

Synthesis and characterisation of chitosan-coated thermosensitive liposomes for triggered release

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Declaration

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me, except where otherwise acknowledged and appropriately referenced.

Wenxin Wang, April 2023

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Abstract

Chitosan has been frequently utilized in many areas including drug delivery system, microcapsules, antibacterial agents, blood anti-coagulants and ophthalmology. Most of the liposomes modified by chitosan were used for oral, ocular and pulmonary delivery. Very little research has been undertaken to investigate the effect of chitosan coating on the characterization of liposomes for intravenous injection. HaT (Hyperthermia-Activated Cytotoxic) liposome was selected as the plain liposome formulation in this thesis, which has demonstrated faster release both *in vitro* and *in vivo* and superior ability to inhibit tumour growth and lower toxicity to healthy tissues than LTSL (Lysolipid-Containing Thermosensitive Liposome) after intravenous injection followed by hyperthermia. The aim of this thesis is to develop chitosan coated HaT liposomes encapsulated with calcein disodium salt and doxorubicin, respectively and compare their release characteristics with those of plain HaT liposomes. TEM images of plain liposomes and low, medium, and high MW chitosan coated liposomes (freshly made and stored ones) were compared. Among all three concentrations (0.1%, 0.2% and 0.3% (w/w)), 0.1% (w/w) medium molecular weight chitosan coated HaT liposomes displayed the best overall performance with the lowest increase on both zeta potential and particle size after storage. Therefore, it was selected for the investigations of release property and shelf life of chitosan coated HaT liposomes. The coating of 0.1% (w/w) medium molecular weight chitosan can increase the stability of HaT liposomes encapsulated with calcein disodium salt or doxorubicin at 37 °C and enhance the shelf life of HaT liposomes both at room temperature and at 4 °C after storage. At 40 and 42 °C, the release percentages of chitosan coated liposomes were still substantial with a slight decrease compared to plain HaT liposomes. Finally, several commonly-used pharmaceutical kinetic models were selected to model the release curves of both calcein and doxorubicin encapsulated liposomes.

Abbreviations

AD	Alzheimer's disease
AIC	Akaike's Information Criteria
AML	Acute Myeloid Leukemia
ANT	Anthracyclines
ApoA-1	Apolipoprotein A-1
APRPG	Alanine-Proline-Arginine-Proline-Glycine Polypeptide
ARKS	AIDS-related Kaposi's sarcoma
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
Azo	Azobenzene
BBB	Blood Brain Barrier
BH	Berberine Hydrochloride
BIC	Bayesian Information Criterion
BLM	Bleomycin
Brij S20 (Brij 78)	Polyoxyethylene (20) Stearyl Ether
CCL	Liposomes Coated With 0.1% (W/W) Medium Molecular Weight Chitosan and Encapsulated with Calcein
CD	Circular Dichroism
CF	Carboxyfluorescein
CHEMS	Cholesterol Succinate Monoester

Cou	Coumarin
CPL	Plain Liposomes Encapsulated with Calcein
cRGD	Cyclic Arginylglycylaspartic Acid
CsA	Cyclosporin A
DCL	Liposomes Coated With 0.1% (W/W) Medium Molecular Weight Chitosan and Encapsulated with Doxorubicin
DDS	Drug Delivery System
DLP	Dendritic Lipopeptide
DLS	Dynamic Light Scattering
DMPC	1,2-Dimyristoyl-Sn-Glycero-3-Phosphocholine
DNA	Deoxyribonucleic Acid
DNR	Daunorubicin
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	Dioleoyl Phosphatidylethanolamine
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
DOX	Doxorubicin
DPL	Plain Liposomes Encapsulated with Doxorubicin
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol
DSC	Differential Scanning Calorimetry
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-mPEG ₂₀₀₀	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino(polyethyleneglycol)-2000]

DSPE-PEG	1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly (ethylene glycol)
DSPG	1,2-Distearoyl-sn-glycero-3-phosphoglycerol
EE	Encapsulation Efficiency
EMA	European Medicines Agency
EPC	L- α -phosphatidylcholine
EPR	Enhanced Permeation and Retention Effect
EYPC	Egg Yolk Phosphatidylcholine
Fab'	Fragment of Antigen Binding
FDA	Food and Drug Administration
FPL	Folate Modified Poloxamer-Coated Liposomes
FR	Folate Receptor
FTIR	Fourier Transform Infrared Spectroscopy
FUS	High Intensity Focused Ultrasound
GA	Glycyrrhetic Acid
GBM	Glioblastoma
GEM	Gemcitabine
GFP	Green Fluorescence Protein
GLUT1	Glucose Transporter Subtype I
GRGDS	Glycine-Arginine-Glycine-Aspartic-Serine Polypeptides
HaT	Hyperthermia-Activated Cytotoxic Liposome
HaT-DOX	HaT Encapsulated with Doxorubicin
HaT-GEM	HaT Encapsulated with Gemcitabine

HaT-OXA	HaT Encapsulated with Oxaliplatin
HCC	Hepatocellular Carcinoma
HePC	Hexadecylphosphocholine
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HPLC-SEC	High Performance Liquid Chromatography- Size-Exclusion Chromatography
HSPC	L- α -phosphatidylcholine, hydrogenated (Soy)
ICD	Immunogenic Cell Death
ILs	Immunoliposomes
IP	Isoelectric Point
LCHL	Low Molecular Weight Chitosan Coated Liposomes
LCST	Lower Critical Solution Temperature
LID	Lidocaine Hydrochloride
Lipo-PEG	PEG-Modified Liposomes
LTSL-DOX	LTSL Encapsulated with Doxorubicin
LTSLs	Lysolipid-Containing Thermosensitive Liposomes
LUVs	Large Uni-Lamellar Vesicles
lyso-PC	Lysophosphatidylcholines
MAB	Monoclonal Antibody
MFH	Magnetic Fluid Hyperthermia
MLVs	Multilamellar Vesicles
MPPC	1-tetradecanoyl-2-hexadecanoyl-sn-glycero-3-phosphocholine
MRgFUS	Magnetic Resonance-Guided Focused Ultrasound

MTO	Mitoxantrone
MVLs	Multivesicular Liposomes
MW	Molecular Weight
MWCO	Molecular Weight Cut-Off
NER	Nebulisation Stability
NGR	Asparagine-Glycine-Arginine Polypeptides
Ost	Osthole
OX	Oxaliplatin
OXA	Oxaliplatin
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Poly (Ethylene glycols)
PHC	Palmitoyl Homocysteine
PK	Pharmacokinetics
PL	Poloxamer-Coated Liposomes
PLD	Pegylated Liposomal Doxorubicin
PLLs	Polyplex-Loaded Liposomes
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PTSLs	Polymer-Modified Thermosensitive Liposomes
R ² (COD)	Coefficient of Determination
RCE	Rabbit Conjunctival Epithelium
RES	Resveratrol

RES	Reticuloendothelial System
REV	Reverse Phase Evaporation Method
RGD	Arginine-Glycine-Aspartic Acid Polypeptide
RIF	Rifampicin
ROS	Reactive Oxygen Species
RR	Release Ratio
RSS	Residual Sum of Squares
SANS	Small Angle Neutron Scattering
scFv	Single-Chain Fragment Variable
SEC	Size-Exclusion Chromatography
SIC	Schwarz Information Criterion
Spiro	Spiropyran
SSE	Sum Of Squared Estimate of Errors
SSR	Sum Of Squared Residuals
Stealth TSLs	Stealth Thermosensitive Liposomes
SucPG	Succinylated Polyglycidol
SUVs	Small Uni-lamellar Vesicles
T _c	Phase Transition Temperature
TEM	Transmission Electron Micrograph
TER	Transepithelial Electrical Resistance
TF	Transferrin
TFR	Transferrin Receptor

TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor
TPP	Triphenylphosphonium
TSLs	Thermosensitive Liposomes
TTSLs	Traditional Thermosensitive Liposomes
UCST	Upper Critical Solution Temperature
UV	Ultraviolet
UVs	Uni-Lamellar Vesicles
Vem-TD-Lip	Peptide-Modified Vemurafenib-Loaded Liposome
VIP	Vasoactive Intestinal Polypeptide
VSV-G	Vesicular Stomatitis Virus G Protein
WG	Wogonin

Contents

Chapter 1. Introduction	29
1.1 Commonly used ingredients for liposome preparation	31
1.2 Basic approaches and principles of liposome preparation	32
1.2.1 Thin film hydration method	33
1.2.2 Solvent injection techniques	34
1.2.3 Reverse phase evaporation method (REV)	36
1.2.4 Freeze-drying method	38
1.2.5 Freeze thaw method	39
1.3 Developments in liposomes	39
1.3.1 Stealth liposomes (passive targeted, long circulating liposome)	40
1.3.2 Active targeted liposome	43
1.3.2.1 Receptor targeted liposome	44
1.3.2.1.1 Transferrin-modified targeted liposomes	44
1.3.2.1.2 Folic acid-modified targeted liposomes	46
1.3.2.1.3 Peptide-modified targeted liposomes	48
1.3.2.1.4 Glycosyl-modified targeted liposomes	49
1.3.2.1.5 Glycyrrhetic acid-modified targeted liposomes	50
1.3.2.2 Mitochondria-targeted liposomes	51
1.3.2.3 Antibody-targeted liposomes	53
1.3.3 Environment-responsive liposomes	54

