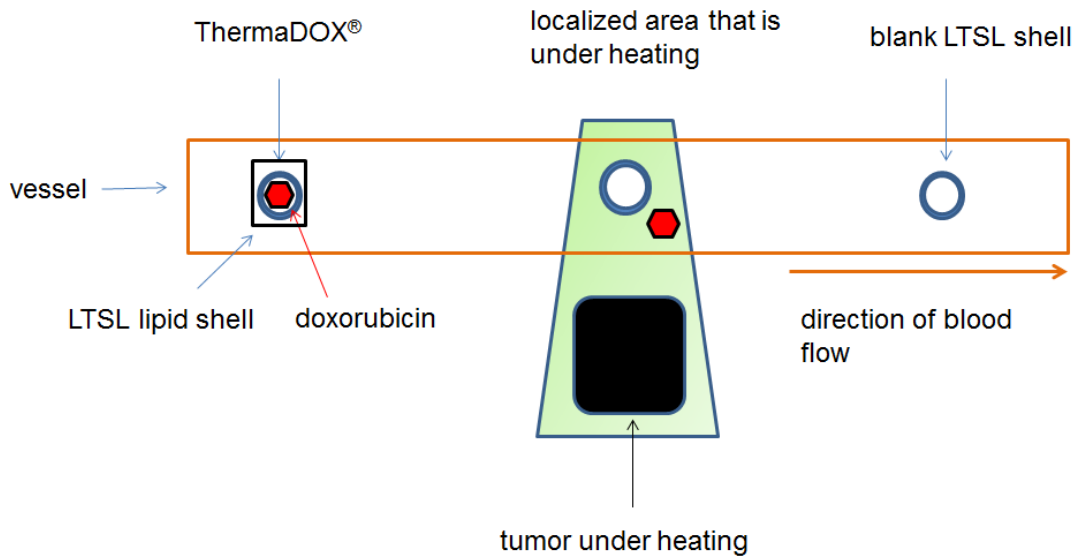


Figure 2.5. Schematic that demonstrates the main function of ThermaDox®. Prior to the administration of ThermaDox®, the targeted tumour site is heated using external source. Encapsulated doxorubicin is then released within blood vessels that feed the tumour (Chen et al. 2004). Also shown in the figure is the release mechanism of doxorubicin (i.e. released at the solid-liquid boundary of liposome upon heating).

Intravascular release refers to a strategy in which the main site of doxorubicin release occurs within blood vessels in the tumour (Chen et al. 2004). Prior to the treatment, the tumour region is heated to a designated temperature, once thermal equilibrium is established, LTSL is then injected intravenously, aiming to destroy the endothelial and tumour cells located at the perivascular regions. Using water bath as a method of

heating, the combinatorial effects of various LTSL and intravascular release strategy have been studied intensively and accepted as the method of choice (Chen et al. 2004, Mills and Needham. 2005). It offers two major advantages to the patients:

- The accumulated amount of doxorubicin at the heated tumour area is much higher compared to extravascular release (Needham et al. 2000).
- Released doxorubicin can damage the micro-vessel that nurtures the tumour (Chen et al. 2004).

The first advantage is due to the combined effect of a higher initial plasma vesicle concentration and higher permeability of free doxorubicin (Kong et al. 2000). The second advantage stems from the release of doxorubicin within the micro-vessel that nurtures the tumour, which has been reported to lead to a spontaneous reduction of blood flow in affected vessels and a subsequent destruction of endothelial and perivascular tumour cells around the affected area (Chen et al. 2004). As heating by water bath cannot be applied *in vivo*, the feasibility of clinically available heating methods (*e.g.* radiofrequency ablation or high intensity focused ultrasound, *etc.*) has been extensively studied both *in vitro* and vivo, with satisfactory results.

2.3.1.3. Failed clinical trials of LTSL for tumour treatment

Possibly as a result of impressive *ex vivo* data (Kong et al. 2000), a stage 3 ThermoDox® clinical trial (HEAT trail) was initiated in the US in 2008 to treat liver cancer using radiofrequency ablation (RFT) as the heating source. Unfortunately, it has recently been reported that ThermoDox® in combination with RFA did not benefit the patients compared to the reference group and failed to reach the goal of the trial. Prior to the initiation of such a high profile clinical trial, highly variable effectiveness of ThermoDox® intravascular release strategy on various animal tumour models had already been reported by the same group (Yarmolenko et al. 2010). In this study, variations of blood flow pattern were regarded as the potential causes of failure (Yarmolenko et al. 2010), as high blood flow fluctuations in the peripheral region of the tumour has been reported (Durand. 2001). Reduced blood flow may partially block the entrance of the affected micro-vessel, making it unavailable for doxorubicin to either pass through and enter the tumour or damage the vessel. However, it is possible that the widely reported LTSL instability may have played in part in the failure of HEAT trial.

As mentioned above, Lysolipids have been claimed to be the crucial component that enables LSTL vesicles to release encapsulated substances rapidly upon heating as a result of its single hydrocarbon chain structure. Unfortunately, there have been growing reports of lysolipid-protein interactions and various groups have linked such undesirable phenomena to the widely reported LTSL instability at 37^{o}C (Al-Jamal et al. 2012, Li et al. 2013). What is more, recent studies have demonstrated the desorption tendency of lysolipids from their originally incorporated vesicles upon the presence of liposome-alike substance such as red blood cells or other lysolipid-free vesicles at physiological temperature (Sandström et al. 2005, Banno et al. 2010). Using L-α-phosphatidylcholinecontaining liposomes to mimic red blood cells, ThermoDox® has been reported to have lost 50% of its originally incorporated lysolipid after 10 min incubation (Sandström et al. 2005). Accompanied with such lysolipid desorption is a dramatic loss of doxorubicin, with more than 50% loss of the encapsulated doxorubicin molecules within 5 min of the addition of red blood cells (Banno et al. 2010). The above *in vitro* data strongly suggest that there is a possible relation between the desorption of lysolipid and inferior *in vivo* stability of incorporated vesicles.

However, it has to be emphasized here that the extent of doxorubicin leakage from LSTLs *in vivo* has shown considerable variation among publications. Using serum from a tumour bearing mouse, more than 50% of the originally encapsulated doxorubicin molecules have been found to be lost from LSTL after 1 hour incubation at physiological temperature (Al-Jamal et al. 2012). On the other hand, LSTLs incubated in a healthy mouse serum is much more stable, with less than 20% loss after 1 hour incubation (Li et al. 2013). In addition, LSTLs have shown different sensitivities to serum from different animal species and it has been reported that mouse serum results in the highest level of doxorubicin leakage, while LSTL is least susceptible to calf serum (Hossann et al. 2012).

2.3.2. Polymer-based thermal sensitive nanoparticles

While liposome may be the first nanoparticle system that has been shown to be a suitable drug carrier for heat activated release of encapsulated material, the usage of thermal sensitive polymers has a long history in industry (Schild. 1992), mainly as surfactant or viscosity modifier. Poly-N-isopropylacrylamide, (PNIPAM, show in Figure 2.6) - the first synthesized thermal sensitive material, is a polymer that has a unique solubility characteristic; its solubility reduces upon temperature rise, a property contrary to the behaviour of most polymers (Schild. 1992). The temperature at which such a transition occurs is called the lower critical solution temperature (LCST); below the LCST, the polymer is in its extended form and remains soluble in aqueous media. Once temperature exceeds LCST, it becomes hydrophobic and shrinks (precipitates) in water (Kohori et al. 1999).

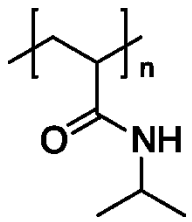


Figure.2.6. Molecular structure of PIPAAM, the first thermal sensitive material.

As the LCST of PIPAAM ($34^{O}C$) is close to the physiological temperature of human body, it should be a good candidate as a thermal responsive material. The major weakness of PIPAAM, however, is the lack of an ideal structure that offers a desirable LCST (> $40^{0}C$) and rapid release behaviour. In the late 90s, the team behind the development of polymeric nanoparticles composed of doxorubicin-conjugate developed a PNIPAM-based micelle with doxorubicin being encapsulated in the interior of micelle (Kohori et al. 1999). While the resultant product offers a desirable LCST ($40^{0}C$), the slow release behaviour makes it difficult to be used as a thermal sensitive doxorubicin carrier for drug delivery (Kohori et al. 1999).

2.4. Tissue plasminogen activator-encapsulated TSL for thrombolysis

Compared with the long historical background of cancer-targeting DDS, the development of DDS for tPA delivery did not start until the 2000s and is still much less developed than for cancer-treating DDS. In chronological order, 4 types of tPA-incorporated DDS have been developed:

- tPA-covalently conjugated carrier in 2003 (Murciano et al. 2003)
- Ultrasound sensitive tPA-liposome complex in 2007 (Tiukinhoy-Laing et al. 2007)
- Magnetic guided ultrasound responsive polymer tPA DDS in 2008 (Kaminski et al. 2008)
- Shear-activated tPA-conjugated nanotherapeutics in 2012 (Korin et al. 2012)

2.4.1. Development of tPA-covalently conjugated carrier

As has been stated in the introduction, patients that receive a pure tPA infusion have a high risk of experiencing undesirable side effects as a result of undesirable pharmacokinetic behaviour of the protein (*e.g.* high adherence to tissue and haemostatic blood clots, *etc*). Aiming to prevent such unwanted penetration of free tPA molecules into haemostatic fibrin and to increase the half-life, tPA molecules have been covalently conjugated with nano- or micro- sized carriers. Among various formulations that follow such a strategy, tPA-conjugated red blood cells (*i.e.* tPA/RBC) is one of the most well-known and studied delivery system. Thanks to the micron-size of red blood cells and the strong bond between tPA molecules and the carrier, the half-life of tPA/RBC has been dramatically enhanced compared to pure tPA infusion and the other tPA-conjugated vesicle systems (Ganguly et al. 2005). In addition, compared with free tPA molecules, tPA/RBC have lower affinity to haemostatic fibrin, thereby reducing the risk of internal bleeding (Ganguly et al. 2005). The core mechanism of tPA/RBC is illustrated in Figure 2.7.

Ironically, the thrombolytic efficiency of tPA/RBC also suffers as a result of its large particle size. Due to the higher drag force it may experience under blood flow (proportional to the radius of the particle), the micron-sized tPA/RBC is less likely to

accumulate onto the targeted blood clot compared with free tPA molecules (Korin et al. 2012).

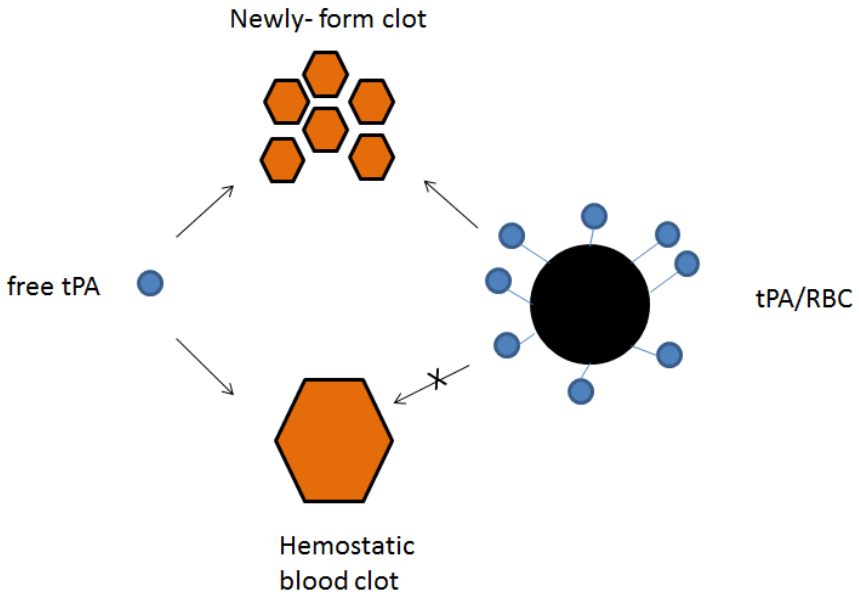


Figure. 2.7. Schematic that demonstrates the concept of tPA/RBC (adopted from Murciano et al. 2003). The main function of tPA/RBC is to dissolve the newly formed blood clot in circulation without damaging the already formed blood clot. Surprisingly, tPA/RBC is also proved to be useful to lysis blood clot that causes embolism.

2.4.2. Ultrasound sensitive tPA-liposome complex

Echogenic tPA associated liposomes (tPA-ELIP) were initially designed to enable liposomes to release encapsulated tPA using low intensity ultrasound as a trigger source (*i.e*. diagnostic ultrasound) (Tiukinhoy-Laing et al. 2007). Air bubbles are co-encapsulated into the liposome as an ultrasound contrast enhance agent (Huang et al. 2001), while a certain percentage of tPA molecules are associated with the liposome membrane to grant the liposome blood clot-targeting capability (Tiukinhoy-Laing et al. 2007). Both *in vivo* and *in vitro*, tPA-ELIPs have been shown to be able to accumulate at the blood clot site and release the encapsulated tPA molecules by ultrasound.

However, an anionic lipid has been used in the formulation to increase the “apparent tPA encapsulation”. As a result, only a small proportion of tPA molecules (around 15%) is truly encapsulated in the interior of the liposome, with the rest being either loosely associated with, or incorporated into the liposome membrane (Smith et al.2010). This deviates from the initial concept of tPA-ELIP (*i.e*. to locally “release” tPA molecules by ultrasound). As a result, it may be reasonable to speculate that, rather than burst release of encapsulated tPA at the blood clot site, the observed superior thrombolytic rate enhancement was merely a combined effect of improved pharmacokinetic profile of tPA-ELIP (*i.e.* longer $t_{1/2}$ and blood clot target capability) and enhanced tPA efficiency against blood clots under ultrasound exposure (Everbach et al. 2000).

2.4.3. Magnetic guided ultrasound responsive polymer tPA DDS

Diagnostic ultrasound (i.e. low-intensity ultrasound with high duty cycle) is used in magnetic guided ultrasound responsive polymer tPA DDS to release the incorporated rt-PA through the degradation of polymer in response to acoustic power (Kaminski et al. 2008). The latest progress in this area involves the establishment of a magnetic-guided and tPA incorporated PEG-PLA microsphere (Kaminski et al. 2008). Although *in vitro* experiments demonstrate the release of tPA triggered by ultrasound, the rate of tPA release is not satisfactory (took more than 1 hour for complete release, Kaminski et al. 2008).

2.4.4. Shear-activated tPA-conjugated nano-therapeutics

Shear-activated tPA-conjugated nano-therapeutics (tPA-Ns) is an amazing design that utilizes the shear-stress sensitivity of its fabricated micro-particle counterpart. PLGA nanoparticles are initially fabricated into micro-aggregates by a spray-drying technique; tPA-molecules are then coated onto the aggregates through covalent bonding (Korin et al. 2012). Upon injection, the micro-particles spontaneously break up into tPA-conjugated nanoparticles at the blood clot site due to the high shear stress of the local environment. The combined effect of smaller particle size of tPA-NS and high surface exposure of tPA molecules grants the nanoparticle high blood clot adherence functionality (Korin et al. 2012). In addition, since the surface area is much smaller than

a single micro-particle aggregate, the majority of tPA molecules will not be exposed to haemostatic blood clot, making it an even safer and blood clot specific formulation in comparison to the existing tPA-liposome counterparts. The concept of shear-activated tPA-Ns aggregates is illustrated in Figure.2.8.

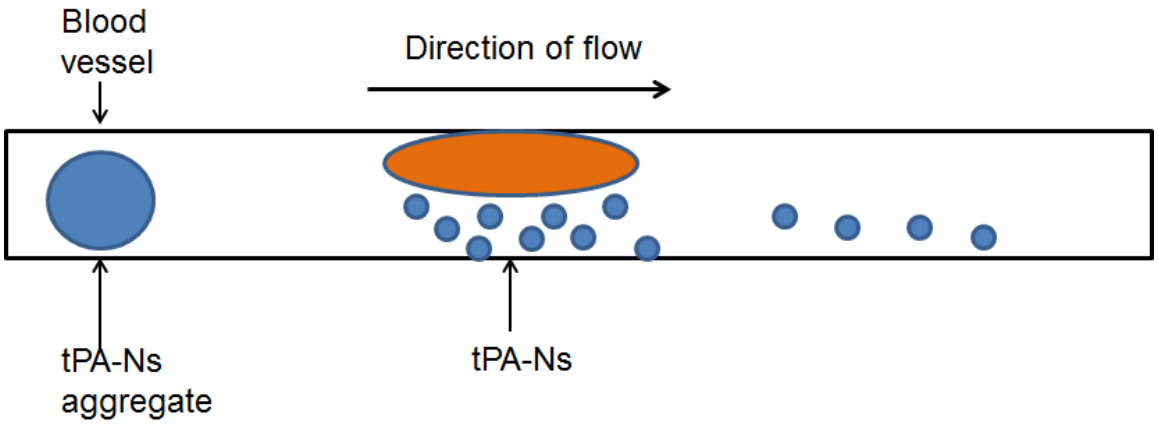


Figure 2.8. Schematic that demonstrates the concept of shear-activated tPA-Ns aggregates (adapted from Korin et al. 2012). Micro-particle is broken into nanoparticles when passing through the blood clot.

Its major shortcoming lies in its inability to lysis blood clots that totally block the vessel, a circumstance that is commonly encountered in patients. As a high shear-stress tunnel is needed to break up tPA-Ns aggregates, a total blockage of a vessel will prohibit the transformation of micro-particles into tPA-Ns.

2.5.Summary

As the cytotoxicity of doxorubicin is proportional to its local concentration, the most desirable doxorubicin-DDS is to maximize the concentration of free doxorubicin in tumour within a short period of time, whilst limiting doxorubicin exposure to normal enzymes or cells. Currently, EPR-based nanoparticles and LTSL are two main types of DDS that aim to achieve these desirable features. However, the current generation of EPR-based doxorubicin-DDS may stimulate the acquirement of multidrug resistance due to its long accumulation time (Al-jamal et al. 2012, Sterpetti et al. 2006). Compared with EPR-based doxorubicin-DDSs, LTSL has advantage over the other systems as it is able to release most of the encapsulated doxorubicin at desirable area within a short

treatment time (Needham et al. 2000), reducing the chance of multidrug resistance development. However, the presence of lysolipids reduces the stability of LTSL in circulation, resulting in premature leakage of doxorubicin at physiological temperature (Al-jamal et al. 2012).

Similar to doxorubicin, the extent of blood clot lysis is proportional to the local concentration of tPA. Hence, the ideal DDS for tPA should have a similar pattern as its doxorubicin counterpart (i.e. rapidly release tPA at targeted blood clot within a short period of time, whilst limiting tPA availability in circulation). Currently, tPA-ELIP and tPA-Ns are two types of DDSs that are designed to meet these requirements. While tPA-ELIP has a long history of development (Tiukinhoy-Laing et al. 2007, Laing, et al. 2012), only a small population of tPA is shielded from circulation environment and this reduces the selective targeting nature of DDS toward desirable pathological blood clot. Currently tPA-Ns is the only tPA-DDS that is able to selectively release majority of tPA molecules at pathological blood clot area (Korin et al. 2012). However, the presence of high shear stress is the pre-condition of tPA release (Korin et al. 2012). As a result, such DDS may not be able to unblock vessel that is totally blocked, which is common in patients suffering from acute ischemia stroke. Other types of tPA DDS either lack the ability to selectively target pathological blood clot (RBC/tPA) or have insufficient tPA release rate (magnetic guided ultrasound responsive polymer tPA DDS).

Chapter 3. Development of simple and stable thermal sensitive liposomes for the treatment of solid tumour

3.1. Introduction

TTSL composed of DPPC: HSPC cholesterol and DSPE.2000 has been reported to have much lower thermal sensitivity and anti-tumour effect compared with lysolipid containing TSL (Needham et al. 2000). However, a recent study by De Smet et al on the once neglected TTSL formulation demonstrated it as a worthy liposomal carrier of doxorubicin (De Smet et al. 2011). The TTSL prepared by this group was found to rapidly release 90% of the encapsulated doxorubicin after 5 min heating at 42^{o}C (De Smet et al. 2011, 2013).However, while its anti-tumour efficiency is yet to be determined, the combination of intravascular release and their TTSL vesicles led to a significantly lower release of doxorubicin in the tumour area compared with LSTL (Al-Jamal et al. 2012). As a result, whether TTSL of this type can be used as an alternative to the currently unstable LSTL for tumour treatment is still unknown. As sterically stabilized DPPC liposomes have been shown to be able to rapidly release the encapsulated molecules (Woo et al. 2008), one of the aims of the project is to determine whether it is feasible to utilize simple doxorubicin-encapsulated TSL composed solely of DPPC and PEG.lipid (PEG2000.DSPE) at 96:4 molar ratio to replace the current LTSL vesicles. In order to simplify the terminology used in the thesis, TSL in the following text **only** refers to vesicles composed solely of DPPC and PEG2000.DSPE.

As mentioned in section 1.6, encapsulating a higher amount of doxorubicin is a desirable property liposomal doxorubicin: As long as the presence of doxorubicin molecules does not compromise the integrity of liposome membrane, a higher amount of encapsulated doxorubicin per lipid (drug to lipid ratio D/L, *W/W*) can help reduce the % leakage of encapsulated doxorubicin at physiological temperature and increase the amount of

doxorubicin that is available for tumor deposition (Jonhnston et al. 2008). However, thermal sensitive liposomes (including TSL) often have a much lower D/L ratio compared with their non-thermal sensitive counterparts (Chiu et al. 2005). As demonstrated in the table 3.1, the D/L ratio of thermal sensitive liposome (~0.05) is usually 5 times lower than the D/L ratio of non-thermal sensitive liposome (~0.3). In the current project, effort has also been made to increase the doxorubicin loading capacity of TSL.