1.3.3.1 Thermosensitive liposome.....	54
1.3.3.1.1 Traditional thermosensitive liposome (TTSL).....	55
1.3.3.1.2 Lysolipid-containing thermosensitive liposome (LTSL)	57
1.3.3.1.3 Polymer-modified thermosensitive liposome (PTSL)	59
1.3.3.1.4 Stealth thermosensitive liposome (Stealth TSL)	60
1.3.3.1.5 Hyperthermia-activated cytotoxic liposome (HaT).....	62
1.3.3.2 pH-sensitive liposomes	63
1.3.3.3 Photosensitive liposomes	65
1.3.3.4 Magnetic liposomes.....	67
1.3.4 Dual drug-loaded liposomes	69
1.4 Heating approaches for thermosensitive liposomes (TSLs)	73
1.5 Chitosan coated liposomes.....	75
1.5.1 Chitosan coated liposome for oral delivery.....	77
1.5.2 Chitosan coated liposome for ocular delivery	78
1.5.3 Chitosan coated liposome for pulmonary delivery	79
1.5.4 Chitosan coated liposome for intravenous injection	80
1.6 Objectives of this work.....	82
1.7 Thesis outline.....	83
Chapter 2. Development of chitosan-coated thermosensitive liposome	85
2.1 Introduction	85
2.2 Materials and Methods	87
2.2.1 Materials	87

2.2.2 Preparation of plain thermosensitive liposome	88
2.2.3 Preparation of chitosan-coated thermosensitive liposome	88
2.2.4 Preparation of doxorubicin encapsulated thermosensitive liposome	89
2.2.5 Preparation of calcein disodium salt encapsulated thermosensitive liposome	89
2.2.6 Separation of liposomes from the unencapsulated drug by gel filtration	90
2.2.7 Selection of gel filtration media	94
2.2.8 Liposome Characterisation: Zeta potential	96
2.2.9 Liposome Characterisation: Particle size	97
2.2.10 Liposome Characterisation: TEM	98
2.3 Results and discussion	102
2.3.1 The influence of ultrasonic time on the liposome size	102
2.3.2 Isoelectric point of lipids and different MW chitosan	105
2.3.3 The influence of different molar ratio of lipids on particle size and zeta potential of liposomes	110
2.3.4 The TEM graphs of different MW chitosan coated liposomes	113
2.3.5 The comparison of particle size and zeta potential of different MW chitosan coated liposomes	121
2.4 Conclusion	129
Chapter 3 Characterisation of chitosan-coated liposome with calcein disodium salt encapsulation	131
3.1 Introduction	131
3.2 Materials and Methods	133

3.2.1 Materials	133
3.2.2 The determination of calcein fluorescence intensity standard curve	133
3.2.3 Liposome characterisation: Encapsulation efficiency	134
3.2.4 Determination of the measurement assay and calculating equation of release property	135
3.2.5 Liposome characterisation: Release Property	137
3.2.6 Liposome characterisation: Shelf life	138
3.2.7 Determination of fitting models	139
3.2.8 Determination of fitting parameters	150
3.3 Results and discussion	154
3.3.1 The influence of pH on the Calcein fluorescence intensity standard curve .	154
3.3.2 Encapsulation efficiency of calcein encapsulated liposome	161
3.3.3 Release property of calcein encapsulated liposome	164
3.3.4 Shelf life of calcein encapsulated liposome	169
3.3.5 Fitting of release curves of calcein encapsulated liposomes using different pharmaceutical models.....	172
3.3.6 Comparison of fitting parameters	181
3.4 Conclusion	184
Chapter 4 Characterization of chitosan-coated liposome with doxorubicin encapsulation .	186
4.1 Introduction	186
4.2 Materials and Methods	188
4.2.1 Materials	188
4.2.2 The determination of doxorubicin fluorescence intensity standard curve	189

4.2.3 Liposome characterisation: Encapsulation efficiency	190
4.2.5 Liposome characterisation: Release Property	191
4.2.6 Liposome characterisation: Shelf life	192
4.2.7 Determination of fitting models and fitting parameters	193
4.3 Results and discussion	193
4.3.1 The influence of pH on the doxorubicin fluorescence intensity standard curve	193
4.3.2 Encapsulation efficiency of doxorubicin encapsulated liposome	197
4.3.3 Release property of doxorubicin encapsulated liposome	200
4.3.4 Shelf life of doxorubicin encapsulated liposome.....	204
4.3.5 Fitting of release curves of doxorubicin encapsulated liposomes using different pharmaceutical models.....	207
4.3.6 Comparison of fitting parameters	220
4.4 Conclusion	224
Chapter 5 Conclusion	226
5.1 Conclusion	226
5.2 Future work.....	228
Reference	230

List of Figures

Figure 1.1 Schematics of (a) SUV, (b) LUV, (c) MLV and (d) MVL.....	31
Figure 1.2 Illustration of thin film hydration method	34
Figure 1.3 Illustration of solvent injection technique for the formation of liposomes	36
Figure 1.4 Illustration of reverse phase evaporation method	38
Figure 1.5 Illustration of freeze-drying method	39
Figure 1.6 Schematic of a PEGylated (stealth) liposome	42
Figure. 1.7 Schematics of traditional thermosensitive liposomes (TTSLs), lysolipid-containing thermosensitive liposomes (LTSLs), polymer-modified thermosensitive liposomes (PTSLs), Stealth thermosensitive liposome (Stealth TSL) and Hyperthermia-activated cytotoxic liposome (HaT).	55
Figure 1.8 Illustration of the transition of azobenzene between the trans conformation and the cis conformation.....	66
Figure 1.9 Schematic of dual drug-loaded liposomes.....	71
Figure 1.10 Schematic of MRgFUS. Focused ultrasound can heat tumours rapidly transmitted from skin while protecting neighbouring tissues from damage. Two-dimensional maps of ultrasonic heating are displayed by the application of MR thermometry, providing real-time feedback for focused ultrasonic system. MRgFUS can be used for sustained and controlled heating on tumours to trigger drug release from thermosensitive liposomes [12].	75
Figure 1.11 Structure of chitin and chitosan [148]	76
Figure 2.1 The principle of gel filtration.....	90

Figure 2.2 Photograph of the calcein disodium salt encapsulated liposome gel filtration process	92
Figure 2.3 Photograph of the doxorubicin encapsulated liposome gel filtration process	93
Figure 2.4 Molecular weight ranges for gel filtration media [189]	94
Figure 2.5 Figure demonstrating the surface potential, stern potential, and zeta potential as a function of distance of a spherical particle [190].	96
Figure 2.6 Working principle of TEM [195].....	100
Figure 2.7 The particle size of plain liposome after ultrasonication. Data represents mean $\pm$ SD. n=3 independent batches.....	103
Figure 2.8 The particle size of plain liposomes (■), calcein encapsulated liposomes (●) and doxorubicin encapsulated liposome (▲) after various time of ultrasonication. Data represents mean $\pm$ SD. n=3 independent batches.....	104
Figure 2.9 The effect of pH on the zeta potential of 0.1% (w/w) low MW chitosan (■), 0.1% (w/w) medium MW chitosan (●) and 0.1% (w/w) high MW chitosan (▲). Data represents mean $\pm$ SD. n=3 independent batches.....	105
Figure 2.10 The effect of pH on the zeta potential of DPPC (■) liposomes and Brij S20 (●) micelles. Data represents mean $\pm$ SD. n=3 independent batches.	106
Figure 2.11 The isoelectric point (IP) of 0.1% (w/w) low, medium, high MW chitosan, DPPC and Brij S20. Data represents mean $\pm$ SD. n=3 independent batches.	108
Figure 2.12 The zeta potential of 0.1% (w/w) low, medium, high MW chitosan, DPPC and Brij S20 when the pH value is 7. Data represents mean $\pm$ SD. n=3 independent batches.	109
Figure 2.13 The influence of various molar ratio of lipids on zeta potential of freshly made liposomes (■), liposomes after one month storage at 4 °C (▲) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	110

Figure 2.14 The influence of molar ratio of lipids on liposome size of freshly made liposomes (■) and liposomes after one month storage at 4 °C (▲) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	111
Figure 2.15 Photograph of liposomes solution consisted of different molar ratio of DPPC and Brij S20 in deionised water. Numbers on the vials indicate the molar ratio of DPPC in the liposome composition.	112
Figure 2.16 Close view (a) and distant view (b) of TEM images of freshly made plain HaT liposome. The scale bar of TEM image (a) is 100 nm and that of TEM image (b) is 500 nm.	113
Figure 2.17 Close view (a) and distant view (b) of TEM images of plain HaT liposome after one month storage at 4 °C. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 500 nm.	114
Figure 2.18 Close view (a) and distant view (b) of TEM images of freshly made 0.1% (w/w) low MW chitosan coated HaT liposome. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 500 nm.....	115
Figure 2.19 The distant view (a,b) of TEM images of 0.1% (w/w) low MW chitosan coated HaT liposome after one month storage at 4 °C. The scale bars of TEM images (a,b) are both 500 nm.	115
Figure 2.20 Close view (a) and distant view (b) of TEM images of freshly made 0.1% (w/w) medium MW chitosan coated HaT liposome. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 500 nm.....	117
Figure 2.21 The distant view (a, b) of TEM images of 0.1% (w/w) medium MW chitosan coated HaT liposome after one month storage at 4 °C. The scale bars of TEM images (a,b) are both 500 nm.	117