Table 3.1. Summary of reported D/L ratio of liposomal doxorubicin in the literature

Type of formulation	Composition of formulation (mole ratio)	Dox/lipid ratio (*W/W*)	Reference
DPPC-based thermal sensitive liposome	DPPC:DSPE.PEG.2000 (100:4)	0.05	Banno et al. 2010
	DPPC: MSPC: DSPE-PEG2000 (90:10: 4)	0.05	Banno et al. 2010
	DPPC/DSPC/ DSPE-PEG2000 (90:10:5)	0.05	Al-Ahmady et al. 2012
	DPPC:HSPC: Chol:DPPE. PEG2000 (50:25:15:3)	0.06	de Smet et al. 2011
Non-thermal sensitive liposome	EPC:chol (7:3)	0.33	Fritze et al. 2006
	DSPC: chol (7:3)	0.39	Johnston et al. 2008

DPPC represents 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. DSPE.PEG.2000 represents 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000. MSPC represents 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine. DSPC represents 1,2-distearoyl-sn-glycero-3-phosphocholine. EPC represents egg phosphotidylcholine. Chol represents cholesterol.

3.2. Selection of preparation method for doxorubicin-encapsulated vesicle

Up until now, the pH gradient method has been the most widely adopted procedure to achieve a high loading of doxorubicin in liposomal vesicles. Therefore this active loading strategy was selected as the liposome preparation method in this project. In general, the preparation of liposomal doxorubicin large unilamilar vesicles (LUV) consists of the following three steps:

1) Prepare multilamilar liposome vesicles (MLV) and subsequently LUV.
2) Create cross-membrane pH gradient.
3) Incubate pH gradient-established LUV into doxorubicin-containing solution (incubation step).

Proper handling of the first step will not only influence some of the crucial characteristics of the liposome membrane (*e.g.* phase transition temperature, component of lipid, *etc*) but also determine the total amount of lipid that is available for doxorubicin encapsulation. While successful execution of the rest two steps (pH gradient active loading of doxorubicin) will greatly affect the eventual drug to lipid ratio (D/L) of liposomes.

3.2.1. Preparation of multilamilar and large unilamilar liposome and optimization of preparation process

Preparation of dried lipid film followed by hydration is regarded as one of the most commonly used methods for the preparation of MLV containing mixed lipids. At first, lipid organic solutions are mixed at a desired percentage. Solvent is removed using a rotorary evaporator under vacuum to form a dried thin layer. Subsequent hydration of dried lipid film is then carried out using selected buffer (Walde et al. 2001). Due to the amphiphilic characteristics of phospholipids, MLVs are formed with a particle size ranging from 0.5 to 10 μm (Olson, F et al. 1979). Extrusion is then carried out to reduce the size of the vesicles.

Extrusion of liposome is a technique by which MLVs are forced to pass through a filter with a defined pore size to yield liposome particles with diameters close to the pore size

of the filter used. It has been reported that breaking and resealing of the MLV membrane can occur when vesicles are trying to pass through the pores, forming large LUV (Hope et al. 1993). In order to obtain a homogenous LUV population, a 10-cycle extrusion operation is usually recommended (Hope et al. 1993).

As written in the extrusion handbook provided by the extruder manufacturer (Avanti Polar Lipids Inc. Alabaster, AL, USA), the temperature used during extrusion needs to be higher than the phase transition temperature (T_m) of vesicle membrane and failing to do so will not only result in a significant loss of lipid during extrusion, but also increase the force needed for the lipids to get through the membrane. Therefore, in the present study, the temperature during extrusion was carefully controlled and ensured to be above T_m to minimize the lipid loss during extrusion process.

3.2.2. pH gradient active loading of doxorubicin

A schematic that illustrates the general process of pH active loading of doxorubicin is shown in Figure 3.1. In general, the method utilizes the difference in membrane permeability between protonated and depronated forms of doxorubicin (Cullis et al. 1991): prior to the initiation of doxorubicin encapsulation process, a stable cross-membrane pH gradient of 3 is required to be formed by hydrating ddruried lipid film using an acidic (pH =4) phosphate buffer, followed by exchanging the exterior buffer with a basic buffer (pH =7). After the completion of buffer exchange, encapsulation of doxorubicin is initiated by introducing doxorubicin into the LUVs solution (incubation step). Having an acid dissociation constant (pKa) of 8.13, doxorubicin molecules should be in their depronated and protonated form in basic and acidic environment, respectively (Fritze et al. 2006). Due to the higher permeability of deprotonated doxorubicin in the exterior basic environment, doxorubicin is able to pass through the membrane; subsequent protonation of doxorubicin in the acidic interior environment, on the other hand, will prevent the movement of doxorubicin from going out (Harrigan et al. 1993). It is the combined result of continuous inward movement of depronated doxorubicin and caging of protonated doxorubicin within the acidic vesicle interior that leads to the encapsulation of doxorubicin with a high D/L ratio.

During experimental designing, the selection of hydration buffer (*i.e.* acidity of buffer and valancy of the counterion) is crucial as it contributes strongly to the final I D/L of the resultant doxorubicin-TSL. This will be discussed in detailed below.

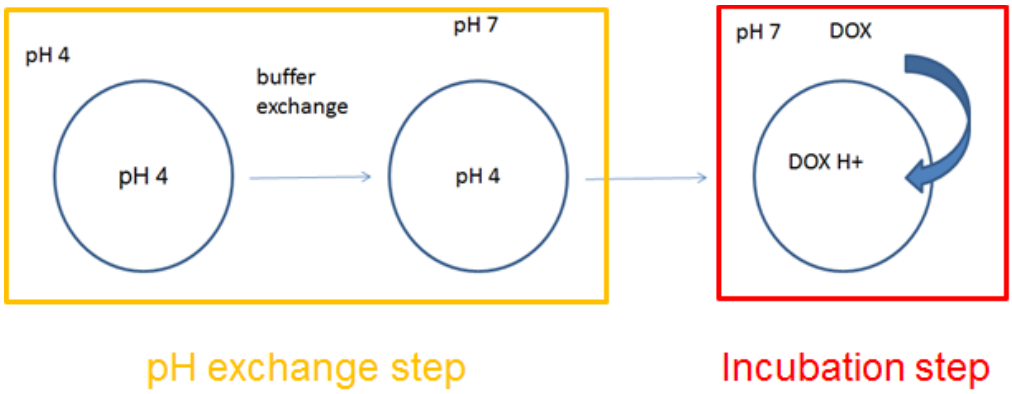


Figure 3.1. Schematic of pH gradient method for doxorubicin encapsulation. During pH exchange step, crosee-membrane pH gradient is established. Incubation step is the crucial step during which doxorubicin molecules pass through the membrane, whereas the previously established pH gradient remains relatively intact.

3.2.2.1. Early development of pH gradient method

As illustrated in Figure 3.1, the establishment of a cross-membrane pH gradient not only regulates the distribution of protonated and deprotonated doxorubicin, but also results in the formation of "deprotonated doxorubicin gradient", increasing the D/L of doxorubicin by catalyzing the inward movement of depronated doxorubicin molecules. Hence, in order to maintain the "deprotonated doxorubicin gradient" and high D/L ratio, the cross-membrane pH gradient needs to be maintained throughout the encapsulation process.

During the early development and optimization of pH active loading, vesicles composed of egg phosphatidylcholine (EPC) and cholesterol were utilized and it was found that such vesicles were unable to sustain their original pH gradient. Unlike most ion molecules, the permeability of small hydrogen protons through membranes composed of EPC and cholesterol is extremely high (Cullis et al. 1991). In response to an already established pH gradient (*i.e.* an H^+ proton gradient), protons have strong outward-membrane tendency in order to establish an electro-chemical gradient (Cullis et al.

1991). Hence, a cross-membrane pH gradient may have been compromised even prior to the initiation of doxorubicin encapsulation. Such a proton depletion state is further accelerated during the doxorubicin encapsulation process as the basic nature of doxorubicin molecules further consumes protons. Hence, in order to compensate for the potential loss of protons during the liposomal preparation process, early effort on pH gradient optimization focused on maximizing protons by increasing the concentration and acidity of the buffer (Harrigan et al. 1993).

In parallel, an ammonium buffer gradient loading was proposed as an alternative version of pH gradient loading strategy (Haran et al. 1993, Fritze et al. 2006, Mayer et al. 1994). Using EPC/cholesterol vesicle as model vesicles and a basic ammonium buffer (*e.g.* ammonium phosphate) as the hydration buffer, a cross membrane ammonium buffer gradient can be established initially after the removal of exterior ammonium buffer. This induces the outward movement of encapsulated ammonium molecules (NH_3) that equilibrates with protonated ammonium buffer (e.g. NH_4SO_4), leaving the relatively impermeable protons (*i.e*. H^+) within the vesicle interior (Mayer et al. 1994). As a result, a pH gradient is spontaneously formed. A simplified schematic that illustrates the principle of ammonium buffer gradient is given in Figure 3.2, using ammonium phosphate as an example. It was later suggested that a combined utilization of the ammonium and pH gradient (i.e. high interior ammonium concentration and low pH vale) would maximize the proton population and increase the D/L ratio of doxorubicin (Fritze et al. 2006), although experimental data were lacking. The loading strategy developed for EPC/cholesterol vesicle, however, cannot be directly applied to maximize the D/L ratio of DPPC: PEG.2000.DSPE vesicles as the behaviour of the two membrane systems during liposomal doxorubicin preparation is fundamentally different.

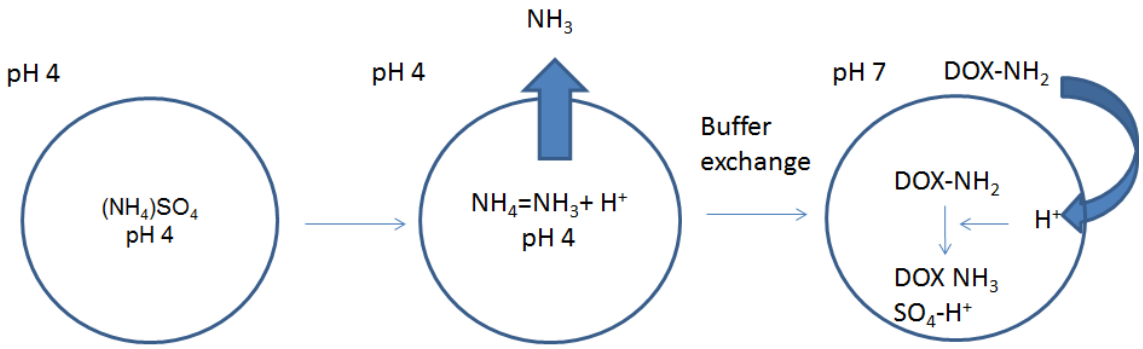


Figure 3.2. Brief description of ammonium gradient method for doxorubicin encapsulation. Ammonium phosphate is used as an example buffer.

3.2.2.2. Selection of acidic buffer for DPPC-based vesicle system for optimal D/L ratio of doxorubicin

Unlike vesicles composed of EPC/cholesterol, the transmembrane movement of H^+ in DPPC vesicles is heavily restricted at room temperature (Chiu et al. 2005). As a result, prior to the initiation of doxorubicin encapsulation, the established pH gradient is unlikely to experience depletion. Hence, an acidic buffer without the addition of an ammonium gradient has been frequently used to prepare doxorubicin-TSL systems (Banno et al. 2010, Chiu et al. 2005, Dos Santos et al. 2004). However, as the H^+ due to doxorubicin accumulation still occurs during encapsulation process, maximization of H^+ through a combination of pH and ammonium gradients is still a preferred strategy. Hence, in this project, an acidic ammonium buffer was selected as the interior buffer to increase the potential D/L ratio of doxorubicin. The selection of counter ion of interior buffer (*e.g.* critic, phosphate, *etc*) will be discussed later.

3.2.2.3. Selection of counterion and its effect on the morphology of liposomal doxorubicin

Although an acidic environment of vesicles is able to restrict cross-membrane movement of protonated doxorubicin molecules, satisfactory vesicle stability cannot be achieved

simply by regulation and maintenance of an established pH gradient. Since the development of pH gradient method, it has been later discovered that the presence of multivalent counterions (*e.g.* citrate, sulfate and phosphate) in the buffer plays an unexpected but significant part in enhancing vesicle stability by encouraging the formation of stable doxorubicin crystals under acidic conditions: Doxorubicin molecules in the presence of a multivalent counterion containing buffer have been reported, using SEM and X-ray diffraction, to be in the form of bundles of fibres that longitudinally align in a hexagonal array even at very low concentrations (<34 μM) (Li et al. 1998). Depending on the valency of the counterion and the acidity of the buffer, doxorubicin is expected to experience two stages of crystallization.

The first stage of crystallization involves the formation of doxorubicin fibres and is the result of the lyotropic behaviour brought by counterions under acidic condition, irrespective of the valency of the counterion. As a result of their lyotropic behaviour (*i.e.* the ability of ion to withdraw water from amphiphilic molecules), the presence of concentrated counterions decreases the solubility of doxorubicin by removing its hydration shell (Fritze et al, 2006), resulting in the stacking movement (*i.e.* formation of fibres) of doxorubicin molecules (Li et al. 1998). As the amphiphilic nature of doxorubicin molecules is a pre-condition of precipitation, the maintenance of buffer acidity throughout the process is crucial for doxorubicin precipitation.

The formation of a bundle of doxorubicin fibres is the second step of counterion-induced doxorubicin crystallization. In addition to the acidity of buffer, the morphology of doxorubicin crystal depends strongly on the valency of the selected counterion: acting as an electrostatic bridging force, multivalent counterions are able to bridge two protonated doxorubicin molecules (under acidic condition) on two adjacent fibres (Li et al. 1998). This eventually leads to the formation of doxorubicin bundles that are composed of fibers (*i.e*. the product of first level of precipitation) that longitudinally align in a hexagonal array (Li et al. 1998). If the buffer only contains mono rather than multivalent counterions (*e.g.* lactobionic acid), the counterion molecules are unable to connect two separate doxorubicin fibres and unable to form bundles (Li et al. 1998). Similar to the formation of doxorubicin fibers, the acidity of buffer needs to be maintained as it ensures the protonation of doxorubicin, which is the precondition of electrostatic force-based connection.

3.2.2.4. Final determination of interior buffer

In order to achieve a high D/L ratio of doxorubicin, an acidic ammonium buffer using phosphate as counterion was selected as the hydration buffer. The addition of an ammonium gradient was expected to increase the population of H^+ protons, increasing the D/L ratio of doxorubicin and fulfilling the pre-condition of doxorubicin crystallization. No preference was made regarding the selection of counterion and phosphate ion was selected due to its availability in the lab.

3.3. Selection of method to determine encapsulation efficiency of liposome

The encapsulation efficiency of liposome prepared in the project was illustrated by the amount of doxorubicin encapsulated in the liposome divided by the amount of lipid (drug/lipid ratio, *w/w*). The total amount of lipids was determined using Stewart assay and the amount of doxorubicin was determined using the same self-quenching mechanism mentioned in section 3.3. 2.

The Stewart assay is a classic biphasic colorimetric phospholipids determination method that utilizes the ability of phospholipids to form a complex with ammonium ferrothiocyanate (Stewart. 1980). Following a simple extraction procedure, the concentration of phospholipids in chloroform phase was determined from the optical intensity at 480 nm wavelength (Stewart. 1980).

The amount of doxorubicin encapsulated was obtained by comparing the fluorescence intensity difference prior to and after total destruction of liposome through the addition of detergent (triton X-100) using the equation 4. The extent of liposome destruction upon triton addition was monitored by the changes of particle size prior to and after the addition of triton X-100.

$$F_{full} - F_0 \quad <4>$$

where F_0 is the amount of encapsulated material detected prior to heating, , F_{full} is the total amount of doxorubicin detected after complete release of doxorubicin using 1 *v/v* % Triton X-100.

3.4. Preparation and analytical process of doxorubicin-TSL

This section describes in detail the preparation and analytical process for nano-sized TSL encapsulated with doxorubicin.

3.4.1. Preparation of doxorubicin-encapsulate liposome

Blank LTSL vesicles (DPPC, MSPC and PEG.DSPE.2000 at a 90:10:4 molar ratio) and a TSL vesicles (DPPC and PEG.DSPE.2000 at 96:4 molar ratio) were prepared using lipid film hydration followed by extrusion (Li et al. 2010). In brief, stock lipid chloroform solutions, with a total of 5 mg lipid were mixed and solvent was then removed using a rotary evaporator to form a dried thin layer. Dried lipid film was hydrated using acidic buffer (300 mM ammonium phosphate, pH 4) at 50^{o}C. Liposome solution was then extruded through a 100 nm filter from MLV to reduce the size of the liposomes at the same temperature. A cross-membrane pH gradient was then established by passing the liposome through a Sephadex G-25-packed column (0.8 cm × 10 cm) using HEPES buffer as an eluent. Stock doxorubicin solution was mixed with the vesicles and allowed to incubate at pre-determined temperature for 1 hour. Liposomes were then separated from any unencapsulated doxorubicin on a Sephadex G-20 column using HEPES buffer as eluent (0.8 cm × 10 cm).

3.4.2. Determination of D/L ratio of liposome

Lipid was quantified using stewart assay. Stock ammonium ferrothiocynate solution was prepared by adding 27.03 g of ferric chloride hexahydrate and 30.4 g of ammonium thiocyanate into 1L of deionized water. Liposome solution (30µl) was added into 5 *ml* of chloroform solution contained in 15 ml plastic centrifuge tube and was vortexed for 30 seconds. 5 *ml* of stock ammonium ferrothiocyanate solution was added into chloroform solution and the mixture was vortexed for another 30 seconds. Mixture was then allowed to settle for 12 overnight. Chloroform layer was extracted and its *UV/Vis* absorbance at 485 nm was determined. When determining the D/L changes of dox-TSL at various incubation temperature, 300µl of liposome solution was added into chloroform rather than 30µl.

Doxorubicin was quantified utilizing its self-quenching characteristic: 20 µl of liposome solution was added into 1ml of HEPES buffer. The amount of encapsulated doxorubicin was calculated by comparing the fluorescence intensities (emission wavelength 480 nm excitation wavelength 555 nm) of sample prior to and after the addition of 1 *v/v* % Triton X100, using equation 4. When determining the D/L changes of dox-TSL at various temperature (figure 4.2), 200 µl of liposome solution was added into 800 µl of HEPES and its fluorescence intensities prior to and after the addition after triton was used to determine the amount of doxorubicin.

3.5. Results and discussion

3.5.1. Influence of incubation temperature on the D/L ratio of dox-TSL

When preparing liposomal doxorubicin using pH gradient method, incubation step is a crucial process (detailed in section 3.2.2) at which deprotonated doxorubicin molecules need to gain access to the interior of liposome, whereas the cross-membrane movement of protons (*i.e*. H^+) is still heavily restricted. In other words, in order to achieve high D/L ratio of the resultant liposomal doxorubicin, membrane of liposome need to have selectively high permeability to neutral molecules *(i.e.* neutral doxorubicin molecules) during the incubation process. As temperature is critical to the permeability of liposome membrane, the temperature during incubation step (*i.e.* incubation temperature) needs to specially selected according to the phase transition temperature (T_m) of liposome membrane. As a rule of thumb, incubation temperature was usually higher than the T_m of liposome membrane (Haran et al. 1993, Mayer et al. 1994, Working et al. 1994). However, in the case of liposome system that uses DPPC as its main component, the selection of incubation temperature has been proved to be difficult: On one hand, little doxorubicin was able to cross though the DPPC membrane in gel phase (~25 ^oC) (Dos Santos et al. 2004). On the other hand, using temperature that exceeds T_m caused premature leakage of H^+ ion, resulting in depletion of pH gradient (Chiu et al. 2005). Consequently, 37^oC (sub-T_m of DPPC membrane) has been mostly used as a practical incubation temperature in various groups during the preparation of liposomal doxorubicin

that use DPPC as main membrane component (Al-Jamal et al. 2012, Banno et al. 2010, Hossann et al.2010. Mills and Needham. 2005. Tagami et al. 2011).

With an intention to increase the D/L ratio of dox-TSLs, the influence of incubation temperature on D/L ratio of the resultant dox-TSL was studied. In addition, it was hoped that such study may provide information regarding the selective permeability changes of DPPC-based membrane to neutral molecule at 37 ^{o}C. The influence of incubation temperature on D/L ratio of the resultant dox-TSLs within 30-36 ^{o}C (1 hour incubation time) was determined and the results are shown in Figure 3.3. It is clear that D/L ratio of dox-TSLs increased with increasing incubation temperature within the range of 30-36 ^{o}C. At 30 ^{o}C, little doxorubicin could be encapsulated into the liposome. Once incubation temperature reached 32 ^{o}C, D/L ratio of dox-TSL increased to 0.018. Increasing the incubation temperature by another 2 ^{o}C further improved the D/L ratio to 0.048. Greater improvement in D/L ratio occurred within 34-36 ^{o}C, where the D/L ratio of dox-TSLs jumped from 0.048 to 0.12.

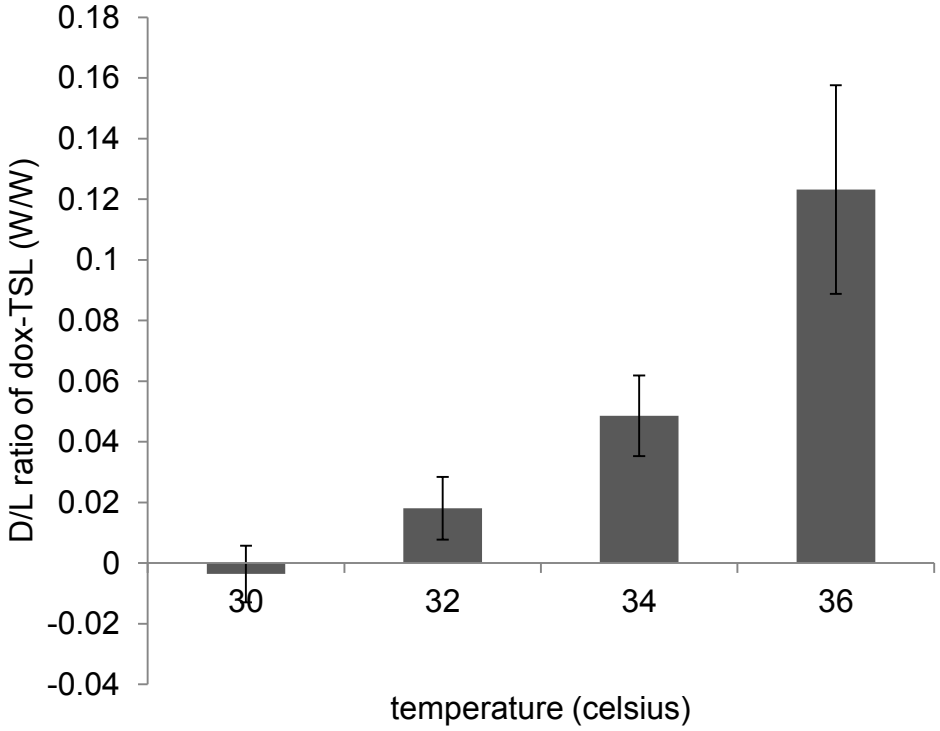


Figure 3.3. Influence of incubation temperature on D/L ratio of dox-TSLs (*n=3*). Incubation process lasted for 1 hour.