Figure 2.22 Close view (a) and distant view (b) of TEM images of freshly made 0.1% (w/w) high MW chitosan coated HaT liposome. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 1 μ m.....	118
Figure 2.23 The distant view (a, b) of TEM images of 0.1% (w/w) high MW chitosan coated HaT liposome after one month storage at 4 °C. The scale bars of TEM images (a,b) are both 1 μ m.	119
Figure 2.24 The distant view (a, b) of TEM images of liposomes samples pre-treated by wrong procedure of negative staining. (a) Liposome sample was over stained, and it is difficult to observe details of these kinds of samples. (b) The carbon layer on the copper grid for TEM was fractured due to the wrong procedure. The scale bars of TEM images (a,b) are both 500 nm.	120
Figure 2.25 The zeta potential of freshly made low MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	121
Figure 2.26 The zeta potential of freshly made medium MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	123
Figure 2.27 The zeta potential of freshly made high MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	124
Figure 2.28 The particle sizes of freshly made low MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	125
Figure 2.29 The particle sizes of freshly made medium MW chitosan-coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.....	127

Figure 2.30 The particle sizes of freshly made high MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean ± SD. n=3 independent batches.....	128
Figure 3.1 The photograph of self-assembly dialysis unit consisting of a Spectrum™ Spectra/Por™ Float-A-lyzer™ G2 dialysis device and a 50 ml plastic centrifugal tube	137
Figure 3.2 Calcein fluorescence standard curves in acetic acid solution (pH=3.0) (●) and HEPES buffer solution (pH=7.4) (■) measured by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) (Slit width=1). Data represents mean ± SD. n=3 independent batches....	155
Figure 3.3. Fitted calcein fluorescence standard curves in HEPES buffer solution (pH=7.4) measured by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA). (a) Calcein concentration ranging from 0 g/L to 3.66 E-3 g/L. (b) Calcein concentration ranging from 2.93 E-2 g/L to 7.5 g/L.	158
Figure 3.4 The photograph of calcein in HEPES buffer solution (pH 7.4) with gradient concentration for plotting calcein fluorescence standard curves (the calcein concentration of the following sample was the half of the previous one and the calcein concentration of first sample was 30 g/l).	159
Figure 3.5 Photograph of calcein in acetic acid solution (pH=3.0) with gradient concentration for plotting calcein fluorescence standard curves (the calcein concentration of the following sample was the half of the previous one and the calcein concentration of first sample was 30 g/l). Samples from No. 1-7 (A) and No. 8-14 (B).....	160
Figure 3.6 Effect of initial added calcein in the liposome preparation process on the amounts of encapsulated calcein inside liposomes. Data represents mean ± SD. n=3 independent batches.	162
Figure 3.7 Effect of initial added calcein in the liposome preparation process on the encapsulation efficiency of calcein. Data represents mean ± SD. n=3 independent batches.	163

Figure 3.8 The percentages of encapsulated calcein released from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean ± SD. n=3 independent batches.....	164
Figure 3.9 The percentages of encapsulated calcein released from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean ± SD. n=3 independent batches.....	166
Figure 3.10 The percentages of encapsulated calcein released from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean ± SD. n=3 independent batches.....	168
Figure 3.11 The percentages of encapsulated calcein leaked from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 4 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean ± SD. n=3 independent batches.....	170
Figure 3.12 The percentages of encapsulated calcein leaked from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 25 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean ± SD. n=3 independent batches.....	171
Figure 3.13 Zero-Order, First-Order, Hixson-Crowell, Hopfenberg, Gompertz and Baker-Lonsdale models are utilised to fit calcein release from plain liposomes (■) at 37 °C. Data represents mean ± SD. n=3 independent batches.....	173
Figure 3.14 Higuchi (A), Korsmeyer-Peppas (B) and Weibull (C) models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium	

molecular weight chitosan (●) at 37 °C. Data represents mean ± SD. n=3 independent batches.	174
Figure 3.15 Higuchi (A), Korsmeyer-Peppas (B) and Weibull (C) models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C. Data represents mean ± SD. n=3 independent batches.	177
Figure 3.16 Higuchi (A), Korsmeyer-Peppas (B) and Weibull (C) models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C. Data represents mean ± SD. n=3 independent batches.	179
Figure 3.17 Higuchi, Korsmeyer-Peppas and Weibull models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C (A), 40 °C (B) and 42 °C (C). Data represents mean ± SD. n=3 independent batches.....	181
Figure 4.1 Schematic of doxorubicin hydrochloride [232]	186
Figure 4.2 Doxorubicin fluorescence standard curves in acetic acid solution (pH=3.0) (●) and HEPES buffer solution (pH=7.4) (■) measured by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) (Slit width=4). Data represents mean ± SD. n=3 independent batches.....	194
Figure 4.3. Fitted doxorubicin fluorescence standard curves in Hepes buffer solution (pH=7.4) measured by Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega). (a) Doxorubicin concentration ranging from 2.98 E-7 g/L to 0.019 g/L. (b) Doxorubicin concentration ranging from 0.039 g/L to 5 g/L.	196
Figure 4.4 Effect of initial added doxorubicin in the liposome preparation process on the amounts of encapsulated calcein inside liposomes. Data represents mean ± SD. n=3 independent batches.....	198

Figure 4.5 Effect of initial added doxorubicin in the liposome preparation process on the encapsulation efficiency of doxorubicin. Data represents mean $\pm$ SD. n=3 independent batches.	199
Figure 4.6 The percentages of encapsulated doxorubicin released from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.....	200
Figure 4.7 The percentages of encapsulated doxorubicin released from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.....	202
Figure 4.8 The percentages of encapsulated doxorubicin released from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.....	203
Figure 4.9 The percentages of encapsulated doxorubicin leaked from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 4 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean $\pm$ SD. n=3 independent batches.....	205
Figure 4.10 The percentages of encapsulated doxorubicin leaked from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 25 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean $\pm$ SD. n=3 independent batches.....	206
Figure 4.11 Zero-Order, Hixson-Crowell, Hopfenberg, Gompertz and Baker-Lonsdale models are utilised to fit doxorubicin release from plain liposomes (■) at 37 °C. Data represents mean $\pm$ SD. n=3 independent batches.....	207

Figure 4.12 Higuchi (A), Korsmeyer-Peppas (B), Weibull (C) and First-order (D) models are utilised to fit doxorubicin release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C. Data represents mean ± SD. n=3 independent batches.....	210
Figure 4.13 Higuchi (A), Korsmeyer-Peppas (B), Weibull (C) and First-order (D) models are utilised to fit doxorubicin release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C. Data represents mean ± SD. n=3 independent batches.....	213
Figure 4.14 Higuchi (A), Korsmeyer-Peppas (B), Weibull (C) and First-order (D) models are utilised to fit doxorubicin release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C. Data represents mean ± SD. n=3 independent batches.....	216
Figure 4.15 Higuchi, Korsmeyer-Peppas, Weibull and First-order models are utilised to fit doxorubicin release from plain liposomes (■) at 37 °C (A), 40 °C (B) and 42 °C (C). Data represents mean ± SD. n=3 independent batches.....	218
Figure 4.16 Higuchi, Korsmeyer-Peppas, Weibull and First-order models are utilised to fit doxorubicin release from liposomes coated with 0.1% (w/w) medium molecular weight chitosan (■) at 37 °C (A), 40 °C (B) and 42 °C (C). Data represents mean ± SD. n=3 independent batches.....	220

List of Tables

Table 1.1 Summary of the approved liposomal drugs for cancer therapy by the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA), among which Depocyt®, Marqibo®, and DaunoXome® have been removed from the market [40] [41]. ...	42
Table 1.2 Summary of latest clinical progress of Thermox®	58
Table 2.1 The summary of the resolution and magnification of several microscopes and the naked eye	99
Table 2.2 The summary of merits and restrictions of TEM	101
Table 3.1 The summary of frequently used kinetic models in pharmaceutical research [205], where Q represents the amount of released drug at time t , Q_0 is the initial amount of drug in the formulation or delivery system, k is the release constant.	139
Table 3.2 Interpretation of drug transport and drug release mechanism [210]	147
Table 3.3 The R^2 (COD) of each fitted curves using Higuchi, Korsmeyer-Peppas and Weibull models. Abbreviations: CPL, plain liposomes encapsulated with calcein; CCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with calcein.	181
Table 3.4 The BIC values of each fitted curves using Higuchi, Korsmeyer-Peppas and Weibull models. Abbreviations: CPL, plain liposomes encapsulated with calcein; CCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with calcein.	182
Table 3.5 The RSS of each fitted curves using Higuchi, Korsmeyer-Peppas and Weibull models. Abbreviations: CPL, plain liposomes encapsulated with calcein; CCL, liposomes	

coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with calcein.	183
Table 4.1 The R^2 (COD) of each fitted curves using Higuchi, Korsmeyer-Peppas, Weibull and First-order models. Abbreviations: DPL, plain liposomes encapsulated with doxorubicin; DCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with doxorubicin.....	220
Table 4.2 The BIC value of each fitted curves using Higuchi, Korsmeyer-Peppas, Weibull and First-order models. Abbreviations: DPL, plain liposomes encapsulated with doxorubicin; DCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with doxorubicin.....	221
Table 4.3 The RSS of each fitted curves using Higuchi, Korsmeyer-Peppas, Weibull and First-order models. Abbreviations: DPL, plain liposomes encapsulated with doxorubicin; DCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with doxorubicin.....	222

Chapter 1. Introduction

Bangham et al. first invented a drug delivery system (DDS) which is known as a liposome in 1965 [1]. Liposomes are microbubbles with one or more aqueous chambers wrapped by one or more amphiphilic molecular bilayer membranes. Usually a phospholipid bilayers are considered as one of the most outstanding nanoparticles due to their superior properties [2]. Drugs can be encapsulated, or embedded, in liposomes for delivery in the human body. As drug carriers, liposomes have the characteristics of being non-toxic, harmless, non-immunogenic, can be highly selective, they can exhibit sustained release and show prolonged drug action time, and are easy to degrade *in vivo*. Liposomes can also reduce adverse drug reactions, improve the therapeutic index of drugs, pharmacokinetic properties, and drug distributions *in vivo*. For instance, it can increase the solubility of lipophilic drug in aqueous active solutions, reduce contact between toxic drugs and healthy tissues and allow various chemical molecules to modify on its surface [3]. By coating the liposomal surface with some antibodies, liposome can merely be recognised by specific receptors, achieving targeted delivery. The active releases from liposomes can be triggered by both internal stimuli (e.g. enzyme and change of pH) and external stimuli (e.g. temperature, ultrasound, magnetic field and light) [4].