Compared with TSLs prepared by other groups (to be detailed in table 4.2), it is clearly demonstrated that an optimized incubation process (36 ^{o}C for 1 hour) significantly increased the amount of doxorubicin encapsulated in the TSL vesicles, increasing the D/L ratio of dox-TSLs from normally reported 0.05 to 0.123 (doxorubicin/ lipid *w/w*). On the other hand, D/L ratio of dox-TSLs prepared in the current project is still significantly lower than the D/L ratio of non-thermal sensitive liposome (~0.33). Given the short incubation time during current incubation process (1 hour), it is possible that extending the incubation time might further increase D/L ratio of dox-TSLs.

3.5.2. Potential mechanism of selective permeability change of TSL membrane with temperature

According to the result demonstrated in figure 3.3, it is demonstrated that the TSL membrane was almost impermeable to neutral doxorubicin (i.e. unprotonated doxorubicin) at 30 ^{o}C. Within the range of 30-34 ^{o}C, TSL membrane became progressively more permeable to neutral doxorubicin molecules and its permeability drastically increased at 34-36 ^{o}C. The observed change in permeability coincided with the occurrence of ripple-phase of DPPC membrane reported by Xiang et al (Xiang et al. 1998), who found that ripple-phase of DPPC membrane started to occur at ~32 ^{o}C and finished at ~36 ^{o}C. Similar phase transition behaviour was also reported by another group (Heimburg et al. 1998). In addition, the observed permeability change of TSL to neutralized doxorubicin is also consistent with the permeability change of DPPC membrane to neutralized acetic acid within a temperature range of 30-35 ^{o}C (Xiang et al. 1997). As shown in Figure 3.4, the permeability coefficient of neutralized acetic acid progressively increased from $1.7x10^{-5}$ to $7.8x10^{-5}$ cm/s within temperature range of 25-34 ^{o}C. When temperature increased to 35 ^{o}C, the permeability coefficient of neutralized acetic acid significantly increased and reached $20x10^{-5}$ cm/s (Xiang et al. 1997).

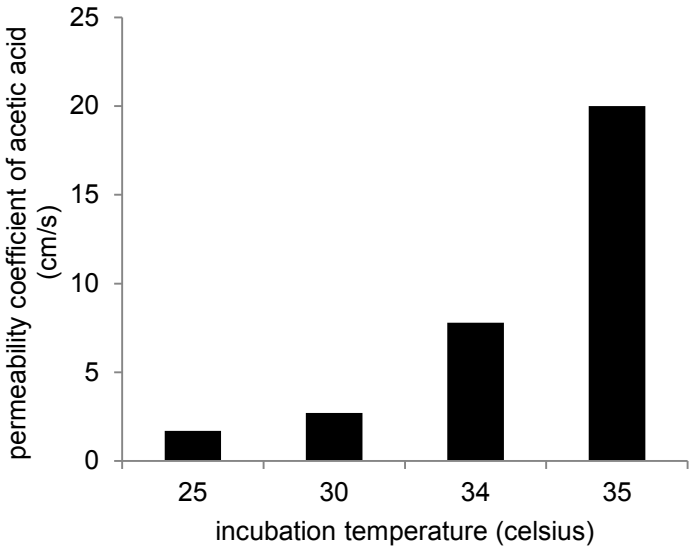


Figure 3.4. Permeability changes of DPPC membrane to unprotonated acetic acid molecules within temperature range of 25 to 35 ^{o}C. The Graph was reconstructed using the data reported by Xiang et al. 1997.

On the other hand, dithionte ion, a protonated molecule with a much smaller molecular weight than doxorubicin has been determined to be unable to cross TSL membrane after 1 hour incubation at 37 ^{o}C (Mills and Needham. 2005). As a result it was speculated that the transmembrane pH and buffer gradient were largely retained within this temperature range (*i.e*. 30-36^{o}C). As the occurrence of ripple phase is currently the only observable membrane behaviour change of DPPC-based liposome within 30-36^{o}C (Heimburg. 1998, Matsuki, et al. 2005, Needham et al. 2000, Pili et al. 2009, Shibata et al. 2001, Wu et al. 2011, Xiang et al. 1997), it is likely that during the permeability enhancement process, the unique property of ripple phase results in the lifting of rate-limiting hurdle of unionized doxorubicin molecules, whereas the obstacle that restricts the cross-membrane movement of ionized molecules remains largely intact. However, the actual property of ripple phase of sterically protected LUV is still without consensus.

3.5.2.1. Unsynchronized movement of phospholipid in ripple phase

For some types of lipid such as phosphatidylcholines (e.g. DPPC), the melting of the lipid membrane has been considered to take two rather than one steps: Prior to the occurrence of phase transition (*i.e.* from solid to liquid phase), a meta-phase termed ripple phase (P_β), characterized by periodic one-dimensional undulation on the surface of the lipid bilayer is formed (Janiak et al. 1979), as illustrated in Figure 3.5.

Figure 3.5. Demonstration of various stages of lipid membrane upon temperature rise (Vist and Davis. 1990).

Since the occurrence of ripple phase has been linked to the analgesic effect of human membrane, the P_β has been under considerable investigation. While the preparation method and structure of vesicles account for the occurrence of P_β (Heimburg et al. 2000), the environment (*e.g.* salt concentration, pH etc) has also proved to be crucial to the states of ripple phase (Bartucci et al. 1990). In addition, during early studies of P_β, the unsynchronized movement of lipid within the membrane has been detected using various techniques. Using X-ray diffraction and DSC, the packing of lipid during P_β of MLV vesicles composed of DPPC was found to be largely unchanged compared with the solid-state, whereas the long-axis rotational rate of hydrocarbon chain however was markedly increased (Lewis and McElhaney. 2011). More recently, using temperature controlled infrared spectrometer, the mobility of different positions of the phospholipids at P_β has been determined by Wu et al (Wu et al. 2011): Upon the transformation of membrane from the gel phase to P_β, the loosening of hydrogen bond structure at C=O interfacial area has been found to be the noticeable variation, whereas the lipid tail packing state has barely changed (Wu et al. 2011). Three positions of lipid and membrane are illustrated in figure 3.6.

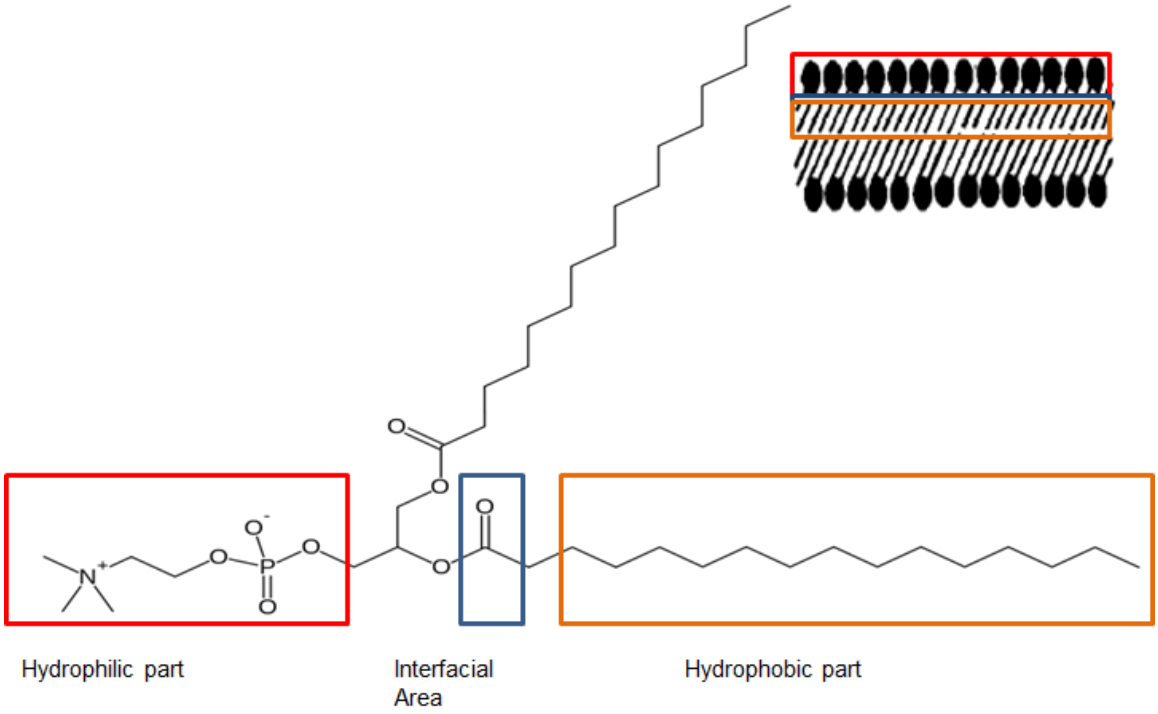


Figure 3.6. Demonstration of C=O (carbon and oxygen double bond) in the DPPC molecules. This chemical structure is the component that forms the interfacial area of the membrane, as shown on the left.

It is likely that the temperature at which the loosening of hydrogen bond network at the interfacial area was initiated coincided with the starting point of permeability acceleration of deprotonated doxorubicin. The complete loosening of the interfacial area (i.e. the completion of gel to ripple phase transition) also matches the temperature at which the maximum D/L ratio is reached. Hence, it is hypothesized that, prior to the loosening of the highly packed hydrocarbon tail region, the hydrogen-bond network at the interfacial region is the rate limiting factor for transmembrane movement of non-ionic molecules such as deprotonated doxorubicin. Such drastic changes at the interfacial region, however, have little influence on the rate of transmembrane-movement of ionic and hydrophilic molecules, as the rate limiting factor lies in the tight hydrophobic part , which remains intact within the 30-36^{o}C region (*i.e.* comfort temperature zone). Still, we cannot rule out the possible relation between the potential occurrence of liquid phase at P_{β} in nanosized vesicles and the increase of permeability of membrane.

3.5.2.2. Relation between the potential occurrence of liquidation at ripple phase in nanosized vesicle and the permeability change

As discussed above, membrane transition from the solid to liquid phase is usually regarded as a two stage process, where the former transition (solid phase to P_β , marked as pretransition temperature, T_p) results in the subtle change of the lipid packing and the latter transition (P_β to liquid phase, marked as T_m,) leads to the melting of the lipid. However, since the initial discovery of continuous enthalpy reading between two transitions (Heimburg. 1998), it has been proposed that both T_m and T_p are related to one membrane change (*i.e.* lipid melting) (Heimburg. 2000). The occurrence and scale of cooperative movement, on the other hand, depend on the preparation method of the lipid: using DPPC as a membrane component, it has been found that unilamilar vesicles with 100nm particle size show a higher cooperativity than its multilamilar counterpart (Heimburg. 2000). However, it is possible that partially-liquefied membrane during P_β is unlikely to account for the existence of “comfort temperature zone”: As has been reported, the fully-liquefied membrane permit high transmembrane movement of both ionic and unionized molecules (Mills and Needham. 2005). Given the low permeability of ionic molecules at the “comfort temperature zone”, it seems unlikely that the property of “partially-liquefied” membrane share the same property as its “fully-liquefied” counterpart.

3.5.3. Potential instability of doxorubicin-TSL as a result of ripple phase

Similar to all other thermal sensitive liposomes (Al-Ahmady et al. 2012, Gaber et al. 1995, Kim et al. 2013, Lindner, et al. 2004, Needham et al. 2000, Tagami et al. 2011), doxorubicin-TSLs prepared in this project is the product of two initially separate liposome systems:

- DPPC-based thermal sensitive liposome in which more than 50 mole % membrane is composed of DPPC phospholipid. 42^{o}C is the temperature at which encapsulated solute is released at the maximum rate.

- PEG.2000-based stealth liposome consisting of 5 mole % of membrane.

Historically, the selection of DPPC as the main component of thermal sensitive liposome is due to the lack of advanced heating method that can avoid irreversible damage to normal human tissues brought about by higher temperatures. Since its first application in drug delivery systems, DPPC has been adopted as the main component of all thermal sensitive liposome formulations (Al-Ahmady et al. 2012, Gaber et al. 1995, Kim et al. 2013, Lindner, et al. 2004, Needham et al. 2000, Tagami et al. 2011). On the other hand, addition of PEGlyated lipid to the membrane is the only method that has been applied to prolong the *in vivo* circulation time of DPPC-based liposome. Till date, whether the protective functionality of PEGlyation may be compromised by the usage of DPPC as main membrane component is yet unknown.

However, it has been found that the *in vivo* stability of dox-TSLs is inferior to the traditional stealth liposome (PEGlayted liposome with HSPC being its main component): using D/L ratio as a parameter, Woo et al demonstrated that dox-TSLs lost 40% of encapsulated doxorubicin within 1hr after injection (Woo et al. 2008). On the other hand, in the same incubation condition, stealth liposome showed little doxorubicin leakage within 1hr after injection (Al-Jamal et al. 2012). As both TSL and stealth liposome have a same level of PEG population (~5%), the compromised PEG protection functionality in TSL at physiological temperature may result from the selection of DPPC as the main component of membrane: The protective functionality of PEGlyation stems from its ability to form an additional layer outside of the original membrane, preventing undesirable protein adsorption and resultant acceleration of solute leakage (Allen et al.

2002). The level of protection PEGlyation offered, on the other hand, relies on the density of PEG on the membrane surface. Upon heating, the membrane becomes more loosely packed and such transition may dilute the density of PEG on the surface and compromise its resultant protection functionality.

In the project, it has been demonstrated that the transition of membrane from the solid to ripple phase may significantly alter the resistance of TSL against unprotonated doxorubicin and it is likely that such a change is also responsible for the protection offered by PEGlyation. As a result, finding the potential relation between the presence of ripple phase and the functionality of PEG layer will become the focus of future work.

3.6. Summary

The results presented in this chapter demonstrate that the formulation developed can encapsulate doxorubicin successfully into thermal sensitive liposomes. The D/L ratio of the dox-TSLs prepared in the present study is 2 times higher than most dox-TSLs reported in the literature. In order to achieve a high D/L ratio, Incubation temperature during vesicle preparation process was found to be critical. A hypothesis was proposed that the ripple phase of DPPC membrane may have contributed to the selective permeability increase of unprotonated doxorubicin molecules during incubation process. It is also proposed that the functionality of PEG layer on dox-TSLs may be compromised at physiological temperature as a result of ripple phase.

Chapter 4. Characterization of dox-TSLs and determination of their thermal sensitivity

4.1. Introduction

As described in chapter 3, dox-TSLs were successfully prepared in the current project and their D/L ratio was 2 times higher than the D/L ratio of dox-TSLs in the existing publications. In addition to D/L ratio, particle size and phase transition temperature of liposomes are two properties that usually determined during the development of novel thermal sensitive liposomes. This is summarized in table 4.1. Particle size contributes to the half-life of vesicles in circulations and 100nm is currently considered as a desirable particle size for circulation of thermal sensitive liposome (to be detailed in table 4.2). Phase transition temperature (T_m) of liposome membrane is another property that most research groups determined during thermal sensitive liposome development. According to the current theory, it is believed that thermal sensitive liposomes reach their highest permeability to ionic molecules upon heating to the T_m, as a consequence of the maximization of solid/liquid interfacial area (Needham et al. 2013). As a result, T_m of liposomes needs to be compatible with the external heating source that triggers the release of encapsulated material. As mentioned in section 3.5.3, 42 ^oC is currently the desirable T_m of liposome (to be detailed in table 4.2). In the current studies, both properties of dox-TSLs were determined and compared with dox-TSLs in the existing publications. It needs to be emphasized in here that, unlike some publications (summarized in table 4.1.), zeta potential (*i.e.* electro potential) of dox-TSLs was not measured in the current studies as the stability of DSPE.PEG.2000-based liposome (*e.g.* dox-TSL, *etc*) rely on the presence of PEG layer rather than electro potential of membrane (mentioned in section 3.5.3).

On the other hand, the performance (*i.e.* thermal sensitivity) of dox-TSLs upon heating is the most important properties of dox-TSLs. In the present studies, the thermal sensitivity of dox-TSL was determined using two heating method and potential doxorubicin release mechanism was also discussed.

Table 4.1. Summaries of property determination during novel thermal sensitive liposome development in existing publications

Component of formulation	Selected Publication	Properties determined		
		Phase transition temperature	Particle size	Zeta potential
DPPC:MPPC:SPE.PEG.2000 (85:10:5)	Anyarambhatla and Needham. 1999	√	√	
DPPC:DSPC:DSPE.PEG.2000 (80:15:5)	Hossann et al 2007	√	√	√
EPC, Chol (7:3) with various molar ratio of poly(EOEOVE)-OD[1]	Kono et al . 2010	√	√	
DPPC/ Brij®S20[2]	Tagami et al. 2011	√	√	
DPPC/DSPC/ DSPE-PEG2000 (90:10:5) with various molar of Lp-peptide[3]	Al-Ahmady et al .2012.	√	√	√

1. poly(EOEOVE)-OD represents poly[2-(2-ethoxy)ethoxyethyl vinyl ether (EOEOVE)].
2. Brij® S20 represents PEGylated single-chain surfactants attached with C-18 fatty acid chain.
3. Lp-peptide represents a type of lipid-anchoring temperature-sensitive amphiphilic peptide . The amino acid sequence [VSSLESKVSSLESKVSKLESKKSKLESKVSKLESKVSSLESK]- NH2

4.2. Selection of characterization method for doxorubicin encapsulate vesicle

4.2.1. Determination of particle size of liposome

Photon Correlation Spectroscopy (PCS) is based on a dynamic light scattering mechanism and the time decay of the near-order of the particles caused by the Brownian motion is used to evaluate the size of nanoparticles via the Stokes-Einstein relation (Sympa TEC note, available on www.sympatec.com). Brownian motion is defined as the random movement of the particles in a fluid that results from the collision of surrounding solvent molecules and is usually measured in terms of a diffusion coefficient. Light scattered by the particles in the sample produces a speckle pattern due to the addition and cancelation of scattered light. The intensity of fluctuation of speckle pattern provides information on the diffusion velocity (i.e. Brownian motion) of the particles (DSL technical note, available online from http://www.malvern.com).

However, direct application of the frequency spectrum is inadequate to determine the diffusion rate of particles due to the decaying of diffusion velocity with time (DLS technical note, available at http://www.malvern.com). Through the application of a correlator, the correlation function G of the scattered intensity can be constructed based on the decay time of the sample (Duffy. 2009). By treating autocorrelation function as a single exponential decay, the diffusion velocity of the sample is obtained, from which the diffusion coefficient of the particle size can be obtained (Duffy. 2009).

4.2.2. Determination of phase transition temperature of liposome

The total area of the solid-liquid interface reaches its maximum at the phase transition temperature. For LTSL, a significant reduction of permeability has been reported when the temperature deviates from the phase transition temperature (Mills and Needham. 2005). Hence, in order to study the maximum permeability of liposomes, it is necessary to determine the phase transition temperature of lipid membrane and this can be achieved by using differential scanning calorimetry (DSC). DSC is a thermal analytical technique that measures the difference of heat flow required to increase the temperature of a sample when compared to that of a reference material (Gill et al. 2010). The melting of crystalline liposome involves the absorption of energy, which can be determined by DSC in the form of heat flow against temperature (Anyarambhatla et al. 1999).

4.3. Selection of assay to study thermal sensitivity of doxorubicin-encapsulated liposome

Doxorubicin molecules that are encapsulated in the interior of liposomes cannot interact with tumour cells until they permeate through the liposome membrane. In the case of thermal sensitive liposomes, the extent of doxorubicin release depends on the permeability of membrane and its variation with temperature (*i.e.* thermal sensitivity of liposome). This molecule-releasing process is similar to the dissolution process of the drug in its conventional formulations that have a visible form (*e.g.* tablet, granulated powder in capsule, *etc*).

4.3.1. Incompatibility of dissolution test with thermal sensitive liposome

In pharmaceutical industry, dissolution tests are used to measure the rate of mass transfer from its physical dosage form to molecular form under a certain temperature and solvent composition (Ravindran. 2011). In dissolution study, drug formulation is physically separated from the released drug molecules, with the latter being extracted from the bulk solution and quantified using appropriate quantification method. The selection of equipment to study the dissolution process of conventional drug formulations is regulated by the Food and Drug administration (FDA) in US (Dissolution Methods Database, available from http://www.fda.gov/). Currently, however, the principle of dissolution test is not compatible with thermal sensitive liposome due to the lack of appropriate separation material.

As none of the FDA-approved separation method is able to separate free drug molecules from nano-sized liposome formulation (e.g. metal basket, glass frit, metal disk, etc), dialysis bag (usually made of polymerized cellulose) has been used in the literature as a separation method to study the release behaviour of liposome formulation (Fugit and Anderson. 2014, Modi and Anderson. 2013): During the assay, liposomes are introduced into either side of a dialysis bag. Due to their relatively small size, free drug molecules are able to pass through the dialysis bag and then extracted from the medium, whereas liposomes remain trapped by the dialysis bag. In US and Europe, dialysis bag has been recommended as a separation tool to study the dissolution process of nano-sized formulation by various professional organizations, including the

European Federation of Pharmaceutical Scientists and American Association of Pharmaceutical Scientists (Burgess et al. 2004).

Theoretically, dialysis bag-based dissolution test should be an ideal assay to study the thermal release behaviour of doxorubicin-encapsulated thermal sensitive liposomes. In practice, however, the diffusion process of doxorubicin molecules through the current generation of dialysis bag has been found to be very slow, making it impossible to determine the thermal sensitivity of doxorubicin-encapsulated thermal sensitive liposome. Using a dialysis bag with cut-off pore size of 6-8 KDa molecular weight (MW), doxorubicin molecules (1.2 g/mL) have been determined to slowly diffuse through dialysis bag and the whole diffusion process took 5 hours to complete (Manocha, and Margaritis, 2010). On the other hand, thermal sensitive liposomes can release majority of the encapsulated doxorubicin within seconds (Needham et al. 2013). Till now, no publication has been found in the literature that reports the use of dialysis bag to study the release behaviour of thermal sensitive liposome.

Fortunately, in the case of doxorubicin-encapsulated liposome, the self-quenching nature of the molecule makes it possible to study the release behaviour of doxorubicin without the need to physically separate free molecules from liposome formulation.

4.3.2. Selection of self-quenching based doxorubicin quantification method

Doxorubicin possesses a strong self-association tendency that leads to the quenching of fluorescent intensity due to the stacking and subsequent shielding of the fluorophore (Montero et al. 1993, de Smet et al. 2013). As a result, an obvious reduction of fluorescence intensity is expected when the concentration of doxorubicin in solution exceeds 50 μm (Kitaeva et al. 2004). After being encapsulated, the internal concentration of doxorubicin is expected to be much higher than 50 μM and is likely to reach 30 mM, if a 10% encapsulation efficiency (initial doxorubicin to lipid ratio is set at 3:1) has been achieved (Li et al. 1998). Upon the release of encapsulated doxorubicin,

an enhancement of fluorescence intensity would occur if the eventual concentration of doxorubicin is set below 50 μM. Hence, the fluorescence intensity difference prior to and after doxorubicin release can be used to calculate the amount of encapsulated doxorubicin *without the need to physically separate free molecules from its liposome formulation. This can be simply expressed as*:

$$F_I - F_0 \quad <1>$$

where F_0 is the amount of encapsulated material detected prior to heating, and F_I is the amount of encapsulated material detected after heating.

Since the invention of doxorubicin-encapsulated thermal sensitive liposome (Gaber et al. 1995), self-quenching based quantification method has become a predominant method to study the release behavior of doxorubicin-encapsulated thermal sensitive liposome, as summarized in Table 4.2. Due to its ease of use and wide application, fluorescent-based method was selected as the basic principle to determine the release behavior of thermal sensitive liposome in the current study. Unlike the method adopted in most publications, however, it was the amount, rather than percentage release of doxorubicin that was used as the parameter to compare the thermal sensitivity of different liposome formulations, which will be described in detail in the next section.

Table 4.2. Summary of prior studies where the thermal sensitivity of doxorubicin-encapsulated thermal sensitive liposomes was determine based on the self-quenching nature of doxorubicin

Group (Country)	Notable publication by group	Parameters used to determine thermal sensitivity of liposome at 42 ^{o}C
Needham (U.S)	Needham et al, 2005 Needham et al. 2000 Needham et al . 2013	% release of encapsulated doxorubicin
Lindner (Germany)	Li et al . 2013 `Hossanet al. 2010.	
Kono (Japan)	Kono et al . 2010	
Tagami (Japan)	Tagami et al. 2011	
Kostarelos (UK)	AL-AHMADY et al .2012.	
Banno (Canada)	Banno et al . 2010	Amount of doxorubicin released per lipid (W/W)
Chiu (Germany)	Chiu et al. 2005	

4.3.3. Selection of parameter to determine the thermal sensitivity of doxorubicin-encapsulated liposome

In majority of publications (except 2 publications, detailed in Table 4.2), the relative thermal sensitivity and stability of thermal sensitive liposome formulations have been determined using equation 2:

$$\%\ \text{release} = \frac{F_I - F_0}{F_{full} - F_0} \times 100\% \qquad <2>$$

where F_0 is the amount of encapsulated material detected prior to heating, F_I is the amount of encapsulated material detected after heating, F_{full} is the total amount of doxorubicin detected after complete release of doxorubicin using 1 *v/v* % Triton X-100.

The % release of encapsulated doxorubicin molecules after pre-determined heating process was compared among formulations. A higher % release was considered as an indication of higher thermal sensitivity of liposome membrane. Groups worldwide that have used the percentage release to measure the thermal sensitivity and stability of doxorubicin-encapsulated thermal sensitive liposomes have also been included in Table 4.2. However, as a drug that is able to crystalize at low concentration threshold (*i.e.* 34 μM), the thermal sensitivity of liposomes cannot be accurately quantified through the % release of encapsulated doxorubicin, despite the popularity of this method.

Based on Fick's law, Lodish et al derived an expression for biological application that relates the permeability of biological membrane with the movement of molecules across a unit area and time (*i.e.* flux) (Lodish et al. 2000):

$$Flux = -P\,C \qquad <3>$$

where P stands for the permeability of biological membrane, and C represents the concentration gradient of target molecules across the membrane.

According to the equation, if the permeability of membrane remains constant, the flux of solute will also remain constant as long as the concentration gradient of solute does not change. In the case of doxorubicin-encapsulated liposome prepared by the pH-gradient method, crystalized doxorubicin is the major physical form of encapsulated doxorubicin

due to their high concentration (>150 mM internal doxorubicin concentration). As crystalized doxorubicin (Dox_{crys}) and free doxorubicin molecules (Dox_{free}) can rapidly transform into one another (Li et al. 1998), a reduction in Dox_{free} during doxorubicin release process will result in an increase in Dox_{crys}, assuming liposome membrane is impermeable to Dox_{crys}. This makes the concentration of Dox_{free} (C_{dox}) constant as long as Dox_{crys} exists (behaving as a supersaturated solution). Consequently, the amount of doxorubicin molecules that permeate through the liposome membrane in a given time frame will also remain constant irrespective of the doxorubicin/lipid ratio (D/L ratio, *W/W*) and provide a clearer picture of the thermal sensitivity of liposome, when compared with % release. This is clearly demonstrated in the paper published by Johnston's group (Johnston et al. 2008), where doxorubicin was encapsulated in liposomes composed of distearoylphosphatidylcholine (DSPC) and cholesterol. Using 2mM ammonium chloride as incubation medium, % release of doxorubicin-encapsulated LUVs at three D/L levels (0.39, 0.1 and 0.047 W/W) was used as an indicator of formulation stability and it was concluded that higher D/L ratio was associated with better overall stability, as demonstrated in Figure 4.1.A. However, when comparing the value of $flux_{dox}$ for the three formulations, different conclusions could be made. Shown in Figure 4.1.B is the $flux_{dox}$ (mg) vs. time based on the same data obtained by Johnston et al., and it demonstrates that more doxorubicin is released from high D/L ratio formulations. In addition, formulations with a low and medium D/L ratio have a similar outward doxorubicin flux and hence membrane permeability, which is consistent with the supersaturated condition of encapsulated doxorubicin solution. Abnormal morphology of doxorubicin crystal was found to occur at higher D/L ratio which may be responsible for increased membrane permeability (Johnston et al. 2008).

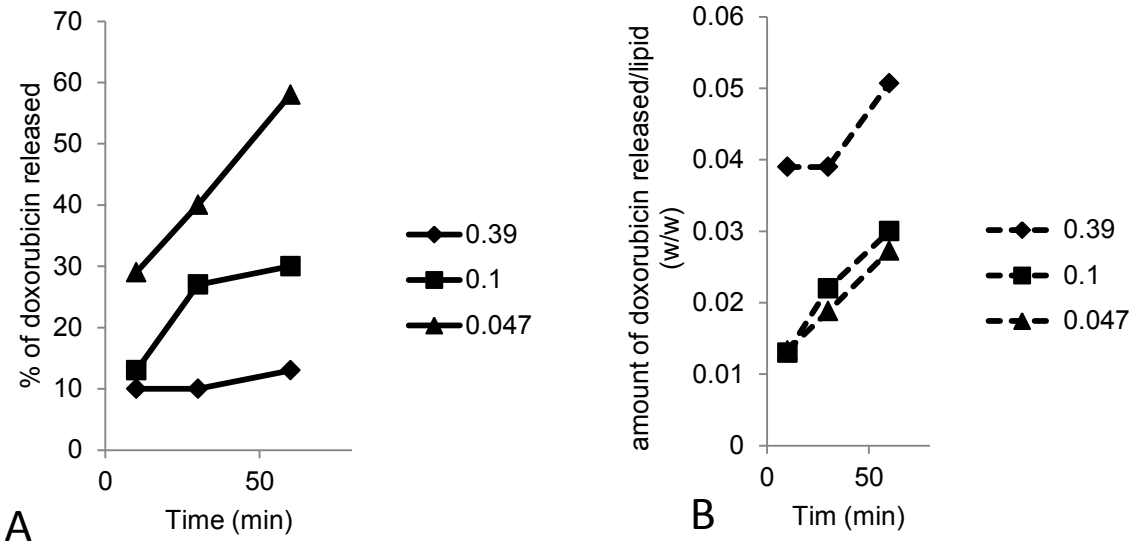


Figure 4.1. Influence of D/L ratio on stability of liposomal doxorubicin using % release (A) or amount of released doxorubicin/lipid (w/w) (B). Both graphs were reconstructed using the data reported by Johnston et al (Johnston et al. 2008).

In summary, the amount of doxorubicin released from liposome (mg of doxorubicin/lipid w/w) will be used in the present project to determine the thermal sensitivity of liposome upon heating.

4.3.4. Selection of heating method to determine the release behavior

To mimic the preferred vesicle delivery strategy (intravascular release, detailed in section 2.3.1.2), an ideal heating method to determine the response of thermal sensitive liposome is to directly immerse liposomes in buffer solution that is at a uniform hyperthermia temperature, in conjunction with real time-monitoring of fluorescent changes of buffer solution. However, a fluorescent detection system that is able to simultaneously detect fluorescent signal of incubated solution upon introduction of another substance without affecting its temperature is not readily available in the Department. Therefore, the thermal sensitivity of liposome solution was determined using two separate heating methods: progressive heating and direct heating.

4.3.4.1. Progressive heating method

In this assay, Liposomes-containing vial was immersed in a temperature-equilibrated water bath. Change of fluorescent signal was then used to determine the amount of doxorubicin release at a fixed time point during heating. In this heating assay, thermal sensitivity of liposomes during the heating process (heated from room temperature to 42 ^{o}C) was measured.

4.3.4.2. Direct heating method

In this heating assay, a small volume of liposome solution (20µl) was directly injected into a relatively large volume of buffer solution (1.5 ml) that had been temperature-equilibrated for 15 min. After a short period of time (10 seconds), the whole buffer system was then quickly transferred to an ice bath and its fluorescent reading was recorded. Compared with the progressive heating method, direct heating provides clinically relevant data as it mimics the intravascular release strategy that is used to thermally trigger the release of thermal sensitive liposome in clinical trials. In addition, temperatures above the phase transition temperature of liposome membrane were used as incubation temperature during direct heating. This would make the performance of liposome more clinically accessible not only to HIFU that is able to accurately heat tissue at a well-controlled temperature range, but also RFA , a heating method that has a wider temperature distribution when used as an external heating source (detailed in section 1.4).

4.4. Analytical process of doxorubicin-TSL

This section describes in detail the analytical process for the properties and performance of liposomal doxorubicin.

4.4.1. Determination of particle size of liposome

Liposome solution (20 µl) was diluted in buffer solution (1 ml, HEPES buffer) and put in a BrandTech® disposable UV Cuvette. Cuvette was then placed in a sample cell of PCS (Zetasizer Nano S, Malvern). Refractive index was set at default liposome solution and incubation temperature was set at 25 ^{o}C. Detection angle was set at 173 ^{o}. Z-average size (hydrodynamic diameter, nm) and pDI (polydispersity index) are the two parameters used in this project to describe the particle size of liposomes. The data was calculated automatically, according to the intensity distribution of measured solution.

4.4.2. Determination of phase transition temperature of TSL membrane

Stock lipid chloroform solutions, with a total of 10 mg lipid were mixed in round-button flask and solvent was then removed using a rotary evaporator to form a dried thin layer. Dried layer was then scratched from the flask (around 1.5 mg) and was added into aluminium pan made for DSC measurement. 100 *wt* % (~1.5 ml) of deionized water was added into the dried lipid and pan was clip-sealed. The sample-containing pan was then introduced into the sample cell of differential scanning calorimeters (Dsc q 2000, TA instrument). Empty sample aluminium pan was used as reference sample. The mixture was allowed to hydrate at 60 ^{o}C for 1 hour. Sample was then cooled to 30 ^{o}C and its heat flow was measured at a heating rate of 1^{o}C/minute from 30 ^{o}C to 60 ^{o}C.

4.4.3. Determination of release behaviour of doxorubicin-TSL

4.4.3.1. Progressive heating assay

Liposome solution (100 µl) was diluted in buffer solution (5 ml, HEPES buffer) and each 1 ml sample was placed separately in a small glass vial. Liposome containing vial was then immersed in a temperature-equilibrated water bath. The vial was taken out from the water bath at pre-determined time points and then quickly transferred into an ice bath to arrest release. All samples were then allowed to equilibrate with room temperature

(~25^{o}C) prior to measurement of fluorescence intensities (480 nm excitation and 555 nm emission) using a fluorometer (LS-55 spectrometer, Perkin Elmer). The amount of doxorubicin released was determined using equation 1. The total amount of encapsulated doxorubicin was obtained through the addition of 1 *v/v* % Triton X100 using equation 1.

4.4.3.2. Direct heating assay

1.5 ml of HEPES buffer was placed in a 15ml plastic centrifuge tube and immersed in a water bath that was already equilibrated at a desired temperature. Buffer was then heated for 15 min before 20 µl of liposome solution was added into the buffer solution. Mixture was heated for 10 seconds and was quickly transferred into a glass vial stationed in an ice bath to stop heating. The percentage doxorubicin release was calculated based on the fluorescence intensity of the sample after heating to that treated with 1 *v/v* % Triton X100.

4.**5 Results and discussion**

4.5.1 Characterization of dox-TSL and dox-LTSL

The D/L ratio, particle size of dox-TSLs as well as the phase transition temperature of TSL membrane were determined and relevant results are summarized in Table 4.3. The properties of dox-TSLs prepared by other groups are also included in Table 4.3 for comparison. Compared with TSLs prepared by other groups, it is clearly demonstrated that an optimized incubation process (36 ^{o}C for 1 hour) significantly increased the amount of doxorubicin encapsulated in the TSL vesicles, increasing the D/L ratio of dox-TSLs from normally reported 0.05 to 0.123 (doxorubicin/ lipid *w/w*). On the other hand, the achieved D/L ratio enhancement seems to have little effect on the hydrodynamic diameter of dox-TSLs in comparison with dox-TSLs with lower D/L ratio. The validity of liposome destruction method (adding 1% *V/V* triton into the solution) was determined by changes in dox-TSLs size prior to and after the addition of triton. Prior to the addition of triton, hydrodynamic diameter of dox-TSLs was 110 nm. After the addition of triton, the particle size of the same dox-TSLs solution dramatically reduced to 10.3 nm. This indicates that dox-TSLs were destroyed and formed into micelles upon the addition of

triton, releasing encapsulated doxorubicin. The total release of encapsulated doxorubicin was also visually noticeable through colour changes of the liposome solution, as demonstrated in Figure 4.2.

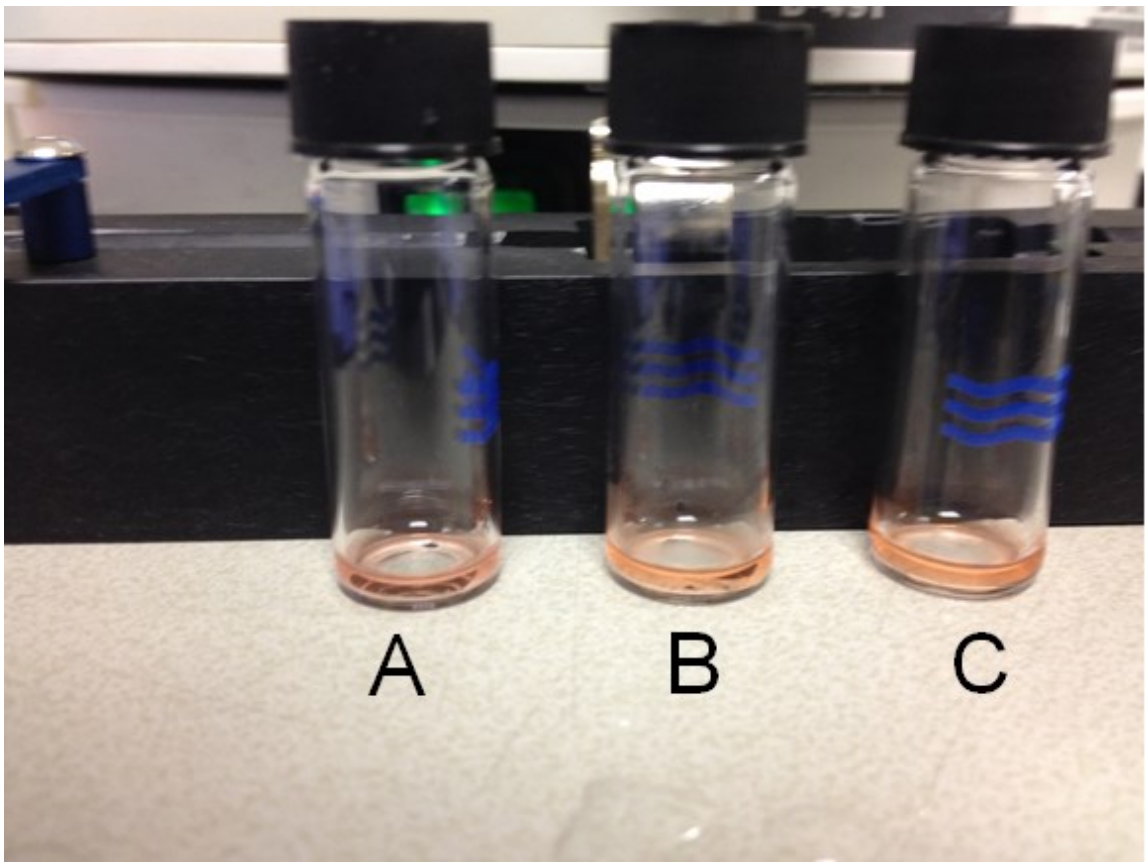


Figure 4.2. Physical appearance of intact dox-TSLs (A), dox-TSLs after the addition of triton *X-100* (B) and dox-TSLs after 2 minutes of progressive heating assay. Intact dox-TSL have unique pink colour. dox-TSLs solution that was either lysed or heated has an orange colour that resembles pure doxorubicin solution.

Table 4.3. Characterization of liposomal doxorubicin prepared in the present study and comparison with publications in the literature.

Composition	Dox/lipid ratio (*W/W*)	Particle size hydrodynamic diameter (nm)	Polydispersity index	T_m $^{\$}$(^{o}C)	Reference
DPPC:DSPE.PEG.2000 (96:4)	0.123 ±0.034*	110 ±1.002*	0.18 ± 0.005*	42.179 ± 0.0012^	Present study
DPPC:DSPE.PEG.2000 (100:4)	0.05	N/A		N/A	Banno et al. 2010
DPPC:DSPE.PEG.2000 (95:5)	0.05	N/A		N/A	Chiu et al. 2005
DPPC:DSPE.PEG.2000 (96:4)	N/A	N/A		41.7	Needham et al. 2013
DPPC: MSPC: DSPE-PEG2000 (86:10:4)	0.139 ±0.032	110±1.185	0.22±0.015	N/A	Present study
DPPC: MSPC: DSPE-PEG2000 (90:10: 4)	0.05	N/A		N/A	Banno et al. 2010
DPPC: MSPC: DSPE-PEG2000 (86:10:4)	0.05	101.5 ± 0.9	0.06 ± 0.01	41.5	Al-Jamal et al. 2012

The results are expressed as mean ± standard deviation. * Measurement was don done with *n=3*. ^ Measurement was don done with *n=2*. $^{\$}$ T_m stands for phase transition temperature of liposome membrane,

The phase transition temperature (T_m) of TSL membrane was determined to be 42.1799 ^oC. The T_m of pure DPPC membrane was also determined to be 42.2226 ^oC and the addition of 5% of DSPE.PEG2000 lipid had little effect on the overall T_m of lipid membrane. Detailed DSC graphs of TSL and DPPC membrane are given in Appendix VII and VIII, respectively.

Dox-LTSLs prepared in the current study had similar characteristic to their dox-TSL counterpart and the relevant data are also presented in Table 4.3. Due to time constraint, however, phase transition temperature of LTSL membrane was not determined.

4.5.2. The determination of dox-TSL release behaviour

4.5.2.1. Release behavior of dox-TSL and dox-LTSL using progressive heating method

Whether the TSL vesicles developed in this project are good doxorubicin carriers or not depends on their ability to rapidly release the encapsulated doxorubicin upon heating. It needs to be emphasized here that as a result of their ultrafast release rate (within seconds, to be illustrated in section 4.5.2.5), the thermal sensitivity of TSLs and LTSLs is not expected to be compared with that obtained by directly immersing liposome solution into temperature-equilibrated buffer solutions. Rather, the doxorubicin release behavior of liposomes during progressive heating provided an opportunity to compare the relative performance of TSLs and LTSLs as a result of the slow heating process. The release behaviors of both formulations in progressive heating were determined and are presented in Figure 4.3.