Hydrophilic drugs can be encapsulated into the aqueous core of liposomes while phospholipid membrane bilayer can incorporate hydrophobic drugs [5]. Compared to intravenous injection of free drugs, liposomes can prolong the half-life of encapsulated drug by protecting the drug from rapid metabolism [6]. Moreover, by reducing the amount of drug within the healthy tissues, the side effects can be minimized [7]. Liposomes can be used as nano-drug carriers by taking advantage of EPR (enhanced permeation and retention) effect to increase the anti-tumour drug concentration in tumour tissues and cells. Due to the

controlled release characteristics of liposomes, drug dosage levels can be reduced, thereby decreasing drug cytotoxicity. The characteristics of liposomes such as particle size, zeta potential and membrane fluidity can be modified by adjusting the lipid formulation [8]. Therefore, liposomes have high value in anti-tumour applications and have been studied intensively by researchers worldwide.

The size of liposomes has a substantial influence on its half-life, and drug encapsulation are affected by the number of bilayers in the liposome and their size. Therefore, liposomes are classified according to the number of lipid bilayers they contain and their sizes. Small unilamellar vesicles (SUVs) have particle size of around 20-80 nm. Due to its small particle size, the encapsulation efficiency is relatively small. Large unilamellar vesicles (LUVs) are large single-layer vesicles with high encapsulation efficiency for water-soluble drugs, and the particle size ranges from 0.1 to 1 μm . Large unilamellar liposomes have large particle sizes, are relatively stable, and have widely applications. Multilamellar bilayer vesicles are called multilamellar liposomes (MLVs), with particle sizes ranging from 1 to 5 μm , which is demonstrated in Figure 1.1 (c). Multilamellar liposomes contain mostly more than five layers, and each bilayer can be loaded with drugs. Multivesicular liposomes (MVLs) are multivesicular and non-concentric liposomes with a particle size of 8-12 μm , as shown in Figure 1.1 (d) [9]. The function is that when the small vesicles rupture, the drugs contained in the vesicles leak out, and the drugs in the remaining vesicles will not be affected, which can achieve the purpose of controlled release.

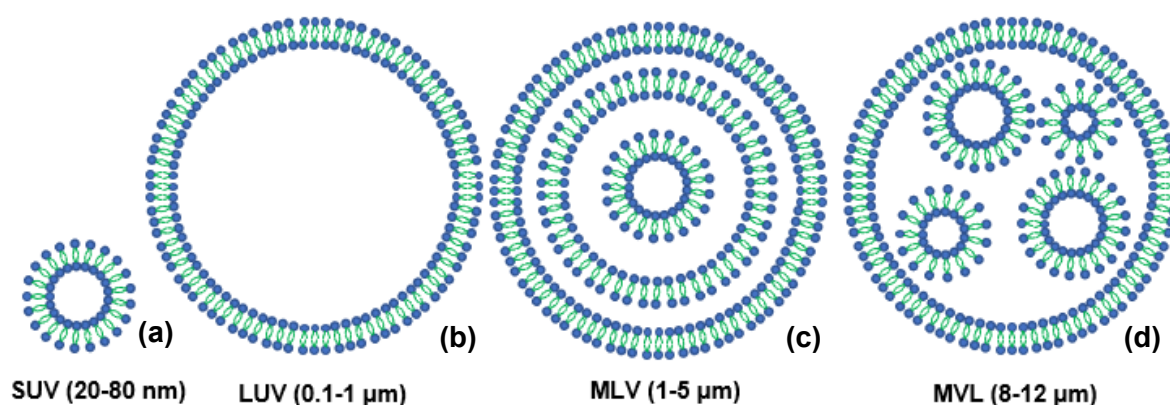


Figure 1.1 Schematics of (a) SUV, (b) LUV, (c) MLV and (d) MVL.

1.1 Commonly used ingredients for liposome preparation

Membrane materials for the preparation of liposomes are mainly lipids and lipoids, including phospholipids and cholesterol. Phospholipids are the constituents of cell membranes with good biocompatibility and low toxicity. Commonly used phospholipid materials can be divided into negatively charged phospholipids (such as phosphatidic acid), neutral phospholipids (such as phosphatidylcholine), and positively charged phospholipids (such as alkylphosphocholine) [10]. Cholesterol, as one of the essential components in biological membranes, is a neutral lipid that can regulate the fluidity and permeability of bilayers and is used to stabilize bilayers. It is more lipophilic than hydrophilic and can self-assemble with phospholipids to form liposome films in organic solvents. Furthermore, the embedment of cholesterol enhances the hydrophobic drug loading efficiency of liposomes. The addition of cholesterol to cationic liposomes makes liposomes an effective gene transfer carrier because it improves the stability of liposomes, reduces its toxicity, and significantly improves its transfection activity [11]. Phospholipids and cholesterol are the basic materials for preparing liposomes. For various functionalized liposomes, some pharmaceutical excipients can be added to the membrane material according to research design or therapeutic use.

Liposome materials are not heat-resistant and undergo a phase transition with temperature, and the temperature at phase transition is called the transformation temperature (T_m). The physical properties of liposomes also change dramatically with changes in temperature. When the temperature is lower than the phase transition temperature, the liposome is in a colloidal state, the structure is relatively stable, the permeability is small, and the acyl side chains are arranged in an orderly state; when the temperature is higher than the phase transition temperature, the liposome lipids are in fluid or liquid crystalline state, the structure tends to be unstable, the mobility increases, the permeability increases, and the acyl side chains change from an ordered arrangement to a disordered one [12]. The temperatures of the tumours (37.2-38.4°C) [13] are higher than those of normal tissues (36.5-37.5°C) [14], so the liposomes at the tumour site can undergo a phase transition due to the increase in temperature, releasing the loaded anti-tumour drugs, thereby enhancing the anti-tumour activity.

1.2 Basic approaches and principles of liposome preparation

At present, there are many preparation methods for liposomes. Conventional methods include thin film dispersion method [15], reverse evaporation method [16], ethanol/ether injection method [17] [18], surfactant removal method [19], pH gradient method [20], emulsification method [21], ammonium sulphate gradient method [22]. Although the principles of the above-mentioned methods are different, the common point is that the lipids need to be distributed in the organic solvent first, and then dispersed in the water phase. In addition to the above conventional methods for the preparation of liposomes, new technologies such as freeze-drying [23], aerosol carrier [24] and hot-melt approaches [25] can also be used.

A liposome formulation with superior quality should have excellent biocompatibility, targeting ability and stability. Moreover, it should be able to decrease the elimination rate of drugs *in*

vivo and reduce the drug toxicity. Nevertheless, liposome formulations commonly have some defects like weak stability and low encapsulation efficiency. This can be modified by selecting appropriate liposomal structure and size, which can be controlled by the preparation methods. During the liposomal preparation process, multiple preparation approaches can be selected or improved based on the actual circumstances in order to achieve the desired effect.

1.2.1 Thin film hydration method

The thin film hydration method is also known as the thin film evaporation method. This method is mainly applied for the preparation of MLVs or LUVs, and is a simple and commonly used preparation method [15]. Lipids are firstly dissolved in chloroform or another suitable organic solvent. Rotary evaporator with reduced pressure is utilized to remove organic solvent. A thin lipid film is formed on the inner surface of a round bottom flask. MLVs with various particle sizes can be obtained by adding buffer solutions into the round bottom flask to hydrate the thin film. Finally, ultrasonication can be used to transfer MLVs into SUVs with reasonably homogeneous particle sizes. Liposome size reduction techniques such as film oscillation dispersion method and extrusion through polycarbonate filters. can also be used to homogenize particle sizes, turning MLVs into LUVs or SUVs. The method is suitable for the encapsulation of both hydrophobic drugs and hydrophilic drugs. The former has a high drug encapsulation efficiency, while the latter has a relatively lower encapsulation rate [26] [27].

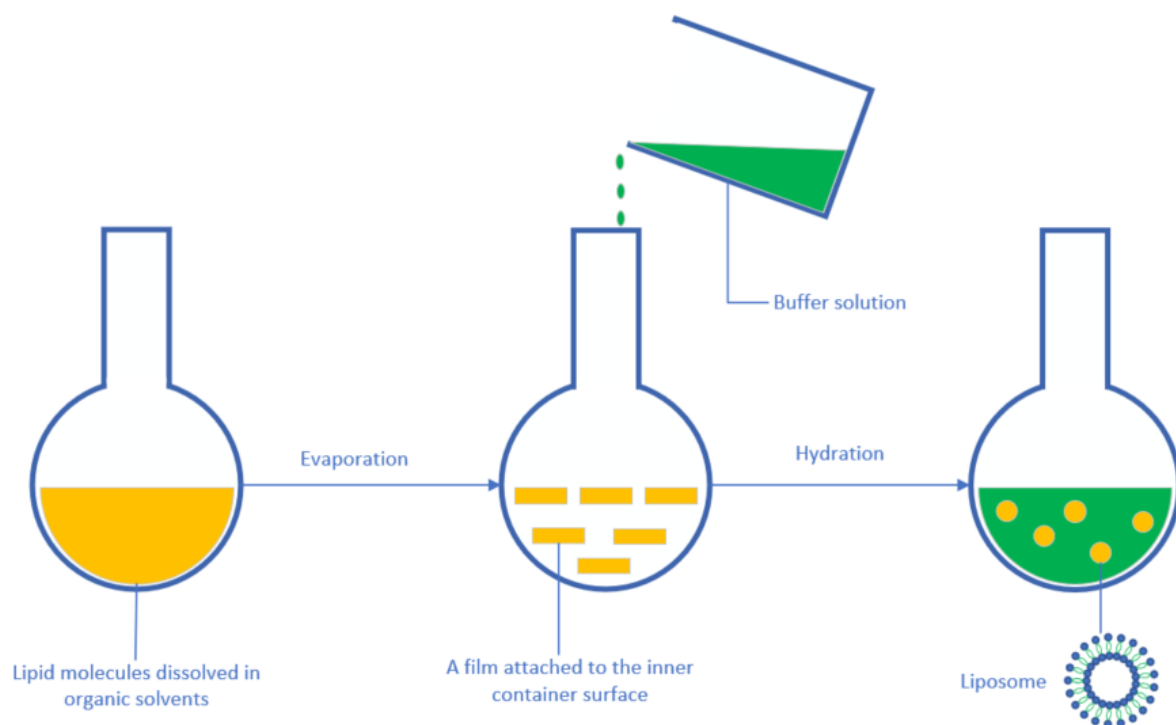


Figure 1.2 Illustration of thin film hydration method

1.2.2 Solvent injection techniques

Solvent injection method can also be used in the preparation of liposomes. The method can be divided into the ethanol injection method and the ether injection method, and are mainly applied to unilamellar liposomes preparation [28]. In this method, lipids dissolved in organic solvents (ethanol or ether) are rapidly injected into an aqueous medium to form the liposomes.

Ethanol injection is a preparation method developed by Batzri and Korn in 1973. In this method, lipids are mixed with ethanol or another hydrophilic organic solvent. Then the mixture is slowly injected into water or buffer solution with mixing. Liposomes are formed when the organic solvents concentration is decreased. Nevertheless, some organic solvent inevitably remains in the liposome solution. Hence, dialysis is necessary to remove the residual organic solvent, which results in nonuniformity of liposome size. The second

problem is that some lipids are not easily soluble in ethanol, and ethanol may cause some drugs to denature and lose efficacy [17] [29]. A variety of factors can affect the production of liposomes by ethanol injection, such as: stirring speed, injection speed, ethanol volume, lipid concentration, water phase osmotic pressure [30].

Ether injection method is a commonly used solvent injection method, which is similar in principle to ethanol injection. Although diethyl ether is not miscible with water, it is a better solvent for many lipids than ethanol. The lipid solution in diethyl ether is injected into a buffer solution at 55~60 °C. With the evaporation of diethyl ether, liposomes with small particle size are formed. The final lipid concentration is not limited in this method, but the concentration of injected lipid needs to be accurate. Although this method still has the problem of residual organic solvent, but compared with the ethanol injection method, the residual solvent is less, due to the lower boiling point of diethyl ether. However, this method also has limitations. The biological toxicity of ether is relatively high, and there are potential safety problems for the researchers [18].

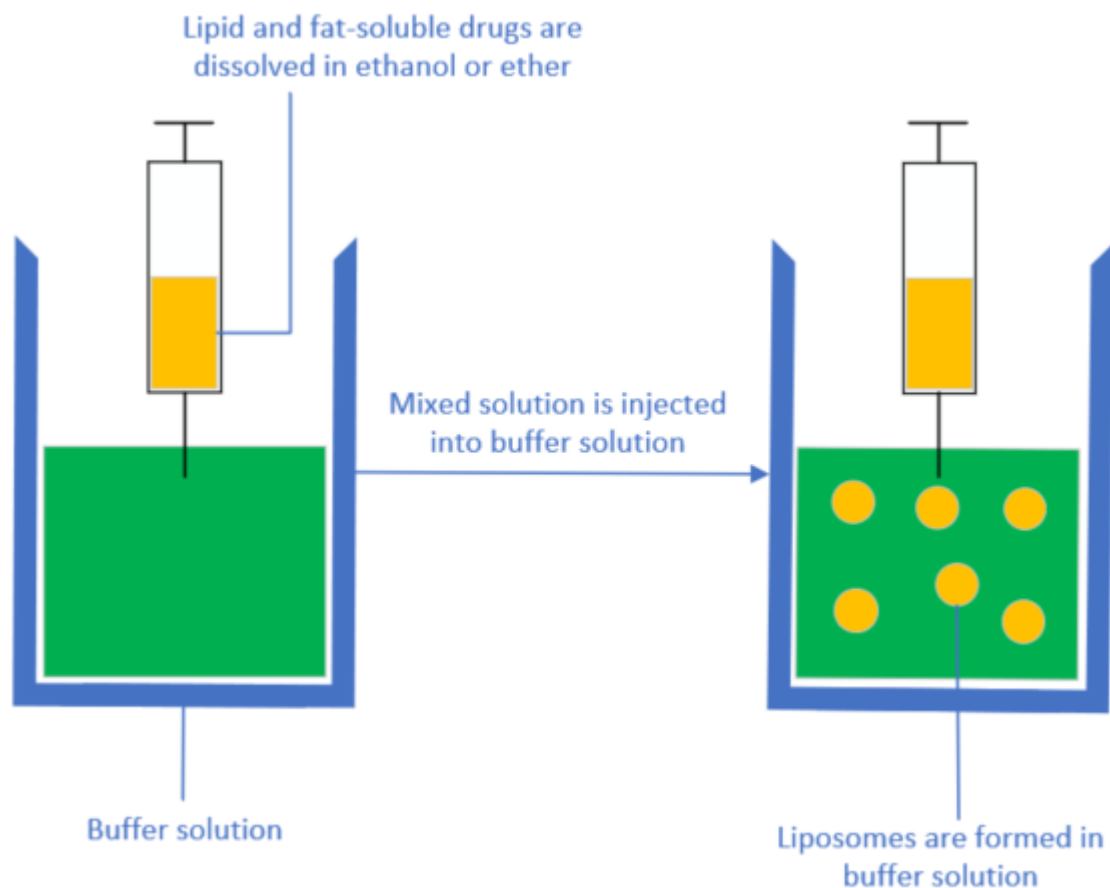


Figure 1.3 Illustration of solvent injection technique for the formation of liposomes

1.2.3 Reverse phase evaporation method (REV)

The first two methods described in this chapter have better encapsulation efficiency for lipid-soluble drugs than water-soluble drugs in most cases and are easy to form MLV (>500 nm). Although SUVs (20-100 nm) can be acquired by methods such as sonication or membrane extrusion, the volume of the aqueous phase contained within the liposome is very small. The reverse phase evaporation method (REV) was first proposed by Szoka and Papahadjopoulos in their 1978 paper and is mostly used in the preparation of LUVs. To be specific, firstly, lipids are dissolved into an organic solvent, then buffer solution with dissolved drug is added into it. After ultrasonication, a stable W/O emulsion is formed. Next,

the organic solvent is removed from the emulsion by decreasing the pressure. Buffer solution is added again to dissolve any lipid gel attached on the inner surface of the container. A much higher aqueous volume to lipid ratio can be achieved by using this method compared to other preparation procedures [16]. Although the reverse evaporation method can better retain the water phase, it also has certain other limitations. Drugs dissolved in the aqueous phase will cause direct contact with organic solvents and the ultrasonication makes the method unsuitable for encapsulating fragile molecules such as peptides. In the preparation process, it is easy for organic solvents to remain, which may interact with lipids and drugs [30].

If supercritical CO₂ is utilised to replace the organic solvent, the problem of the residual organic solvent can be overcome. In that case, a new approach is developed, which is called the supercritical reverse evaporation method. Otake et al. conducted further research on the basis of the supercritical reverse evaporation method, and using dioleoyl phosphatidylcholine as the membrane material, successfully prepared glucose encapsulated liposomes with improved encapsulation efficiency [31]. However, this method requires supercritical conditions for the CO₂, particularly high pressures and this limits the applicability of the method.