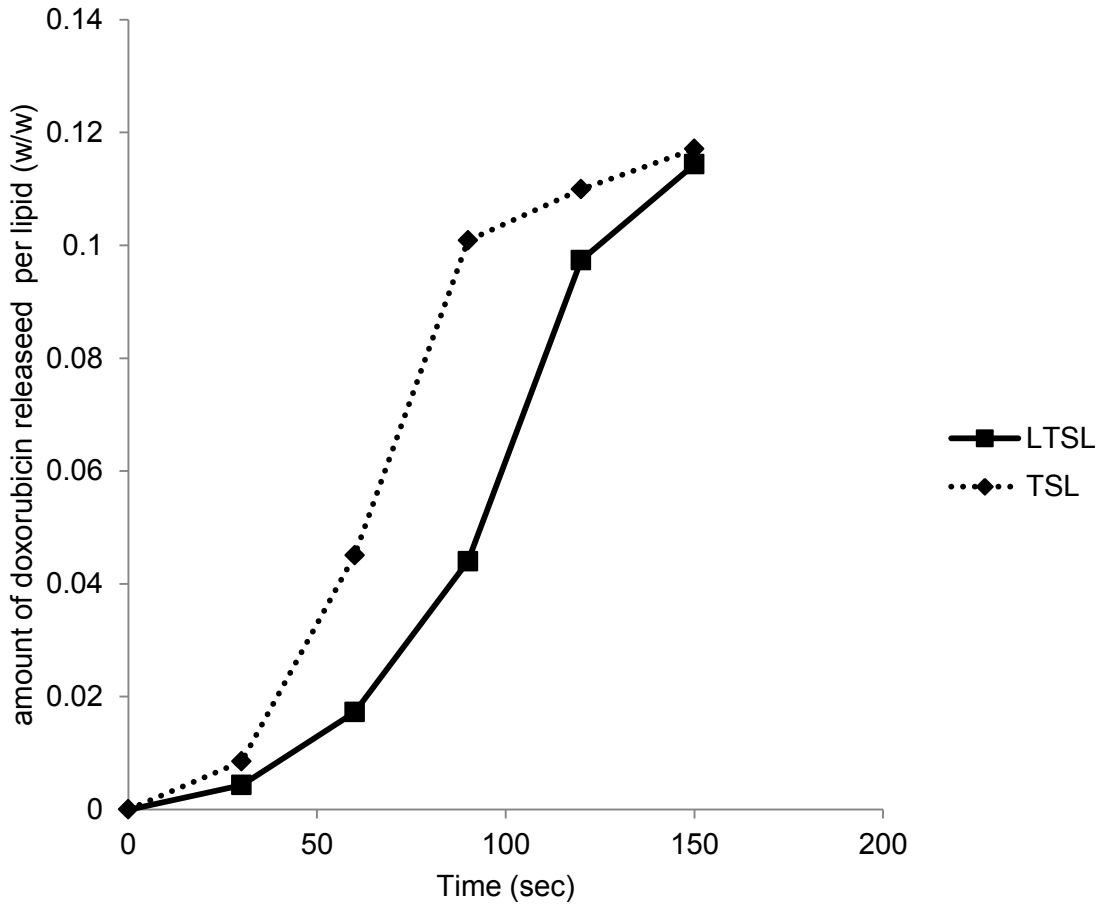


Figure 4.3. Amount of doxorubicin released per lipid (w/w) from dox-TSL *(n=3)* and dox-LTSL *(n=5)* at 42°C using progressive heating method. Standard deviation was too small to be shown on the graph.

Unexpectedly, dox-TSLs prepared in this project was able to release majority of the encapsulated doxorubicin during 2.5 minutes heating at a rate that was faster than their LTSL counterpart: After 1.5 minutes of heating, TSLs were able to release 0.1 mg of doxorubicin per mg of lipid, while LTSLs only released around half that amount of doxorubicin (0.056 mg of doxorubicin per mg of lipid). After 2 minutes of heating, around 88 % of encapsulated doxorubicin was released from TSLs as was evident from the color changes of the solution (Figure 4.2). Based on the obtained results, it seems that the addition of 10% of lysolipid (MPSC) stabilized the liposome membrane upon heating and slowed the release of encapsulated doxorubicin. While unexpected, it was not the first time that lysolipid-free thermal sensitive liposome was reported to have higher

thermal sensitivity than its LTSL counterpart. This is further discussed in the next section.

4.5.2.2. Reported superior thermal sensitivity of TSL in comparison with LTSL

A selection of existing publications that reported the superior thermal sensitivity of lyso-lipid free thermal sensitive liposome in comparison with LTSL have been summarized and listed in Table 4.4. Using a simple HEPES buffer as incubation medium, Dox-TSLs prepared by Chiu et al released 6 μg of doxorubicin/ mg of lipid after 30 minutes incubation at physiological temperature (*i.e.* 37 ^{o}C), whereas Dox-LTSLs released less than 2.5 μg of doxorubicin/ mg of lipid under the same incubation condition (Chiu et al. 2005). Similarly, using fetal calf serum as incubation medium, dox-TSLs prepared by Li et al were able to release 0.104 mg of doxourubicin/ mg of lipid after 5 min heating at 41 ^{o}C, while Dox-LTSLs were only able to release 6.5 μg of doxorubicin/ mg of lipid (Li et al. 2013). Cisplatin molecules, another chemotherapy agent, were also found to be able to permeate through TSL membrane three times faster in comparison with LTSL when heated at 37 ^{o}C (Woo et al. 2008). Hence, according to the data presented in Table 4.4, TSLs appear to have higher thermal sensitivity within the temperature range of 37 to 42 ^{o}C. This conclusion is consistent with the results obtained in the progressive heating method (*i.e.* heating the glass vial that contains liposome to 42 ^{o}C using water bath). As doxorubicin and MSPC can incorporate into the DPPC-based membrane, it is possible that MSPC acts to “protect” the membrane from doxorubicin incorporation, which would otherwise compromise the thermal response of liposome. This will be discussed in section 4.5.2.4.

Table.4.4 summary of prior studies that demonstrate lysolipid-free thermal sensitive liposome having faster release rate than LTSL at various temperature points

Group	Membrane composition/ Encapsulated material	Temperature and incubation time	Incubation media	Amount of TSL mg/ml	Amount of LTSL
Li et al.2013	Doxorubicin	37 (60 min)	50 % fetal calf serum	0.04	0.03
		41 (5 min)	99 % fetal calf serum	0.104	0.065
Chiu et al. 2005		37 (30 min)	HEPES buffer	0.006	0.0025
Woo et al.2008	Cisplatin	37 (30 min)	HEPES buffer	0.00375	0.00125
Hossann et al. 2010	Carboxy-fluorescent	42 (5 min)	5% glucose/ fetal calf serum (FCS) 1:1 (vol/vol)	0.054	0.045

4.5.2.3. Existing reports on interaction of doxorubicin and lysolipid with DPPC membrane

Apart from its ability to interact with DNA, being able to disrupt membrane of target cell is another vital cytotoxic mechanism of doxorubicin. This has been determined both in artificial liposome system as well as in pathological tumor cell models. In 1989, Dupou-Cezanne et al carried out a study to determine whether doxorubicin molecules would interact with liposome composed of egg phosphatidylcholine (egg PC) and cholesterol (Dupou-Cezanne, et al. 1989). By measuring energy transfer of paired fluorescent probes, doxorubicin was found to penetrate the lipid bilayer with its dihydroanthraquinone group being inserted into the hydrocarbon part of liposome. Later in 1992, Awasthi et al measured the partition of radio-labeled doxorubicin molecules in lipid membrane and cytosol part of fibroblast cells (Awasthi et al. 1992). The ratio of membrane/cytosolic concentration was above 30 and such parameter correlated well with cell's susceptibility to doxorubicin (Awasthi et al. 1992). In the theoretical field, pioneering simulation work was performed by Yacoub et al to study the potential interaction site of DPPC membrane with doxorubicin molecules (Yacoub et al. 2011): Using molecular dynamic simulations, changes in DPPC membrane structure were studied in the presence of doxorubicin molecules at 50 ^{o}C. Unlike its egg PC counterpart, DPPC molecules also interact with the polar site of doxorubicin (*e.g.* hydroxyl, ketone*, etc*) through electrostatic force, as illustrated in Figure 4.4. Consequently, DPPC-doxorubicin interactions reduce the area occupied by each phospholipid molecule from 63 Å to 57 Å.

On the other hand, as a phospholipid analogue, lysolipid was expected to incorporate into the DPPC-based membrane in a similar way to its phospholipid counterpart (Mills and Needham. 2005). While lacking experimental work, coarse-grained molecular dynamic simulation has been carried out to study the structural change of DPPC bilayer upon the addition of MPPC (16-carbon lysolipid) (Winter and Schatz. 2010). Interestingly, similar to the effect of doxorubicin, the incorporation of 10 mol % of MPPC also reduced the area per phospholipid molecule from 65 Å to 60 Å (Winter and Schatz. 2010). Considering the similar effect of doxorubicin and lysolipid on DPPC membrane, why insertions of doxorubicin increase the thermal sensitivity of DPPC membrane?

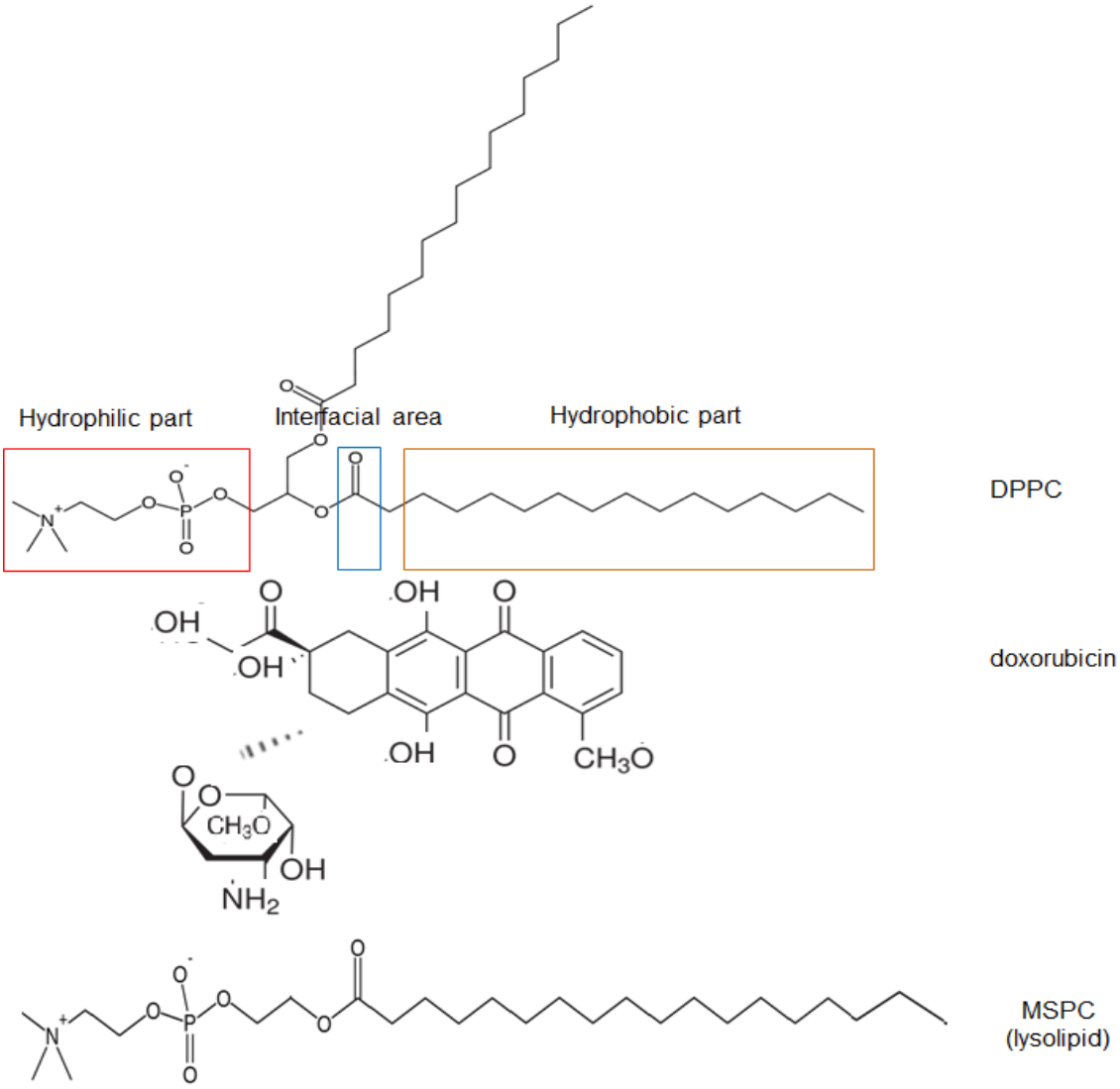


Figure 4.4. Potential interaction of doxorubicin and lysolipid (MSPC) with DPPC lipid. Dihydroanthraquinone group of doxorubicin was found to interact with hydrophobic tail of egg PC (Dupou-Cezanne, et al. 1989). Simulation studies showed that the polar group of doxorubicin could interact with hydrophilic part of DPPC membrane (Yacoub et al. 2011). Lysolpid interacts with DPPC lipid as its two-fatty acid chain counterpart (Winter and Schatz. 2010).

4.5.2.4. Potential mechanism behind the superior thermal sensitivity of dox-TSL

In the presence of lysolipid-free liposome analogues (*e.g.* red blood cell), lysolipids tend to leave their originally-incorporated DPPC membrane and migrate to lysolipid-free membrane. This membrane-membrane movement of lysolipid was first reported by Sandstrom et al and was defined as desorption tendency of lysolipid (Sandstrom et al. 2005): After incubating LTSLs (donor membrane of lysolipid) for 1 hour in a liposome-containing medium, where the liposome was composed of egg PC (acceptor membrane of lysolipid), LTSLs lost 50% of their originally incorporated lysolipids. On the other hand, LTSLs suffered little lysolipid reduction after 1 hour-incubation in a simple buffer solution (donor membrane of lysolipid was absent) (Sandstrom et al. 2005). Later on, the physiological impact of lysolipid desorption on dox-LTSLs stability was studied by Bann et al (Banno et al. 2010): Using blood as incubation medium (with red blood cell as acceptor membrane), it was found that more than 50% of the encapsulated doxorubicin was lost after 5 minutes incubation at 25 ^{o}C, which was accompanied by 50% reduction of membrane-incorporated lysolipids. On the other hand, LTSLs incubated in serum (in the absence of red blood cell) lost less than 5% of encapsulated doxorubicin. What can be learned from these two papers is that, the presence of acceptor membrane (*e.g.* egg PC liposome, red blood cell, *etc*) creates a lysolipid concentration gradient that enables the membrane-membrane migration of lysolipids (from donor membrane to acceptor membrane). In addition, desorption of lysolipids creates vacancies in donor membrane, resulting in leakage of encapsulated material. Without an acceptor membrane (as in the case of simple buffer solution), there would be no concentration gradient of lysolipid, hence no migration of lysolipids.

As described in section 4.5.2.3, doxorubicin molecules have the ability to incorporate into DPPC-membrane (Dox_{mem}). Unlike their membrane-bound lysolipid counterpart, as long as the concentration of Dox_{mem} is higher than either side of the membrane, Dox_{mem} can leave their originally incorporated membrane and enter the bulk solution. In other words, desorption movement of Dox_{mem} does not require the presence of an acceptor membrane. Similar to the desorption movement of membrane-bound lysolipids, desorption of Dox_{mem} may also create vacancies in donor membrane, resulting in leakage of encapsulated agent.

As lysolipid and doxorubicin incorporate into membrane in a similar fashion, the pre-existence of membrane-bound lysolipids may reduce the amount of doxorubicin that can be incorporated into the membrane (both molecules compete for the same incorporation site). Therefore, it is reasonable to suggest that dox-LTSLs have less membrane-bound doxorubicin molecules when compared with their dox-TSLs counterpart. During progressive heating, the thermal release behavior of dox-TSLs and dox-LTSLs were determined in simple buffer condition (*i.e.* no acceptor membrane was present). Hence, membrane-bound lysolipids of dox-LTSLs have no desorption tendency and effect on the release behavior of encapsulated doxorubicin. On the other hand, both dox-TSLs and dox-LTSLs have membrane-bound doxorubicin molecules that are able to desorb from their originally-incorporated membrane during the heating process. As a result of their higher concentration of Dox_{mem}, dox-TSLs might create more vacancies after desorption of Dox_{mem}, thereby increasing their apparent thermal sensitivity in comparison with their dox-LTSLs counterpart, which has less Dox_{mem}. This proposed theory is demonstrated in Figure 4.5

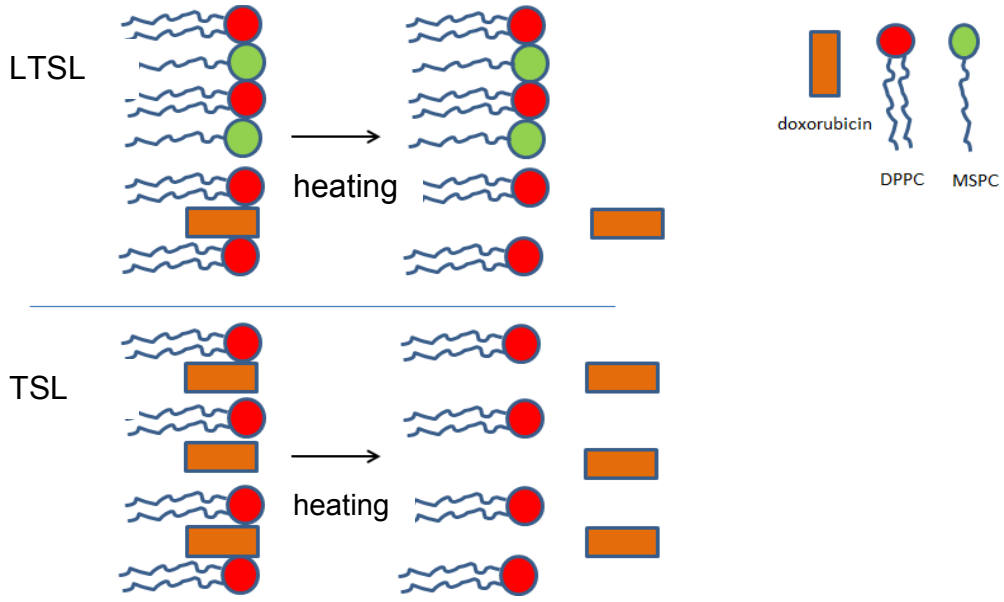


Figure 4.5. Proposed theory that explains why thermal sensitivity of dox-TSLs is higher than that of dox-LTSLs. Only doxorubicin molecules are incorporated in the membrane of TSLs (Dox_{mem}). During heating, desorption of Dox_{mem} from the membrane leaves vacancies that may accelerate the release of doxorubicin upon heating. In the case of dox-LTSLs, existence of lysolipids reduce the amount of Dox_{mem} in comparison with dox-TSLs and this reduces the thermal sensitivity of dox-LTSL upon heating.

4.5.2.5. Percentage release of doxorubicin by direct heating method

While the results obtained by progressive heating method provide a relative thermal responsive behaviour of dox-TSLs and dox-LTSL when heated from 25 ^{o}C to 42 ^{o}C, it does not represent the actual release behaviour of dox-TSLs upon heating under *in vivo* conditions. In order to observe the thermal sensitivity of dox-TSLs in a more clinically relevant environment, the percentage release of doxorubicin from dox-TSLs and dox-LTSLs at 45 and 48 ^{o}C was obtained using direct heating assay and the results are illustrated in Figure 4.6

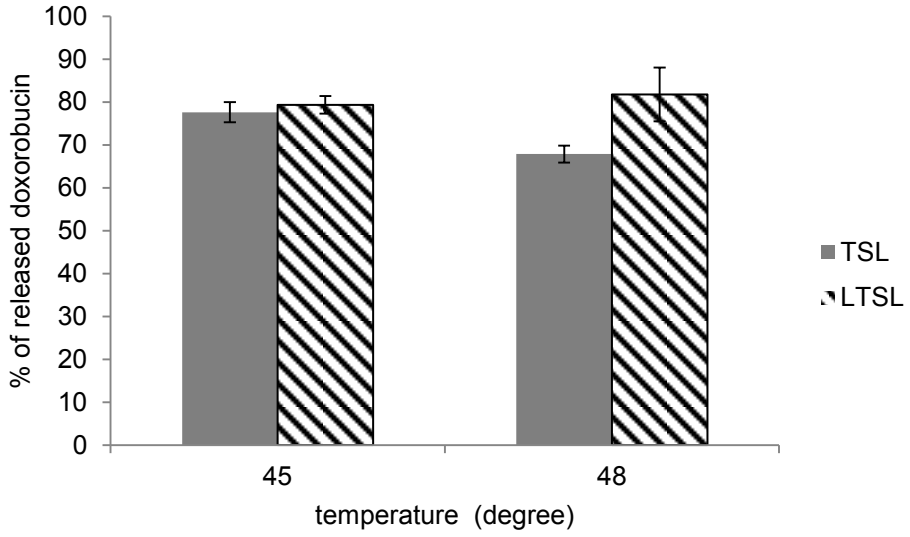


Figure 4.6. Percentage released of encapsulated doxorubicin from dox-TSLs and dox-LTSLs using direct heating assay (*n=3*). A small volume of liposome solution (20 µl) was directly introduced into a larger volume of buffer (1.5 ml) that was temperature equilibrated at desired temperatures in the hyperthermia range.

It should be noted that, during direct heating assay, only % release of encapsulated doxorubicin was determined, while the amount of released doxorubicin per lipid was not measured. This was due to the lack of lipid quantification method during direct heating. Hence, Figure 4.6 does not provide information regarding the relative thermal sensitivity between dox-TSLs and dox-LTSLs. On the other hand, the results clearly demonstrate the fast release behaviour of both formulations in an *in vivo* -mimicking environment: Both dox-TSLs and dox-LTSLs released around 80% of the encapsulated doxorubicin within 10 seconds after liposome solution was added into buffer solution that was

maintained at 45 ^{o}C. At 48 ^{o}C, the % of doxorubicin released from dox-TSL reduced to 67%, whereas dox-LTSLs were still able to release 80% of encapsulated doxorubicin. Descpite of the 10% reduction of released doxorubicin from dox-TSLs at 48 ^{o}C, these results demonstrate the ability of dox-TSLs to release considerable amount of doxorubicin after a short period of direct heating at temperature above T_m of liposome membrane (*i.e*. ~42 ^{o}C). This finding does not support the current theory that explains the thermal sensitivity of TSL, which states that thermal sensitivity of liposome at a fixed temperature is directly proportional to the area of the solid/lipid interface of the membrane (Mills and Needham. 2005).

4.5.2.6. Current theory that explains thermal sensitivity of liposome and a potential new explanation

In their pioneering paper (Mills and Needham. 2005), Mills and Needham determined that the permeability of dox-LTSLs to encapsulated doxorubicin increased dramatically from 5 x 10^{-12} to 2.5 x 10^{-11} moles/s when incubation temperature was increased from 37 ^{o}C to 42 ^{o}C (Mills and Needham. 2005). Further increasing the temperature beyond T_m significantly reduced the permeability of dox-LTSLs to encapsulated doxorubicin, dropping almost to zero at 43 ^{o}C (Mills and Needham. 2005). Hence, it was proposed that the maximization of solid/liquid interface in the liposome membrane was responsible for the maximization of membrane permeability at T_m. In addition, any deviation of temperature from T_m would result in a reduction of membrane permeability as a result of reduction of solid/liquid interface area. Under the possible influence of this hypothesis, subsequent studies that involved the development of thermal sensitive liposome rarely determined the release behavior of liposome at temperatures beyond T_m of the membrane.