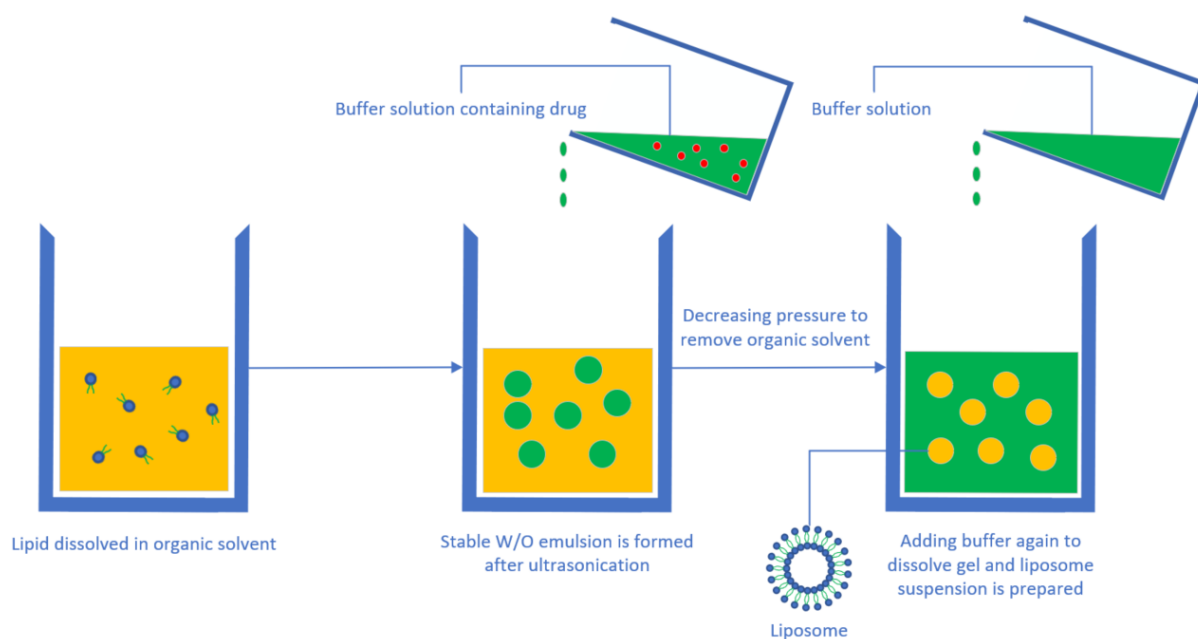


Figure 1.4 Illustration of reverse phase evaporation method

1.2.4 Freeze-drying method

Freeze-drying is mainly used for the production of sterile and submicron small-sized liposomes encapsulated with drug that is sensitive to temperature and is sometimes used for liposome storage. Lipids firstly are dispersed in an aqueous phase. After the freeze-drying procedure, the resulting product is added into the aqueous media which contains the dissolved drug to acquire liposomes. This technology can keep liposomes in a frozen and dry state, which prolongs their shelf life and is more convenient for transportation. Therefore, it has great potential as a solution to the difficulty of long-term storage of liposomes, but sometimes leads to leakage of encapsulated substances during freezing process. In this method, the selection of a suitable freeze protection liquid, which will prevent ice crystal formation, is of great importance to diminish the possible harm to liposomes during the freeze-drying procedure. The most commonly used freeze protection liquid are sugars such as glucose, lactose, mannitol, trehalose [23].

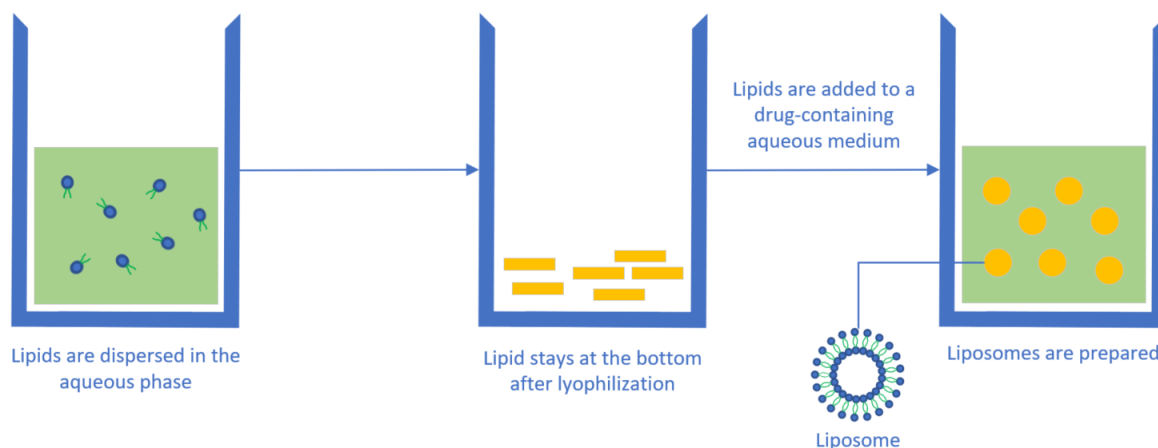


Figure 1.5 Illustration of freeze-drying method

1.2.5 Freeze thaw method

The freeze thaw method is suitable for the preparation of LUVs. Lipids are dissolved into organic solvents before being removed in a rotary evaporator. Then the aqueous solution is added to dissolve the thin film of lipid on the walls of the round bottom flask, forming LUVs. SUVs are obtained by application of ultrasonication. After the addition of drug, the liposome suspension is frozen and thawed repeatedly to form LUVs. The particle sizes of liposomes prepared by this method is not homogeneous, but extrusion can be applied to reduce their diameter and prepare uniform sized liposomes. The most prominent feature of freezing thawing method is its high encapsulation efficiency on account of the high aqueous volume to lipid ratio [32].

1.3 Developments in liposomes

1.3.1 Stealth liposomes (passive targeted, long circulating liposome)

Stealth liposomes are also called long-circulating liposomes or passive targeted liposomes. The early stealth technology refers to incorporating a PEGylated derivative of 1,2-distearoyl-sn-glycero-3-PE (DSPE-mPEG2000) on the surface of traditional liposomes to make it staggered and overlapped to form a steric hindrance. Because liposomes are easily taken up by the reticuloendothelial system (RES), drugs encapsulated in liposomes are found enriching in liver and spleen, rather than their desired site of action. The flexibility and hydrophilicity of this derivative can interfere with the hydrophobic interaction between liposomes and plasma proteins, thereby reducing the absorption of liposomes in the RES. Therefore, the stealth effect and long-circulation function of liposomes in the body are realized. PEGylated liposomes extends circulation time *in vivo* dramatically, increasing amount of drugs concentrating in the target area and, as a consequence, reducing toxicity and side effects [33]. Furthermore, due to the leaky nature of tumour vasculature, nano formulations with long-circulation properties are able to passively target tumour tissue through enhanced permeation and retention (EPR) effects [34].

Anthracyclines (ANT) is a class of aromatic polyketide compound with antitumor effect which was discovered in 1970s, including doxorubicin (DOX), daunorubicin (DNR), epirubicin, aclarubicin, mitoxantrone, pirarubicin and idarubicin. Nevertheless, the dosage of ANT is strictly limited due to its strong cardiotoxicity, especially for senile patients and patients with serious complications like diabetes and coronary heart disease. Encapsulation of ANT in liposomes can reduce cardiotoxicity significantly in the treatment of cancer, and encapsulation efficiency is improved as well [35]. Currently, liposomes encapsulated with DNR and DOX are commonly used in the market.

The world's first anti-tumour liposome product, Doxil® (Doxorubicin liposome injection), was developed using stealth technology. The FDA has approved the application of Doxil® in the treatment of ovarian cancer and Kaposi sarcoma [36]. After the surface modification of PEG, Doxil® presents favourable results in terms of pharmacology, including remarkable extension of circulation time *in vivo*, reduction of the elimination rate by the immune system and low toxicity and adverse effects [37]. The elimination half-life of free doxorubicin is 0.2 h and the AUC is 3.81 µg·h/ mL, while the elimination half-life of Doxil® can reach 41~70 h, and the AUC is 902 µg·h/ mL. At the same time, clinical studies have shown that it can reduce the release of drugs in the myocardium to reduce the cardiotoxicity of doxorubicin [38]. Since then, Sun Pharma has also used Stealth technology to develop Lipodox®, a generic version of Doxil®, Whereas, some other side reactions were discovered and need to be further studied in ANT encapsulated liposomes, such as the hand-foot syndrome, dysesthesia [39].