More recently however, various groups have discovered that the ability of LTSLs and lysolipid-free thermal sensitive liposomes to rapidly release encapsulated material was not compromised at temperature that exceeded T_m of the liposome membrane. A list of existing publications that reported uncompromised release rate of thermal sensitive liposome above T_m is summarized in Table 4.5. Therefore, according to data presented in Figure 4.6 and information summarized in Table 4.5, it is possible that parameters other than the area of solid/liquid interface is responsible for the permeability

enhancement of liposome membrane, a parameter which is not significantly altered by temperatures above T_m. The area occupied by a single lipid (*i.e*. area/lipid) is one of the potential parameters: Using the small angle neutron and x-ray scattering technique, the area/lipid of pure DPPC membrane was found to change little within the temperature range of 50 ^oC to 60 ^oC (Kučerka et al. 2011), whereas a dramatic enhancement of area/lipid was discovered near T_m of membrane (Kučerka et al. 2011). This finding was also theoretically determined by Winter and Schatz using a coarse-grain boundary simulation (Winter and Schatz. 2010). As measurements using direct heating was only made at two temperature points (*i.e.* 45 ^oC and 48 ^oC), a direct correlation between any changes of area/lipid and thermal sensitivity of dox-TSLs cannot be made. In the future, the release behaviour of dox-TSL within the temperature range of 45 ^oC to 50 ^oC can be studied in detail, in order to find the parameters that are responsible for the rapid release behaviour of thermal sensitive liposome.

Table 4.5. List of existing publications that reported thermal sensitivity of liposome being uncompromised at temperatures above T_m of membrane.

Membrane Component (mole percentage)	Encapsulated material	Temperature range at which thermal sensitivity of liposome remained maximum (^oC)	Phase transition temperature of membrane (^oC)	Reference
DPPC:HSPC:MSPC:DSPE-PEG2000 (73.6:18.4:4:4)	Calcein	40-43	N/A	Chen et al. 2014
DPPC: DSPC:DSPE-PEG_{2000} (80:15:5)	Doxorubicin	40-45	N/A	Li et al. 2013
DPPC:MSPC:DSPE-PEG2000 (86:10:4)	Doxorubicin	40-45	N/A	Li et al. 2013
DPPC : DPPG (70:30)	Mitomycin C	42-45	N/A	Hosokawa. 2003

4.6. Conclusion

The results presented in this chapter demonstrate two other properties of liposomes that usually measured during thermal sensitive liposome development. The particle size of phase transition temperature of dox-TSL prepared in the present study is similar to the result reported in the existing publications. Using a progressive heating method, it has been found that dox-TSLs prepared in the present study have higher thermal sensitivity than their dox-LTSLs counterpart. This is consistent with various existing publications. Using a direct heating method, it has been determined that both dox-TSLs and dox-LTSLs are able to rapidly release encapsulated doxorubicin at a heating temperature that exceeds the T_m of lipid membrane. This finding supports the recent findings that T_m is not the only temperature point at which membrane permeability is at its highest.

5. Development of tissue plasminogen activator-encapsulated TSL for thrombolysis

5.1. Introduction

The aim of the second part of this project is to design tPA-encapsulated thermal sensitive liposomes (i.e. tPA-TSL) that are able to encapsulate tPA molecules in the interior of vesicles and to release the encapsulated tPA upon heating. It is also necessary to have an appropriate amount of tPA exposed on the surface of the liposomes for blood clot targeting. Work carried out in this PhD project constitutes a feasibility study of a potentially novel thrombolysis therapy, in which hyperthermia will be introduced at pathological blood clot site, activating the release of encapsulated tPA from tPA-TSL.

As a well-studied tPA-liposome complex, tPA-ELIP has been found to offer effective tPA-fibrinogen interaction (Smith et al. 2010). Hence, even without the application of ultrasound, tPA-ELIPs may still lysis haemostatic blood clots without severely compromising the efficiency of tPA so that the side effect of tPA is not adequately controlled. In comparison, encapsulating a large quantity of tPA into the interior of the liposome prohibits the availability of the majority of tPA prior to the introduction of hyperthermia which is aimed at the targeted clot only. Hence, even if undesirable binding with haemostatic blood clots may still occur, this would be significantly limited since the encapsulated tPA will not be available until its release is activated by localized hyperthermia.

On the other hand, unlike tPA-Ns which are designed to be shear sensitive and require a high shear stress (usually experienced in a narrowed vessel) to trigger the release of tPA (Korin et al. 2012), tPA-TSLs release the encapsulated tPA upon temperature rise and are expected to be insensitive to the morphology of the blood clot they adhere to.

5.2. Development of TSL for thrombolytic agents

This section describes the rationale behind the selection of a pilot thrombolytic agent prior to the utilization of tPA and preparation method of vesicles. Methods to quantify the thrombolytic agent and thermal sensitivity of thrombolytic agent-encapsulated TSL will also be described in detail.

5.2.1. Selection of streptokinase as a pilot thrombolytic agent

Although the development of a tPA encapsulated TSL system was the main goal of the second part of the project, as a result of its high cost, tPA was not initially used to determine the proof of concept of thrombolytic agent-encapsulated TSL. Instead, a much cheaper thrombolytic agent (*i.e.* streptokinase) was selected to determine the feasibility of the concept, as well as the related preparation and quantification methods.

Streptokinase (SK) is a bacteria-produced blood clot reducing enzyme that is able to directly convert plasminogen to plasmin without the presence of fibrin network (Crabbe et al. 1990). Despite of its potent thrombolytic effect, the clinical application of SK has been seriously limited owing to its non-specificity to fibrin-bound plasminogen, which results in systematic conversion of circulating plasminogen to plasmin and increased risk of internal bleeding (Holt et al. 2012). Furthermore, the bacteria nature of SK has been reported to be responsible for the activation of immune response in some patients. This not only diminishes the efficiency of SK after initial injection, but also causes hypotension (Gemmill et al. 1993). It has been recently found, however, by deleting 59 NH_2-terminal residues, the resultant SK molecules, i.e., rSKΔ59, is able to selectively convert fibrin-bound plasminogen as the ability to activate plaminogen in the absence of fibrin is removed (Sazonova et al. 2004, Reed et al. 1999). In addition, it has been suggested that it is the contaminant protein during manufacturing process, e.g. albumin, rather than the SK molecules that causes the activation of immune system (Skopál et al. 2000). Thanks to these fundamental studies, there is a considerable potential to improve the therapeutic efficacy of SK in the future.

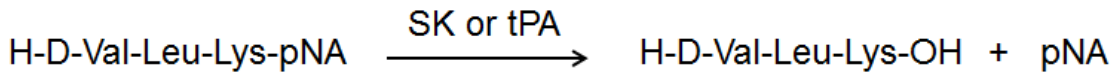
Although the original SK formulation does not have the blood clot targeting function and may not be an ideal thrombolytic agent for clinical use, as a high molecular weight

thrombolytic agent (60 kDa) that is able to convert plasminogen into plasmin, the feasibility of TSL system with SK molecules should be a strong indication of the compatibility of the system with tPA molecules. Hence, the feasibility of TSL and related preparation methods was initially studied using SK as a pilot model. After the successful preparation of SK-encapsulated TSL (i.e. SK-TSL), an attempt would be made to prepare tPA-encapsulated TSL using the optimized preparation method obtained with SK-TSL.

In addition, given the discovery of the 59-NH_2-terminal residues on SK, it is still possible to develop a rSKΔ59 encapsulated TSL that may offer a similar benefit to what is expected from tPA-TSL, and the development of SK-TSL may provide valuable data that can aid such a potential application in the future.

5.2.2. Quantification method for thrombolytic agent

The thrombolytic effect of SK and tPA lies in their ability to produce plasmin through peptide bond cleavage in plasminogen. Although SK and tPA molecules interact with their substrate in different manners, the amount of the end product of the enzymatic reactions (*i.e.* plasmin) is directly proportional to the concentration of thrombolytic agent available (Grierson et al. 1987, Kim et al. 2009). Hence, using plasmin-specific oligopeptide substrate (*i.e.* s-2251™), the conversion rate of plasminogen to plasmin can be used to quantify the thrombolytic agent concentration. The structure of s-2251™ and the related reaction to quantify plasmin are illustrated below:



Where Val represents valine. Leu represents leucine. Lys represents lysine. pNA represents para-nitroaniline. D indicates molecule is D isomer according to spatial configuration of atoms.

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Similar to the specific peptide bond to be targeted on the fibrin network, the peptide bond between lysine and *p*-nitroaniline (*i.e.* pNA) on the s-2251™ can be selectively cleaved

by plasmin molecules (Friberger et al. 1978). Such an amidolytic effect releases NH_2-pNA molecules, a chromogenic molecule having a high absorption coefficient at 405 nm (Budzynski 2001). Therefore the concentration of plasmin, and indirectly, the effective concentration of thrombolytic agent can be obtained based on the enhancement of UV/Vis intensity at 405 nm. It has to be emphasized here that although a detailed study on the substrate-plasmin interaction is beyond the scope of the project, the exclusive selectivity of s-2251™ towards plasmin against other types of protease (*e.g.* tPA and SK) has been determined (Budzynski. 2001). Hence, the break-up rate of the peptide bond in s-2251™ is a reasonable representation of the level of plasmin concentration. Plasminogen and s-2251™ are added in excessive amount to ensure that the concentration of thrombolytic agent is the limiting factor of the assay.

5.2.3. Vesicle preparation method for thrombolytic agent-TSL

Unlike amphiphilic doxorubicin that can be actively loaded into liposomes through pH gradient, until now, proteins can only be passively loaded into liposomes during the formation of vesicles or their water compartments (to be detailed below) (Walde et al. 2001). Leaving aside the special interaction between target solute and phospholipid molecules (Colletier et al. 2002), the encapsulation capacity of a vesicle during passive encapsulation is influenced by the volume of its hydrophilic compartment (*i.e.* internal water space) and its engulfing mechanism (*i.e.* the equilibrating state between phospholipid assemblies and solute molecules throughout the vesicle preparation process). Both parameters are largely determined by the vesicle preparation method adopted.

5.2.3.1. Ideal physical characteristics of vesicle for high encapsulation capacity

In general, the size of the internal compartment of the vesicle, expressed as the volume of entrapped aqueous space per lipid (µl/mg), is affected by the lamilarity (*i.e.* the number of lamellae in the vesicles) and the size of the vesicle. Multilamilar vesicles have an inferior internal water space compared with their uni-lamilar counterparts as a result of the low participation of multilamilar lipid in the internal lamellae and close apposition of adjacent concentric bilayers that further restrict the internal water space (Szoka et al.

1978). On the other hand, larger vesicles have higher internal water space than smaller vesicles as the size of the vesicles is proportional to the ratio of the vesicle surface area to encapsulated volume (Szoka et al. 1978). Hence, in order to maximize the volume of the internal water compartment of vesicles, it is desirable to prepare vesicles with a large particle size and low lamilarity.

5.2.3.2. Available preparation methods for large vesicles with low lamilarity

Currently, ethanol injection (EI), detergent dialysis (DD), freeze-thawing cycle treated MLV (MLV-FAT) and reverse phase evaporation (REV) are the four main preparation methods available to produce large vesicles with low lamilarities (Walde et al. 2001).

EI is considered to be one of the simplest methods to prepare large unilamilar vesicles. In brief, an ethanol solution containing the lipids is injected rapidly into an excess of saline or other aqueous medium to directly form vesicles (Walde et al. 2001). The target solute molecules are encapsulated upon formation of vesicles. The encapsulation efficiency of EI, however, suffers as a result of the limited lipid concentration that can be utilized during the preparation process. This is due to a combination of low lipid solubility in ethanol and a limited amount of ethanol solution that can be introduced into the medium (generally no more than *10v/v%*) (Pons et al. 1993).

During DD preparation, specially selected detergent molecules are mixed with phospholipids and aqueous solution containing the target solute molecules, forming micelles composed of lipid and detergent. Detergent molecules are then removed by dialysis and their departure from the micelle leads to the formation of liposomes (Jiskoot et al. 1986). Similar to EI, the target solute molecules are expected to be encapsulated during the formation of vesicles. Similar to EI, the low concentration of phospholipid is one of its main drawbacks as a result of the low concentration of detergent (and hence phospholipid) aqueous medium can tolerate (Wacker et al. 1998).

MLV-FAT is a method that aims to maximize the encapsulation efficiency of MLV initially prepared by simple hydration through the utilization of freeze-thawing cycle (FAT) (Walde et al. 2001). The multilamilar characteristics of MLVs limit the number of lipid molecules that are available to encapsulate the target solute molecules. Furthermore,

the overall concentration of the solute encapsulated during lipid hydration is inferior to its original medium, further reducing the encapsulation efficiency of MLV (Mayer et al. 1985). By applying FAT, MLVs are repeatedly broken up and reformed, reducing the lamilarity of vesicles (Traïkia et al. 2000). In addition, during the breaking up and reformation of vesicles, solute molecules are in constant equilibrium across the membrane throughout the FAT process. This provides more opportunities for the vesicle to engulf solute molecules at their original concentration and to increase the chance of cross-membrane solute equilibrium, whereas with EI and DD the vesicles only engulf solute molecules once. In REV procedure, vesicles are formed in a two-step organic solvent removal process: initially, lipid-containing organic solvent (immiscible with water) is mixed with water containing the target solute molecules and a reverse micelle emulsion is formed by mechanical methods. In the second step, the organic solvent is removed from the mixture under vacuum (Szoka et al. 1978). While the initial reverse micelle is disintegrated at this step, the remainder of phospholipid molecules fuse around the remaining micelles (becoming bilayers) and eventually form vesicles (Szoka et al. 1978). The schematic of the REV process is illustrated in Figure 5.1.

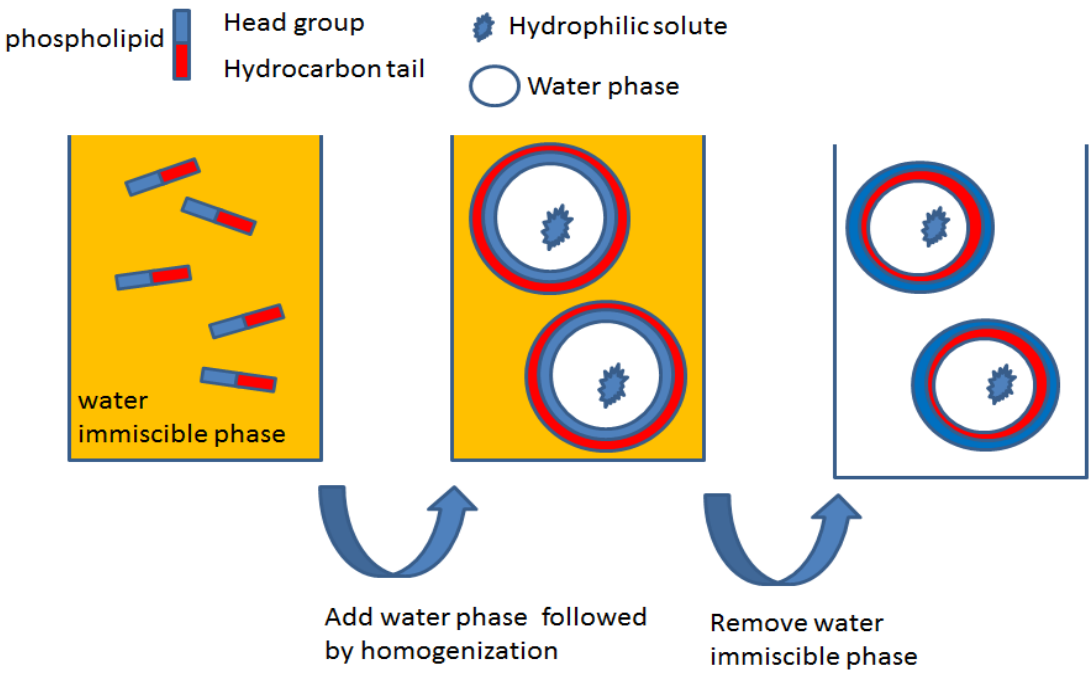

Figure 5.1. A schematic of the preparation process of REV using water-immiscible phase

5.2.3.3. The role of solute-engulfing mechanism in encapsulation capacity of vesicles

Although the physical characteristics of vesicles play a role in determining the encapsulation capacity during passive encapsulation, they only partly account for the encapsulation ability of vesicles. Compared with MLV prepared by simple hydration, the application of FAT has been shown to significantly increase the internal water volume from 0.47 µl/ml to 5 µl/ml (Mayer et al. 1985) and the amount of encapsulated sucrose by 10-fold (Szoka et al. 1978), using EPC/Cholesterol vesicle as a membrane model. On the other hand, while large unilamilar vesicles prepared by fusion method (*i.e.* small unilamilar vesicles are fused into big unilamilar vesicles) have similar internal water volume to those prepared with MLV-FAT, their ability to encapsulate sucrose is only comparable only to that of MLV vesicles (*i.e.* around 5%) (Szoka et al.1978). The significant difference in encapsulation performance among vesicles that have similar physical characteristics indicates the importance of vesicle preparation method as it determines the mechanism of solute encapsulation during vesicle formation. Based on this previous work (Szoka et al. 1978), it is believed that REV is a more promising method for high encapsulation capacity of thrombolytic agent, due to the unique mechanism of solute encapsulation during vesicle preparation or formation of the water compartment of the liposome.

5.2.3.4. REV method for the preparation of thrombolytic agent-TSL

In most of the vesicle preparation methods mentioned previously, solute molecules are encapsulated either during the initial formation (e.g. REV and DD) or re-formation of vesicles (MLV-FAT). Apart from the restriction on lipid concentration, vesicles prepared by EI and DD can only engulf the target solute once (i.e. during the formation of lipid) and it has been reported that such a one-step encapsulation process is not sufficient to engulf the target solute medium at its original concentration (Mayer et al. 1985). Compared with EI and DD, the lipid concentration that can be used during MLV-FAT preparation process is not limited and can be as high as 100 mg/ml (Mayer et al. 1985). In addition, the reformation of vesicles provides an opportunity for vesicles to re-equilibrate with the solute medium, reducing the percentage of vesicles that originally

have a sub-solute concentration. As a result, the ability of vesicles to engulf solute molecules prepared by MLV-FAT is significantly higher than vesicles prepared by the other two methods, making MLF-FAT a method of choice for the preparation of thrombolytic agent-encapsulated TSL. However, as large unilamilar vesicles prepared by the fusion method are unable to engulf more small molecular weight solutes than their multilamilar counterparts, it is still possible that the reformation of vesicles during FAT process is unable to engulf high molecular weight thrombolytic agent (Szoka et al. 1978).

In contrast to the other three preparation methods that engulf their solutes during the formation or re-formation of vesicles, encapsulation of the target solute by REV occurs during the formation of the water compartment (*i.e.* the formation of reverse micelle). During this meta-phase, nearly all the hydrophilic phase, along with all the solute molecules, are considered to be isolated from the organic phase using reverse micelles as a barrier and become a potential internal water compartment with a solute concentration that matches its original hydrophilic environment (Szoka et al. 1978). Although it is possible that the initially formed water compartment may disintegrate during the subsequent formation of vesicles, the addition of adequate lipid molecules can minimize the likelihood of undesirable collapse of the initially formed water compartment and maximize the amount of encapsulated target solutes. From herein the amount of thrombolytic agent encapsulated into the liposome will be referred to as **D/L ratio** (*i.e*. thrombolytic agent/lipid ratio, IU/mg).

In order to maximize the D/L ratio, it is believed that REV offers considerable advantage over MVL-FAT as the target solute concentration in the water compartment is comparable to that in the original hydrophilic phase and no additional effort is needed to maximize its concentration, as is required in the MVL-FAT vesicle preparation process.

5.3. Preparation procedure of thrombolytic agent-encapsulated TSL or LTSL

This section describes in detail the work carried out in order to determine the suitable REV procedure for thrombolytic agents, as well as the relevant analytical process.

5.3.1. Preparation method for thrombolytic agent using reverse phase evaporation method

Stock lipid chloroform/methanol (1:1 *V/V*) solutions were mixed and solvent was then removed using a rotary evaporator to form a dried thin layer. Dried lipid film (12.5 mg of total lipid) was dissolved with chloroform and mixed with tris buffer (20 mM tris, pH 9) containing either SK (10 KIU/ml) or tPA (300 KIU/ml). Mixture was then vortexed for 5 minutes and chloroform was removed from mixture through vacuum. Vesicles were eventually separated from free thrombolytic agent molecules on a Sepharose CL-2B column (0.8 cm × 10 cm) equilibrated with tris buffer.

5.3.2. Quantification of SK and determination of D/L ratio of SK-encapsulated liposome

D/L ratio (IU/mg) of SK-encapsulated vesicles was obtained by total solubilization of the vesicles. At first, liposome was lysised by adding 1 *v/v* % Triton X-100. The amount of SK was obtained by s-2251^{TM}-based amidolytic assay; 100 µl of lysised liposome sample was mixed with tris buffer equilibrated at 30^{o}C using water bath. 20 µl of plasminogen (120 IU/ml) and soybean trypsin inhibitor (SBTI) (0.5 mg/ml) were introduced into the buffer and mixture was allowed to incubate for 5 min. Then, 200 ml of S-2251 ^{TM} (1.98 mg/ml) was added and mixture was further incubated for another 5 min. The mixture was then put in ice bath for 5 min and its UV/Vis reading at 405 nm was obtained to quantify the amount of SK. The percentage of SK that was considered to be encapsulated inside the liposome was obtained by comparing the amount of SK prior to and after the solubilisation of liposome.

5.3.3. Quantification of tPA concentration and determination of D/L ratio of tPA-encapsulated liposome

Similar to SK-encapsulated vesicles, D/L ratio of tPA-encapsulated vesicles was obtained by total solubilisation of the vesicles. At first, liposome was lysised by adding 1 *v/v* % Triton X-100. The amount of tPA was obtained by the same s-2251 ^{TM} -based amidolytic assay with slight modification; 100 µl of lysised liposome sample was mixed with 1 ml of tris buffer at room temperature and was put in the UV/vis spectrometer. Plasminogen and S-2251^{TM} solution were put into the solution. The slope of UV/Vis signal reading at 405 nm between 5 and 10 min was used to quantify the amount of tPA. The percentage of tPA that was considered to be encapsulated inside the liposome was obtained by comparing the amount of tPA prior to and after the solubilisation of liposome.