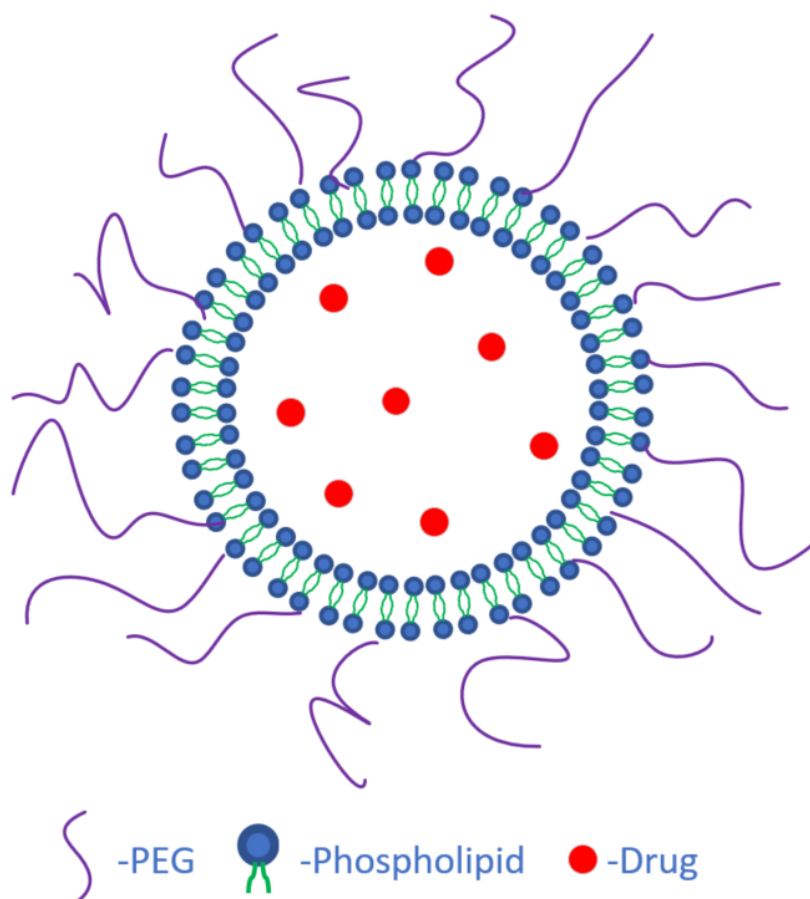


Figure 1.6 Schematic of a PEGylated (stealth) liposome

Table 1.1 Summary of the approved liposomal drugs for cancer therapy by the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA), among which Depocyt®, Marqibo®, and DaunoXome® have been removed from the market [40] [41].

Trade name	Active ingredient	Lipids composition	Company	Approval time
Doxil®	Doxorubicin	HSPC, DSPE-PEG, Cholesterol	Sequus	1995
Myocet®	Doxorubicin	EPC, Cholesterol	Elan	2000
Lipodox®	Doxorubicin	HSPC, DSPE-PEG, Cholesterol	SunPharm	2013

DaunoXome®	Daunorubicin	DSPC, Cholesterol	NeXstar	1996
Mepact®	Mifamurtide	POPC, DOPS	Takeda	2009
Marqibo®	Vincristine	Sphingomyelin, Cholesterol	Talon	2012
Onivyde®	Irinotecan	DSPC, DSPE-PEG, Cholesterol	Merrimack	2015
Vyxeos®	Cytarabine/ Daunorubicin	DSPC, DSPG, Cholesterol	Jazz	2017
DepoCyt®	Cytarabine	DOPC, DPPG, Cholesterol	Pacira	1999

1.3.2 Active targeted liposome

The current clinical application of liposome injections faces many challenges. First, the clinical heterogeneity of the EPR effect leads to massive variability in the accumulation of liposomes in tumours. Second, the physical barrier generated by the tumour's extracellular matrix limits the penetration of liposomes from the perivascular space of the tumour. Third, although the accumulation of liposomal drugs in the tumour is dramatically increased, the amount of drug that can be released from liposomes is restricted, leading to limited therapeutic efficacy. Therefore, in order to more effectively regulate the enrichment and release of liposome drugs at the lesion site, many new liposomes have been developed, such as active targeted liposome, which means the liposome modified by targeted material, making it target the lesion in a directional manner.

1.3.2.1 Receptor targeted liposome

Receptor targeted liposomes are liposomes modified with ligands with specific receptors. Hence, they can be selectively taken up by cells overexpressing the corresponding receptors, thereby enhancing drug accumulation at the lesion site, and reducing off-target toxicity to improve therapeutic efficacy. The most commonly used receptors are folic acid receptors, transferrin receptors, carbohydrate receptors, asialoglycoprotein receptors, glycyrrhetic acid receptors and polypeptide receptors.

1.3.2.1.1 Transferrin-modified targeted liposomes

Transferrin (TF) is a non-heme-binding iron β -globulin involved in iron absorption and cell growth. Transferrin is widely distributed in the body fluids and cells of vertebrates. It is mainly divided into serum transferrin, lactotransferrin and ovotransferrin. Among them, serum transferrin is the most abundant and widely distributed [42]. Transferrin not only participates in iron transport and metabolism, immune regulation, respiration, cell proliferation, but also maintains the balance of iron ions and energy and has antibacterial and bactericidal effects. The main physiological function is to transport iron ions from the site of absorption and storage to erythroblasts to provide raw materials for the synthesis of haemoglobin, and transport iron to the site where iron is needed [43].

Studies have shown that the transferrin receptor (TFR) is widely expressed in the human body, of which primitive red blood cells, brain cells, brain capillary endothelial cells, tumour cells, activated lymphocytes and fibroblasts are highly expressed. The TFR on the surface of tumour cells is 2-7 times that of normal cells, and the affinity between TFR and TF on the

surface of tumour cells is 10-100 times that of normal cells [44]. Transferrin can regulate the amount of intracellular iron. Malignant tumour cells have a great demand for iron, so there are many transferrin receptors on the cell surface. Various common cancers such as bladder cancer, breast cancer, lung cancer, liver cancer and skin cancer are closely related to TF. Clinically, the detection of TFR and TF is helpful for the early diagnosis and identification of tumours. TFR monoclonal antibodies can also directly act on tumour cells to inhibit the growth of tumour cells. TFR can also play a positive role in tumour gene therapy and can effectively improve the transfection efficiency [45].

Glioblastoma (GBM) is one of the most common and aggressive types of brain tumour, which recurs frequently even after full-scale treatment, such as surgery, chemotherapy, and radiation therapy. The typical survival time of GBM after diagnosis is 12 -15 months. Resveratrol (RES), as a natural polyphenol, has an excellent preventive effect on tumours, but its poor physicochemical properties constrain its further applications as a free drug. Numerous researchers have studied RES loaded liposomes or liposomes encapsulated with RES and other drugs such as curcumin, paclitaxel, 5-FU or doxorubicin for treatments of multiple kinds of cancers. Jhaveri et al. loaded RES into transferrin-modified PEGylated liposomes (Tf-RES-L) for the treatment of GBM owing to the superior characteristics of RES-L such as stability, long drug release time *in vitro*, excellent drug loading capacity, and easy expansion. The therapeutic effect of Tf-RES-L was studied in a subcutaneous xenograft mouse model of GBM. The results displayed that Tf-RES-L was more cytotoxic to cancer cells, inhibited tumour growth more efficaciously and increased more mouse survival rate, compared to free RES or RES-L. The biodistribution *in vivo*, pharmacokinetics (PK) properties and the ability to cross the blood brain barrier (BBB) were not investigated in this study, and thus further studies are needed to fully characterize this novel formulation [46].

Osthole (Ost), a coumarin compound, is a potential drug for the treatment of Alzheimer's

disease (AD). However, this drug has properties such as low solubility, low permeability to the blood-brain barrier, and low bioavailability. To expand the clinical availability of this drug, Kong et al. prepared transferrin-modified Ost encapsulated liposomes (Tf-Ost-Lip). The results indicated that this preparation can considerably enhance the permeability of OST to the blood-brain barrier, protect cells from neurotoxicity, and inhibit neuroinflammation, oxidative stress and apoptosis in mice. In addition, the pharmacokinetics and *in vivo* pharmacodynamics results displayed that Tf-Ost-Lip has the function of targeting, long circulation, and sustained release. The remarkable effectiveness of Tf-Ost-Lip against AD both *in vivo* and *in vitro* may be due to two following reasons. First, Tf-modified liposomes can facilitate the permeability of the blood-brain barrier owing to the endocytic mechanism. Second, the presence of hydrophilic substances in liposomes makes it difficult for the reticuloendothelial system to absorb them, hence the half-life of OST *in vivo* is noticeably prolonged [47].

1.3.2.1.2 Folic acid-modified targeted liposomes

Folic acid, as one of the water-soluble vitamin complexes, is a coenzyme of the one-carbon unit transferase system. It plays a key role in promoting cell growth and division. It can promote the maturation of megaloblastic cells in the bone marrow, which cannot be synthesized by body but can only be obtained from the diet. If the human body lacks folic acid, it can cause symptoms such as megaloblastic anaemia. Moreover, folic acid deficiency is positively correlated with the risk of tumour incidence, and folic acid has a dual regulatory effect on the occurrence of tumours. Because folic acid is essential for gene replication, highly expressed tumour cells have more folic acid receptors than normal cells on the surface to take up more folic acid. Therefore, folic acid receptors can be used as the target of folic acid-modified liposomes. Moreover, folic acid has the advantages of low immunogenicity, low price, good affinity with cells, and high stability [48].

The intake and absorption of folic acid by the body are mainly through two pathways: one is to transport folic acid into cells through the transmembrane folate transporter with low affinity; the other is through the folic acid receptor with high affinity to mediate the uptake of folic acid and internalize cells, and the design mechanism of folate ligand-conjugated targeted drug formulations is mainly through the latter pathway [49].

Bleomycin (BLM), as an antitumor antibiotic, has some characteristics such as serious side effects and low half-life *in vivo* that constrains its potential clinical usage. Using the thin film hydration technique, Chiani et al. successfully prepared folate receptor (FR) targeted liposome encapsulated with BLM (FL-BLM). Experimental results demonstrated that FL-BLM significantly improved the anticancer effect and cellular uptake of BLM in HeLa cells of human cervix carcinoma compared to free BLM and free folate form BLM (L-BLM). Annexin-V analysis and flow cytometry revealed that cell cycle arrest was ameliorated at G2/M phase by using FL-BLM. Besides, HeLa cells took in more FL-BLM than human breast carcinoma MCF-7 cells. In summary, this study demonstrated that FL-BLM can be regarded as a promising novel formulation for targeted drug delivery to promote the efficacy of cancer therapy [50].