5.3.4. Thermal sensitivity test of SK or tPA-encapsulated vesicles

The release behaviour of both vesicles at various temperatures was obtained by directly heating liposome solutions in a temperature-equilibrated water bath for 5 min. Liposome solution was then allowed to cool down in a room temperature-equilibrated water bath for another 5 min. The amount of thrombolytic agent detectable after heating is quantified using the amidolytic method described in section 5.3.2.or 5.3.3. The percentage release of thrombolytic agent was calculated using the following equation

$$\%\ \text{thromobltyic agent release} = \frac{F_I - F_0}{F_{full} - F_0} \times 100\%$$

Where F_0 is the amount of thrombolytic agent detected prior to heating, F_I is the amount of thrombolytic agent detected after heating, F_{full} is the amount of thrombolytic agent detected after complete release of thrombolytic agent using 1 *v/v* % Triton X-100.

5.3.5. Determination of phase transition temperature and particle size of tPA-TSL

Stock tPA-TSL solution (10 µl) was added into aluminium pan made for DSC measurement and pan was clip-sealed. The sample-containing pan was then introduced into the sample cell of differential scanning calorimeters (Dsc q 2000, TA instrument). Empty sample aluminium pan was used as reference sample. The heat flow of sample from 30 ^{o}C to 60 ^{o}C was recorded.

Stock tPA-TSL solution (100 µl) was diluted in buffer solution (1 ml, Tris buffer) and was transferred into BrandTech® disposable UV Cuvette. Cuvette was then transferred into sample cell of PCS (Zetasizer Nano S, Malvern). Refractive index was set at default liposome solution and incubation temperature was set at 25 ^{o}C. Detection angle was set at 173 ^{o}. Z-average size (hydrodynamic diameter, nm) and pDI (polydispersity index) are the two parameters used in the project to describe the particle size of liposomes. The data was based on the intensity distribution of measured solution.

5.4. Results and discussion

5.4.1. Feasibility of SK-encapsulated LTSL

SK molecules were encapsulated into LTSL vesicles using REV process and the D/L ratio of SK in the produced SK-LTSL was determined and illustrated in Figure 5.2. As can be seen, 1 mg of lipid was able to incorporate 88 IU of SK molecules. Among the total incorporated SK molecules, 55% was unavailable for plasminogen interaction during s-2251^{TM} based assay and was considered to be “encapsulated”.

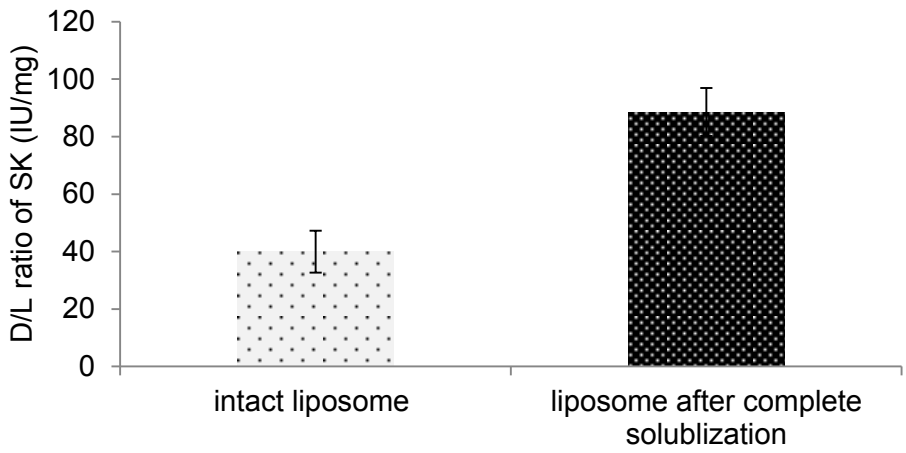

Figure 5.2. D/L ratio (IU/mg) of SK-encapsulated LTSL (*n=3*). Amount of SK was determined using s-2251™ based amidolytic assay. 55% of SK was considered to be encapsulated and was unavailable to convert plasminogen till complete solublization of liposome.

The extent of SK release from LTSL vesicles at various temperatures was determined. As shown in Figure 5.3, vesicles were able to release majority of the encapsulated SK molecules (~90%) after 5 min heating at 45°C. The structure and mechanism of the release behaviour of SK-LTSL vesicles, however, are somewhat different from the doxorubicin-encapsulated LTSL vesicles prepared by extrusion method.

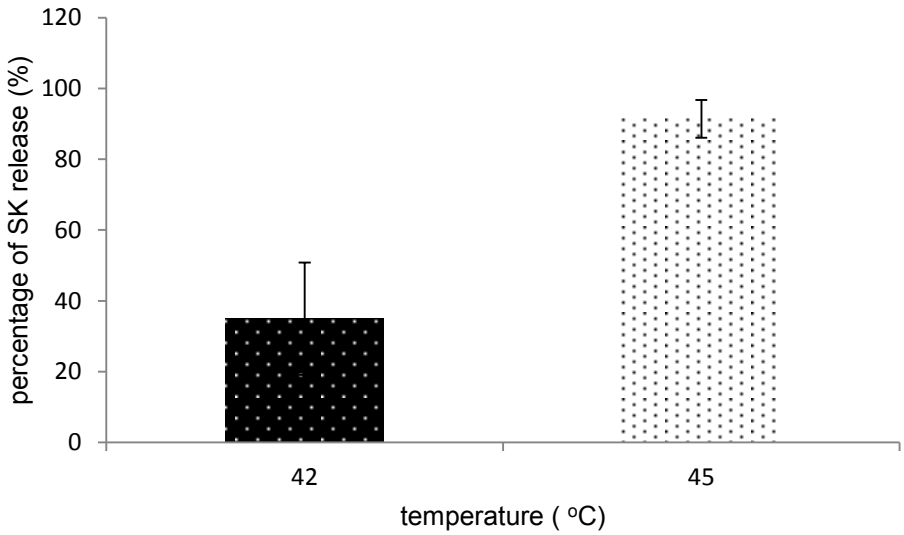

Figure 5.3. The extent of SK release from LTSL at 42 and 45°C (*n=3*). Stock SK-TSL solution was heated at predetermined temperature for 5 min and the amount of SK molecules was determined using s-2251 based assay. Heating at 45 °C for 5 min resulted in complete release of encapsulated SK.

5.4.2. Feasibility of tPA-encapsulated TSL

Having demonstrated the applicability of REV for encapsulation of SK, the same procedure was applied to tPA (the target thrombolytic agent of the project), but the formulation of vesicle was changed from LTSL to a more stable TSL due to the higher MW of tPA, which may destabilize the membrane. When determining the thermal sensitivity of tPA-TSLs, heating time was increased to 7 minutes as it was expected that high MW molecules might hinder their release from TSLs.

The drug to lipid ratio (D/L ratio, IU/mg), particle size and phase transition temperature of tPA-TSLs were determined using the methods described in section 5.3. The obtained results are presented in Table 5.1, which demonstrates that tPA-TSLs were able to encapsulate 5.07 IU (0.016936 mg) of tPA per mg of lipid. According to the measured heat flow, tPA-TSL had a phase transition temperature (T_m) of 41.79 °C, similar to the T_m of dox-TSLs (presented in Table 3.2). Interestingly, the hydrodynamic diameter of tPA-TSLs was significantly larger (2286 nm) in comparison with blank REV-prepared TSLs

(538 nm), suggesting that the incorporation of tPA molecules altered the physical properties of liposome membrane. In addition, tPA- TSLs released 75% of encapsulated tPA upon 7 minutes heating at 45^{o}C.

Table 5.1. Characterization of tPA-TSLs and blank TSLs prepared by REV method

Type of formulation and component	D/L ratio, (IU/mg)	Particle size		T_m (^{o}C)	% of tPA release after heating
		Hydrodynamic diameter (nm)	Polydispersity index		
tPA-TSL DPPC:DSPE.PEG.2000 (96:4)	5.07 (1.10) (n=3)	2286 (34) (n=3)	0.5315 (0.4293) (n=3)	42.1799 (0.00121) (n=2)	75.1 % (11.2) (n=4)
Blank-TSL DPPC:DSPE.PEG.2000 (96:4)	N/A	538[5] (44)	0.7953[5] (0.159)	N/A	N/A

5.4.3. Potential release mechanism of tPA from TSL upon heating

Irrespective of its potential release behaviour, thermal sensitive liposomes prepared by extrusion method (~100 nm) have been reported to retain their morphology after heating (de smet et al. 2013). Different from the small molecular-encapsulated TSLs (*e.g,* doxorubicin, *etc*), thrombolytic agent-encapsulated thermal sensitive liposome prepared here are likely to have experienced irreversible destruction upon heating. This was based on initially observed turbidity changes of the heated SK-LTSL liposome solution during D/L determination assay, in which 100 µl of heated stock liposome solution was added into 1 ml of Tris buffer. It was noticed that the appearance of mixture after the

addition of heated liposome solution was much clearer compared to that of the unheated liposome. This resembled the turbidity reduction of triton X-100 treated liposome solution.

In order to find the potential mechanism of tPA release from tPA-TSLs upon heating, the particle size of tPA-TSLs solution prior to and after 7 minutes heating at 45 ^{o}C was determined and shown in Figure 5.4.A. The results show that the particle size of tPA-TSLs reduced significantly from 2286 nm to 1010 nm after heating, indicating that tPA-TSL might have been destroyed. In contrast to tPA-TSLs, particle size reduction of blank REV-prepared TSLs (TSLs encapsulated with pure buffer solution) was much less significant, reducing from 538 nm to 447 nm. This indicates that the presence of tPA molecules is critical to the irreversible morphology change of tPA-TSL upon heating. Changes in particle size of tPA-TSLs after addition of triton X-100 are presented in Figure 5.4.B as a reference.

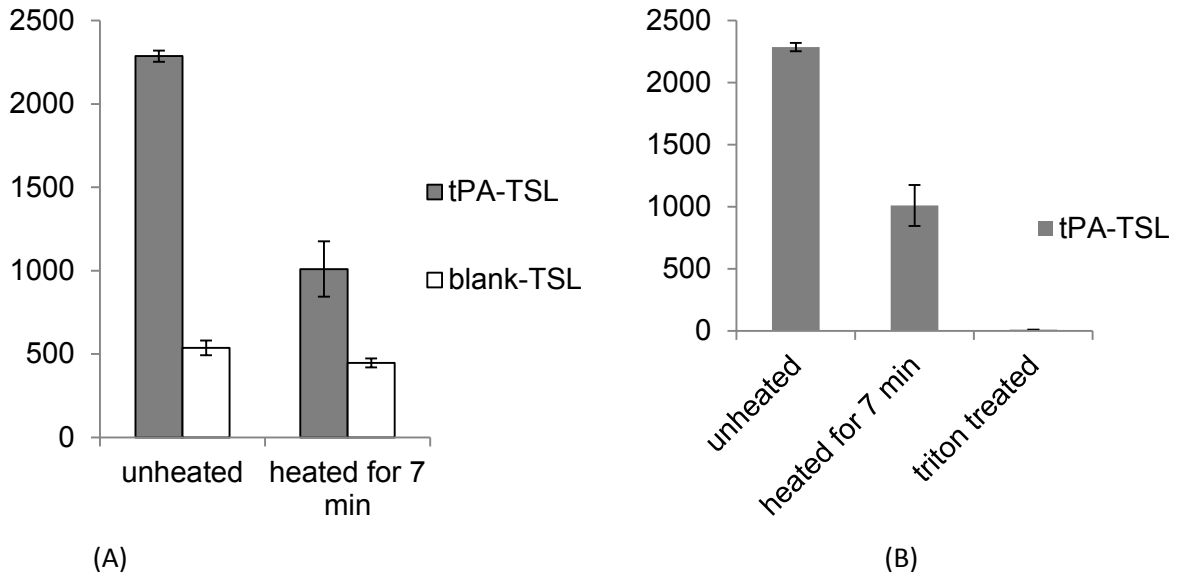

Figure 5.4. Changes in particle size of tPA-TSLs and blank TSLs prior to and after heating at 45 ^{o}C for 7 minutes (A) (*n=3*). Changes in particle size of tPA-TSLs prior to and after the addition of triton X-100 (B) (*n=3*).

It has been well documented that different types of proteins can associate with different sites of the lipid membrane, influencing the properties of the resultant protein-lipid complex: the small ionic permeability of liposome has been reported to be drastically changed, either as a result of liposome-protein association or structural change of protein in response to temperature rise (Kimelberg. 1976). Although we cannot verify the actual state of encapsulated tPA molecules (*i.e.* whether it was truly encapsulated in the interior of the liposome, or it was in a "unavailable state" to the assay), it is speculated that, upon heating, the lipid-associated protein causes irreversible dissociation of REV membrane rafts between protein molecules, which results in the eventual destruction of the liposome.

5.4.3.1. Lipid-protein interaction

Depending on their hydrophobicity, membrane-associated proteins have various preferable lipid-interaction sites and have been initially categorized into three groups. Proteins in group I are charged water soluble proteins that interact solely with head groups of phospholipids (Papahadjopoulos et al. 1975). Groups II proteins are less hydrophilic than group I proteins and interact with both the hydrophilic head group and bilayer face (Papahadjopoulos et al. 1975). Group III are composed of water-insoluble apolipoproteins that interact mainly with the hydrocarbon part of membrane (Papahadjopoulos et al. 1975). Transmembrane proteins were later defined by Kimelberg et al as the fourth type of proteins due to their ability to interact with head group on both monolayers of the membrane (Kimelberg. 1976). As will be discussed below, both tPA and SK interact with the head group as well as the apolar region of the lipid and are considered as group II proteins.

It was initially hypothesized that, by determining the preferable interaction site of the protein, the impact of addition of protein on/into the membrane can be predicted and summarized: while the interaction between group I and the head group of lipid had little influence on the permeability of membrane, penetration of the other types of protein (groups II, III and IV) into the lipid membrane has been predicated to result in a substantial expansion of originally closely packed membrane and enhancement of vesicle permeability (Papahadjopoulos et al. 1975).

It was later discovered that, in addition to the category of the protein, changes in membrane property also depended strongly on the structure of the lipid itself: being considered as a group II protein, gramicidin S protein was only capable of altering the thermaltropic behaviour of anionic lipid and neutral lipid that have strong shielding at their positive sites (*e.g.* DPPC) (Prenner et al. 1999). Such proteins however, have little effect on 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (lipid with a low shielding strength) (Prenner et al. 1999). Two other type II group proteins, cytochrome C and A1 protein also show the reliance of anionic group to alter the property of the membrane (Papahadjopoulos et al. 1975). As a result, the structure of both lipid and protein should be taken into account when predicting the likely alteration of membrane property.

5.4.3.2. Hypothesis of tPA-induced TSL destruction upon heating

Possibly due to the high shielding of amine group, DPPC behaves as a pseudo-anionic lipid and has been reported to interact with group II proteins such as cytochrome c, gramicin S and result in the modification of thermotropic behaviour of liposomes. Given the type II protein nature of tPA molecules, the membrane property of DPPC-based TSL system is likely to be altered as a consequence of the insertion of tPA molecules, resulting in expansion of membrane area.

In summary, the destruction of tPA-TSL upon heating may be the result of the following two combinations:

1. Interaction between protein and lipid membrane
2. Melting of lipid chain upon heating

5.4.4. Hypothesis of structure of tPA-TSL

As defined in section 5.3.3., “encapsulated tPA molecules” is a term used to represent the population of tPA molecules that is not available to plasminogen prior to either heating or destruction of vesicles using Triton X-100. While it is hoped that the unavailability of encapsulated tPA molecules to plasminogen is a result of them being truly encapsulated in the interior of liposomes, given the observed interaction between tPA molecules and membrane, one cannot rule out the possibility that interactions between the hydrophobic active sites of tPA molecules and the apolar-region of the lipid (*i.e.* interface and/or hydrocarbon tail region) may prevent the protein being available to plasminogen (pseudo-encapsulation). The potential protein-PEG layer interaction is another possible pseudo-encapsulation state that may contribute to the unavailability of tPA molecules to plasminogen. Using the current thrombolytic agent quantification method (*i.e.* s-2251^{TM}-based assay), it was not possible to distinguish whether the unavailability of tPA molecules was the results of either “true encapsulation” or “pseudo-encapsulation” of the protein.

5.5. Summary

By the end of the project, it has been demonstrated that both SK and tPA molecules can be encapsulated into DPPC-based vesicles and the encapsulated agents can be released after heating at 45^{o}C. Based on observed particle size change of tPA-TSLs prior to and after heating, it has been hypothesized that thrombolytic agent was released from vesicles as a result of irreversible destruction of liposome upon heating. This mechanism of release is different from TSL systems that encapsulate small MW molecules such as doxorubicin. Such a unique release mechanism is believed to be a combined result of protein insertion into the liposome membrane and melting of lipid chain upon heating.

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6. Conclusions

In this chapter, the major achievements made in this project are summarized, together with critical comments on the current formulation and results obtained. This is followed by a summary of the intrinsic limitations of the analytical methods employed, and suggestions for future work.

6.1. Main conclusions

6.1.1. Development of lysolipid-free doxorubicin-TSL

The most significant achievement in this part of the project is the development of a lysolipid-free TSL formulation for the encapsulation of doxorubicin. By directly heating dox-TSLs into buffers solution that are temperature-equilibrated at hyperthermia (45 ^{o}C), around 80% of encapsulated doxorubicin could be released within 10 seconds. As the formulation does not contain lysolipids, dox-TSLs are free from the risk associated with lysolipid-induced leakage at physiological temperature. Hence, the doxorubicin-TSL produced using this formulation has the potential to become a viable substitute for the current doxorubicin-LTSL formulation.

The second achievement is the discovery that selection of incubation temperature (30^{o}C-36 ^{o}C) contributes heavily to achieving high D/L ratio of dox-TSLs. As a result, the D/L ratio of dox-TSLs prepared in the current study is 2 times the D/L ratio that reported in the existing publications. This is done by increasing the inward movement of doxorubicin molecules into the liposome, while limiting the outflow of H^{+} molecules. Moreover, it has been found that the permeability changes of TSLs to unprotonated doxorubicin within 30^{o}C- 36 ^{o}C temperature range coincides with the initiation of membrane transition from solid phase to ripple phase (at ~32^{o}C) (Heimburg. 1998). This finding may provide us insights into the intrinsic instability of DPPC-based TSL under *in vivo* conditions. Based on the analysis presented in Chapter 3, there is a possibility that the application of DPPC as a membrane component may itself compromise the protective functionality of PEGlyation and hinder the further optimization and application of thermal sensitive liposomes.

A limitation of this study is the lack of detailed information on membrane transition. In the ground-breaking paper that described the application of lysolipid in DPPC-based liposome, Anyarambhatla and Needham provided detailed DSC scan of TSL membrane which clearly demonstrated the occurrence of pretransition of DPPC TSL membrane (from solid phase to ripple phase) by the presence of endothermic peak at ~37 ^{o}C. A similar peak in the DSC scan was also obtained by other groups (Heimburg. 1998, Wu et al. 2011). On the other hand, change in heat flow during pretransition was several orders lower than that during the main transition of membrane. As presented in appendix VII, the observed heat flow changes of main transition in the current study (1.5 MW) was 7 times lower than that observed by Anyarambhatla and Needham (~10 MW) . Therefore, it is highly likely that the lack of membrane transition information in our measurements was due to the low concentration of liposome.

6.1.2. Development of lysolipid-free tPA-TSL

The most significant achievement in this part of the project is the development of DPPC-based thermal sensitive liposome for encapsulation and release of thrombolytic agents, of which SK and tPA have been tested. This is the first time lysolipid-free TSLs have been shown to have the capacity to encapsulate and release high molecular weight therapeutics. It has also been observed that the release mechanism of SK or tPA encapsulated-TSL is different from that of small molecule-encapsulated TSL, It is commonly believed that the after the heating process, small molecule-encapsulated TSLs retained their integrity (de Smet et al. 2013). However, the SK or tPA-loaded TSL vesicles produced in this project are believed to have experienced irreversible destruction upon heating, thereby releasing the encapsulated contents. This is based on the observed particle size changes of liposome solution prior to and after heating.

The tPA-TSL developed in this project is a combined product of 3 separate formulations:

- Liposomes that have fibrin-targeting capability through the direct incorporation of tPA into the liposome membrane (e.g. tPA-ELIP).

- PEG.2000-based stealth liposome containing 5 mole % of membrane.

- DPPC-based thermal sensitive liposome.

While the original intention was to keep all three functionalities described above in one formulation, it is possible that the *in vivo* stability of the tPA-TSLs was compromised by this combination, largely as a result of direct insertion of tPA for fibrin-targeting capability: Upon binding onto the fibrin, the affinity of tPA towards plasminogen increases significantly (Nieuwenhuizen. 1994), which is accompanied by the breakage of tPA from a one-chain protein molecule into a two-chain molecule (Thorsen. 1992). As a considerable amount of proteins might be in "pseudoencapsulation state" with the active hydrophobic part of the protein being inside the membrane, it is possible that the tPA-incorporated membrane may be disrupted due to the conformation change of tPA proteins upon fibrin binding resulting in either premature leakage of tPA prior to heating or loss of thermal sensitivity of liposome. Furthermore, since DPPC is the main-component of the tPA-TSL, the membrane is already in a relatively loose ripple phase, further weakening the disruption resistance of membrane against tPA-conversion.

In summary, similar to the doxorubicin-TSL, the combined effect of DPPC-based thermal sensitive liposome and direct insertion of tPA for fibrin-binding may reduce the TSL stability at physiological temperature.

6.2. Limitations of the analytical methods employed

The analytical methods adopted in this project were selected based on their established application, wide usage and availability. Nevertheless, each analytical method has its own intrinsic limitations that may affect its ability to accurately determine the parameters of interest. These are explained below.

6.2.1. Limitations of doxorubicin quantification method

The self-quenching characteristic of doxorubicin was exploited to determine the D/L ratio of doxorubicin and the release behavior of doxorubicin-encapsulated vesicles at various temperatures: the initial fluorescence intensity (F_0) was used to represent the initial concentration of solute (C_0) and was quantified using the relevant calibration curve. Although widely used in liposome research, this method cannot measure C_0 directly, and the measured F_0 is a result of either:

- Accumulated fluorescence signal of liposome with quenched concentration of solute encapsulated, or

- Accumulated fluorescence signal of liposome with unquenched concentration of solute inside the membrane

It is the stacking of solutes at high concentrations that leads to the shield of fluorophore and the reduction (i.e. quenching) of fluorescence signal. After being encapsulated in the liposome, however, the stacked solutes become isolated from other populations of stacked solutes that are encapsulated in the other vesicles. Due to the disappearance of stacking effect among individual solute aggregates, C_0 increases with increasing liposome concentration. On the other hand, it is also possible that, solutes may be incorporated in the membrane of liposome at unquenched concentration and the F_0 is the accumulated fluorescence reading of the solute that is associated with the membrane. In either of these cases, F_0 is not a good indicator of the initial concentration of solute. In most cases though, the fluorescence signal corresponding to the release of solute is much higher than F_0. Hence, the value for C_0 calculated from F_0 is a reasonable reference point to determine the relative amount of solute release after liposome lysis or heating.

6.2.2. Limitations of heating method for measurement of thermal sensitivity

Throughout this project, water bath (detailed in appendix II) was used to heat the liposome solution to the desired temperature points. However, this simplified heating equipment does not mimic the *in vivo* condition, in which liposome solution, regardless of the temperature at which it is stored prior to administration (e.g. 4^{o}C or 25^{o}C), will first enter the circulation which is at normal physiological temperature (~37^{o}C). Through the circulatory system, liposome is transported to the target area where it is heated (~42^{o}C). Hence, using the heating method adopted here, the initial condition of liposomal membrane prior to heating (in solid phase at room temperature) is different from the expected *in vivo* condition (in ripple phase at 37^{o}C) and it is unknown whether the release behaviour of liposome will be different if the initial condition of membrane is different.

6.2.3. Limitations of s-2251^{TM}-based assay for thrombolytic agent quantification

As described in section.5.2.2, through the utilization of plasmin-specific substrate (i.e. s-2251^{TM}-based assay), tPA and SK were indirectly quantified by the extent of plasminogen-plasmin conversion. This method has been used throughout the project to:

- Determine the D/L ratio of SK-TSL and tPA-TSL by comparing the availability of thrombolytic agent to plasminogen prior to and after the lysis of liposome
- Determine the thermal sensitivity of SK or tPA encapsulated TSL by comparing the availability of thrombolytic agent to plasminogen prior to and after the heating of liposome solution.

While the result of s-2251^{TM}-based assay measures the ability of the the thrombolytic agent-loaded TSLs to convert plasminogen to plasmin, the assay do not provide some of the crucial information about:

- The true nature of tPA or SK unavailability to plasminogen prior to either lysis of liposome or heating of liposome solution.

- The fibrin-targeting capability of liposome and its subsequent stability.

The first failure is a result of complicated protein-liposome interaction, as discussed in detail in section 5.4.4.1. The second failure, on the other hand, is due to the incompatibility of fibrin and s-2251^{TM}-based assay, as addition of fibrin increases the UV/Vis absorption of solution which compromises the ability of s-2251^{TM}-based assay to quantify the plasminogen to plasmin conversion.

6.3. Future work

6.3.1. Determination of PEG functionality and fluidity of membrane

The intrinsic instability of DPPC-based TSL at physiological temperature makes it hard to further optimize the doxorubicin-TSL formulation. Since the instability is due to the compromised PEG functionality in ripple phase. By increasing T_m of the membrane, it is possible that the PEG functionality can be preserved. In the future, the feasibility of this theory can be tested first by determining the stability of DPPC:DSPE.2000 liposome at sub ripple phase temperature (i.e. below 30^{o}C). This can be accompanied by a detailed DSC measurement of the membrane.

6.3.2. Determination of release behavior of TSL at temperatures above T_m

The release behavior of TSL at temperatures above T_m should be carefully studied more thoroughly using direct heating method. This will elucidate the role of liquid-solid interface in enhancing the thermal sensitivity of TSL at T_m. If the release behavior of liposome at temperatures above T_m is comparable to that at T_m, then It may indicate that parameters other than liquid/solid interface is responsible for the rapid release of DPPC-based liposome upon heating.

6.3.3. Determination of tPA-TSL stability at physiological temperature

While the thermal sensitivity of tPA-TSLs upon heating was determined in the present study, the stability of liposomes at physiological temperature was not measured.

However, it is expected that a combined result of tPA incorporation and application of DPPC-based membrane may compromise the stability of tPA-TSL. If tPA-TSLs wre found to be unstable at physiological temperature, effort would be needed to increase the stability of tPA-TSL. The first attempt would be to tighten the packing of membrane through the use of lipid with a higher phase transition temperature. In addition, a different assay, *e.g*. fibrin-containing *in vitro* assay, should be carried out to quantify the amount of released tPA. The degree of tPA leakage and thermal sensitivity could be determined by the reduction of fibrin loss, rather than the UV/Vis absorption change in S-2251 based assay.

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Achievement

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ZHANG, X., LUCKHAM, P. F., HUGHES, A. D., THOM, S. & XU, X. Y. 2011. Development of lysolipid-based thermosensitive liposomes for delivery of high molecular weight proteins. *Int J Pharm,* 421**,** 291-2.

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Appendix I- List of chemicals used and their abbreviations

Function of chemical during assay	Abbreviation in text	Full name of chemicals	Manufacture and catalogue number	Purity
Size exclusion column for liposome/solute separation	Sephadex G-25 column	Disposable PD-10 Desalting Columns	GE Healthcare (7-0851-01)	≥98%
	Sepharose® CL-2B column	N/A	Sigma-Aldrich (CL2B300)	N/A
Lysis of liposome	Triton™ X-100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol	Sigma-Aldrich (X100)	N/A
Chemicals for tPA/SK quantification	Plasminogen	*Plasminogen*, Human Plasma	Merck KGaA, Darmstadt, Germany (528175)	≥95%
	SBTI	Trypsin Inhibitor from *Glycine max* (soybean)	Sigma-Aldrich (T2327)	≥98%
	s-2251	D-Val-Leu-Lys 4-nitroanilide dihydrochloride plasmin substrate	Sigma-Aldrich (V0882)	N/A

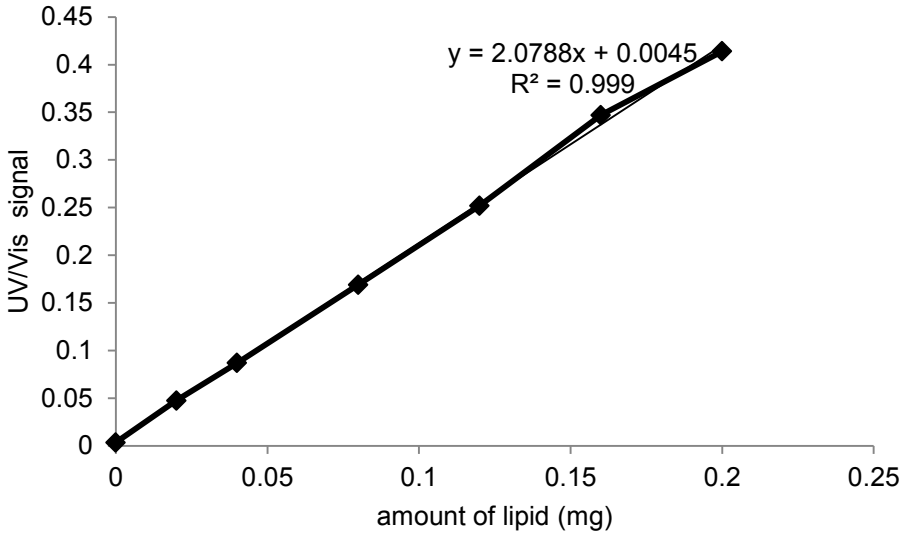
Full name		Full name	Manufacture and catalogue number	Purity
Phospholipid	DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine	Avanti Polar lipid, Alabaster, AL, USA (850355P)	>99
	MPPC	1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine	Avanti Polar lipid, Alabaster, AL, USA (855675P)	>99
	MSPC	1-dipalmitoyl-sn-glycero-3-phosphocholine	Avanti Polar lipid, Alabaster, AL, USA(855775P)	>99
	DSPE.2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000	Avanti Polar lipid, Alabaster, AL, USA (880120P)	>99
	DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine	Avanti Polar lipid, Alabaster, AL, USA (850365C)	>99
	HSPC	L-α-phosphatidylcholine, hydrogenated (Soy)	Avanti Polar lipid, Alabaster, AL, USA (840058P)	>99
MRI contrast agent-incorporated lipid	Gd.DOTA.DSA	Gadolinium (III) 2-{4,7-bis-carboxymethyl-10-[(N, N-distearylamidomethyl-N`-amidomethyl]-1,4,7,10-tetra-azacyclododec-1-yl}-acetic acid	Synthesized by previous research group	N/A
	Gd. PE-DTPA	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (gadolinium salt)	Avanti Polar lipid, Alabaster, AL, USA (791275P)	>99
Other lipid component	Cholesterol	3β-Hydroxy-5-cholestene	Sigma-Aldrich **(**C8667**)**	>99

Solute	Abbreviation of chemical	Full name	Manufacture and catalogue number	Purity
Hydration buffer For doxorubicin and calcein	Ammonium phosphate	Ammonium phosphate monobasic	Sigma-Aldrich (216003)	≥98%
	HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	Sigma-Aldrich (H3375)	≥99.5%
	Sodium chloride	N/A	Sigma-Aldrich (S9888)	≥99%
Hydration buffer For SK and tPA	Tris buffer	Tris(hydroxymethyl)aminomethane	Sigma-Aldrich (252859)	≥99.8%
Target encapsulated solute	Doxorubicin	Doxorubicin hydrochloride	Sigma-Aldrich (D1515)	98.0-102.0%
	Calcein	Bis[N,N-di(carboxymethyl)aminomethyl]fluorescein disodium salt, Fluorescein-bismethyliminodiacetic acid disodium salt	Sigma-Aldrich (21030)	≥90%
	tPA	Tissue Plasminogen Activator, Human	Merck KGaA, Darmstadt,Germany (612200)	≥300 KU/mg protein
	SK	Streptokinase from β-hemolytic *Streptococcus*	Sigma-Aldrich (S3134)	≥3,500 units/mg solid

Appendix II -Information on analytical equipment used in the project

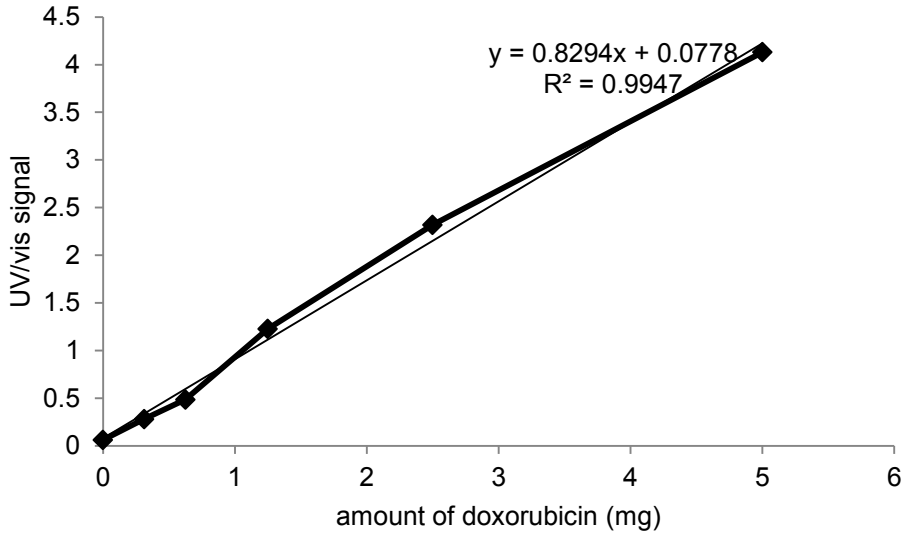
Equipment used for doxorubicin-TSL preparation and determination	Model of equipment (supplier)
Particle size determination	Zetasizer Nano S (Malvern)
Fluscent determination for doxorubicin and calcein release	LS-55 spectrometer (Perkin Elmer)
Water bath for release study	Heating bath for rotary evaporator B-491 (Buchi)
Incubator for encapsulation	Heating bath for rotary evaporator B-491 (Buchi)
Phase transition determination	Dsc q 2000 (TA instrument)
SK/tPA-TSL heating	Heating bath for rotary evaporator B-491 (Buchi)
UV/Vis measurement for S-2251-based assay	UV-Vis spectrophotometer Lambda 25 (PerkinElmer)
Stewart assay for lipid determination	UV-Vis spectrophotometer Lambda 25 (PerkinElmer)

Appendix III- Calibration curve for lipid quantification

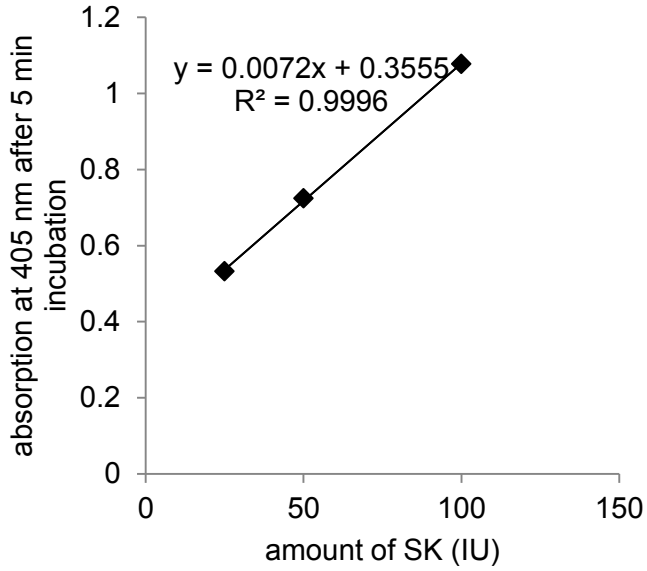


Calibration curve of stewart assay. Calibration curve was used for lipid quantification

Appendix IV- Calibration curves for doxorubicin quantification

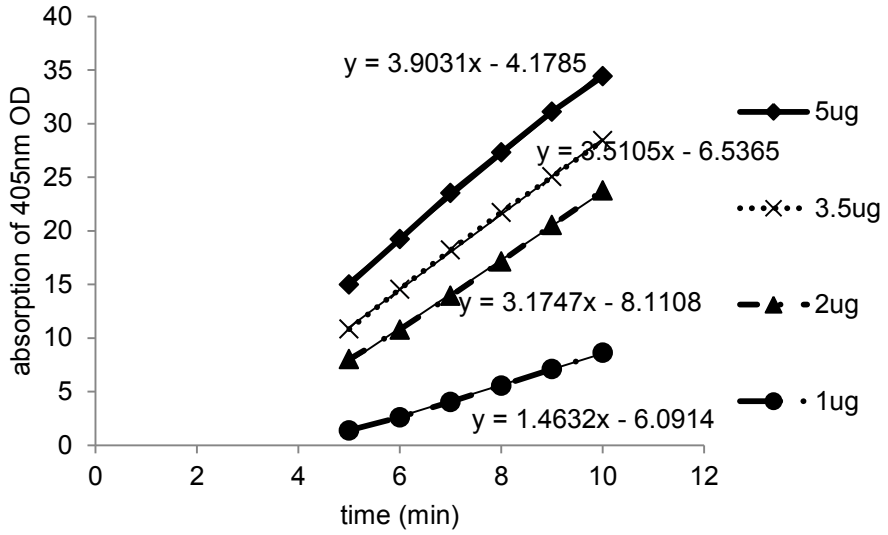


Appendix V -Calibration curve for SK quantification encapsulated in TSL

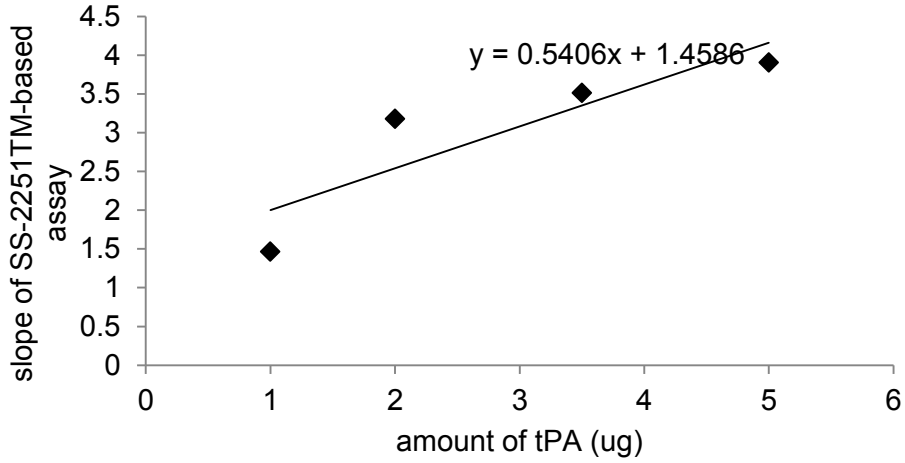


Calibration curve for SK quantification encapsulated in SK-TSL. UV/Vis absorption of solution 5 min after the addition of S-2251^{TM} substrate was used to quantify the concentration of SK.

Appendix VI- Calibration curve for tPA quantification

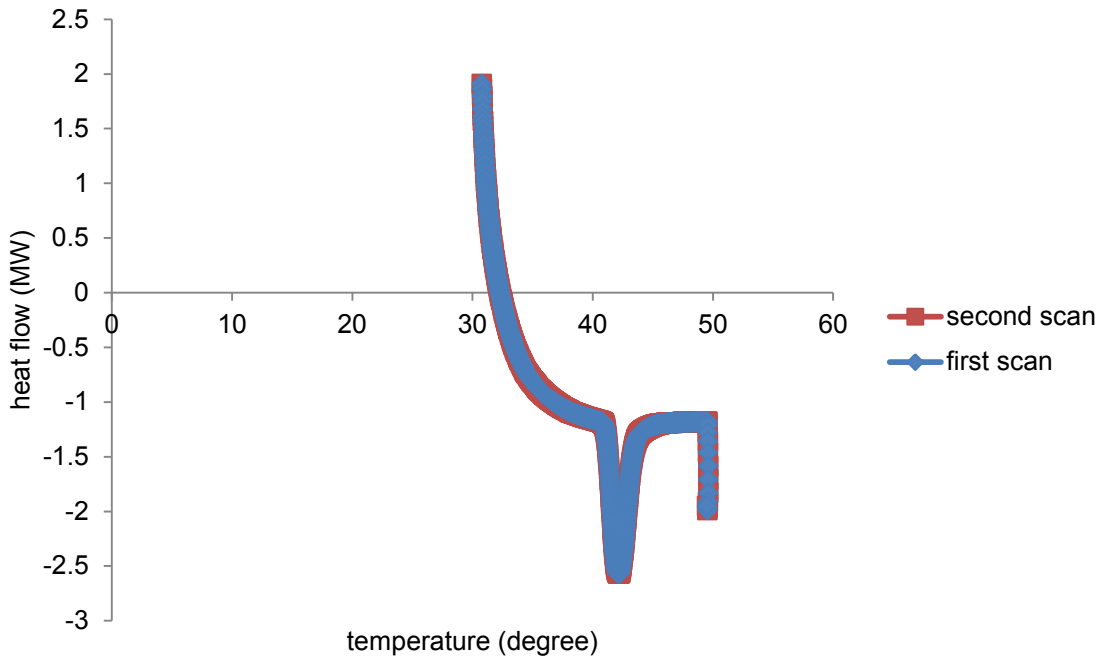


Result of s-2251 [TM] assay with various tPA concentrations. The slopes are used for tPA quantification encapsulated in tPA-TSL containing Gd.lipid.



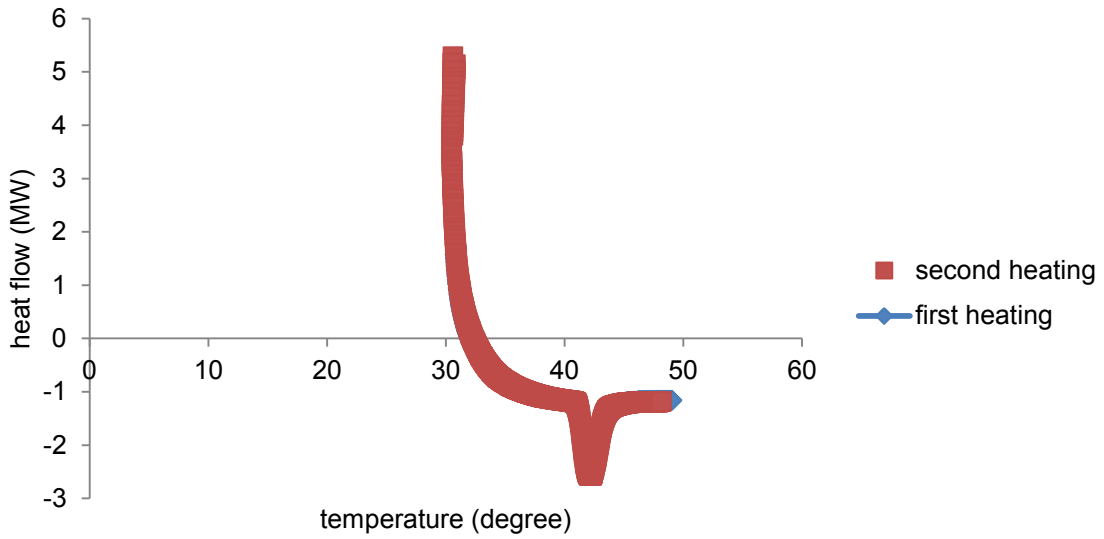
Calibration curve of tPA encapsulated in tPA-TSL containing Gd.lipid. Slopes obtained during S-2251 [TM] assay is used to quantify tPA.

Appendix VII- Heating DSC Thermogram of TSL membrane



Heating DSC Thermogram of TSL membrane DPPC: DSPE.PEG 2000 (molar ratio 96: 4) formulation. Lipid amount is around 1.5mg. Heating rate is 1 ^{o}C/min.

Appendix VIII- Preparation method of liposome gel to prove the feasibility of HIFU heating to release F5



Heating DSC Thermogram of pure DPPC membrane. Lipid amount is around 1.5mg. Heating rate is 1 ^{o}C/min.