Lohade et al. used aminobenzylamine as the linker to bind the folic acid ligand to cholesterol, and then incorporated the cholesterol into the liposome formula to prepare folic acid-modified liposomes encapsulated with doxorubicin. This liposome exhibited high selectivity for tumours, thus increasing cytotoxicity to tumour cells. Biodistribution studies and fluorescence analysis of B16F10 melanoma cells confirmed that folate modified poloxamer-coated liposomes (FPL) had substantially higher tumour cellular uptake than conventional poloxamer-coated liposomes (PL). Besides, FPL revealed superior ability of containing tumour growth compared to PL in B16F10 melanoma tumour-bearing mice [51].

1.3.2.1.3 Peptide-modified targeted liposomes

Polypeptide-modified liposomes are another type of targeted delivery liposomes. The application of polypeptides as modifiers has the advantages of no immunogenicity, good biocompatibility, low toxicity, and easy transformation and optimization. Commonly used polypeptides include vasoactive intestinal polypeptide (VIP), glycine-arginine-glycine-aspartic-serine polypeptides (GRGDS), arginine-glycine-aspartic acid polypeptide (RGD), asparagine-glycine-arginine polypeptides (NGR), and alanine-proline-arginine-proline-glycine polypeptide (APRPG) [52].

Vemurafenib, a FDA-approved drug for the treatment of melanoma, is usually administered orally, which has limited its anti-tumour efficacy, is not easily absorbed by the human body, and is prone to serious side effects. To overcome these shortcomings, Zou et al. successfully developed a peptide-modified vemurafenib-loaded liposome (Vem-TD-Lip) for transdermal administration. These nanoliposome are negatively charged and have over 95% vemurafenib encapsulation efficiency. *In vitro* results exhibited that Vem-TD-Lip can successfully penetrate into cells and be taken up by cells with limited toxicity to healthy cells but has a considerable inhibitory effect on melanoma cells. *In vivo* experiments confirmed that transdermal administration of Vem-TD-Lip was more efficacious in constraining melanoma in mice than by intravenous and oral administration, while reducing damage to healthy tissues and organs such as liver, kidney and lung [53].

Apatinib, as a tyrosine kinase inhibitor, which has the ability to treat a variety of cancers. In one study, cyclic arginylglycylaspartic acid (cRGD) and polyethylene glycol (PEG) were used to modify liposomes (cRGD-Lipo-PEG) for delivering apatinib to human colon cancer tissue.

cRGD-Lipo-PE displayed excellent colloidal stability, high drug encapsulation efficiency, suitable particle size, negatively charged zeta potential and controlled drug release behavior after evaluation *in vitro*. Compared with PEG-modified liposomes (Lipo-PEG), cRGD-Lipo-PEG exhibited a higher intracellular uptake rate leading to a better antitumour effect and tumour cell apoptosis. The *in vivo* results demonstrated that, after intravenous or oral administration, cRGD-Lipo-PEG indicated more noticeable targeting ability, better drug distribution, lower side effects and better inhibitory effect on colon cancer in mice than free apatinib and apatinib loaded Lipo-PEG [54].

1.3.2.1.4 Glycosyl-modified targeted liposomes

Sugars are one of the vital energy sources for the human body and is also a bioinformatic molecule that plays an indispensable role in many physiological processes. Glycosylation can be used for therapy and diagnosis through carbohydrate and receptor recognition. However, glycosylation has been proved to affect the physiological activities of organs by targeting, so the safety is worthy of further verification, and more research is needed for large-scale application in the clinic. Glucose transporter subtype I (GLUT1) and lectin receptor are regarded as the most commonly investigated glycosyl receptors. Glycosylation can be used for targeted transport to liver, brain, and lung. Drug delivery system modified with glucose, galactose and mannose have been shown to ameliorate drug distribution *in vivo* and increase cell uptake, leading to a substantial rise of drug accumulation in target areas. There are still many problems in the targeted drug delivery of glycosylation, and researchers need to conduct more research in the future to expand its clinical application and translation [55].

Glycosyl-modified targeted liposomes have become more and more widely used in recent years [56]. Lidocaine hydrochloride (LID) is an amide local anaesthetic and is a commonly used local anaesthetic in clinical practice. LID takes effect rapidly and stably, but its transdermal absorption effect is poor. The particle size of glycosyl-modified targeted liposomes is smaller than that of ordinary liposomes, and it has more positive charges, which is suitable for human epidermal administration. Using glycosyl-modified targeted liposomes to load lidocaine hydrochloride can significantly improve the transdermal absorption efficiency of LID and increase its permeability [57].

1.3.2.1.5 Glycyrrhetic acid-modified targeted liposomes

Glycyrrhetic acid (GA) is an active ingredient in traditional Chinese medicine liquorice, which has excellent anti-inflammatory, anti-viral and anti-tumour effects. The most common location of liver cancer are the liver parenchyma cells. Studies have shown that there are a lot of GA receptors on the surface of liver parenchyma cells. Therefore, glycyrrhetic acid-modified liposomes can target liver parenchymal cells and exert anti-tumour effects [58].

In one study, glycyrrhetic acid (GA) was used to modify oxaliplatin (OX) loaded liposomes which were prepared by using a thin-film dispersion technique. It can be observed from transmission electron microscopy (TEM) images that OX-liposomes and GA-OX-liposomes have similar particle sizes (90 nm) and are uniformly dispersed as spherical particles. Both have extremely high encapsulation rates (around 94%) and are both negatively charged. *In vivo* and *in vitro* experiments demonstrated that OX-liposomes could substantially prolong the release time of OX compared with free OX, and the GA modification on the liposome surface did not considerably affect its release characteristics. In addition, the release profiles of OX-liposomes fitted the Weibull model. By analysing the drug concentration distribution in various

tissues, it can be found that GA-OX-liposomes are mainly absorbed by the liver after intravenous administration. Finally, in histological studies, GA-OX-liposomes did not give rise to severe side effects such as epithelial cell shedding or necrosis [59].

In another study, for the treatment of liver cancer, one of the most common lethal cancers worldwide, a novel glycyrrhetic acid (GA)-modified Wogonin (WG) encapsulated liposome (GA-WG-Lip) was developed. It was discovered that the modification of GA on the liposome did not affect the high encapsulation efficiency of Wogonin loaded liposome (WG-Lip) itself. Compared with WG-Lip and free WG, GA-WG-Lip exhibited more and faster accumulation and distribution in the liver, longer retention time, and higher tumour inhibition rate. The results revealed that the IC₅₀ value of GA-WG-Lip was 1.46 times higher than that of WG-Lip. This is probably because of the raised receptor-mediated uptake of liposomes in liver tissues. Therefore, glycyrrhetic acid (GA)-modified liposomes can be used as a potential targeted therapy for liver cancer, which deserves more research in the future [60].

1.3.2.2 Mitochondria-targeted liposomes

Many studies have found that mitochondria are involved in the production of reactive oxygen species (ROS), which can regulate apoptosis, and in important processes such as electron transport systems and adenosine triphosphate (ATP) synthesis *in vivo*. Mitochondrial damage disrupts mitochondrial function, leading to the gradual accumulation of defective organelles, and cellular and tissue damage. Therefore, abnormal mitochondrial metabolism in cells may result in the occurrence of many diseases, such as diabetes, Parkinson's disease, Alzheimer's disease, and cancer [61] [62] [63]. Mitochondrial lesions can easily lead to cell cancerization, and tumour metastasis is closely related to mitochondria, so mitochondria are an important research project for anti-tumour. Malignant tumour cells have

the characteristics of rapid division, and division requires a lot of energy, so the content of mitochondria in tumour cells is much higher than that in normal cells. Studies have found that many characteristics of tumour cells, including unlimited proliferative capacity, impaired apoptotic mechanisms, insensitivity to inhibitory growth signals, enhanced anabolism, and reduced autophagy, are associated with impaired mitochondrial function [64]. Since mitochondria play an important role in apoptosis-mediated cell death and tumour cells inhibit apoptosis, mitochondria-targeted induction of apoptosis is expected to be an effective treatment for tumour suppression. In recent years, mitochondria have been used as drug targeting sites and relevant research is also being carried out gradually [65].

Owing to the complex intracellular environment in which mitochondria are located, it has been a great challenge for researchers to target drugs to mitochondria in cells. Wang et al. successfully developed mitochondria-targeted liposomes composed of guanidinium-based dendritic lipopeptide (DLP), which have stronger cell penetration and be able to accumulate more drugs in tumour tissue. It can deliver 3.5 times more drug to intracellular mitochondria compared to current triphenylphosphonium (TPP)-based mitochondria-targeted liposomes. Because mitochondria are extremely sensitive to temperature and reactive oxygen species, photodynamic and photothermal therapy are combined with DLP liposomes for the cancer treatment. Results revealed that this combined therapy can completely eradicate breast tumour cells in mice [66].

At present, the application of active targeting liposomes mostly adopts combined modification methods such as dual ligands, ligand-antibody. This method can significantly improve the aggregation of drugs at pathological sites, and enhance the therapeutic index of drugs, which has obvious anti-tumour advantages [67].

1.3.2.3 Antibody-targeted liposomes

Antibody-targeted liposomes, also known as immunoliposomes (ILs), are a class of liposomes that use antibodies to specifically bind to antigens to improve the binding rate to tumours. Antibodies have low toxicity, high affinity, strong specificity, and easy modification, which are an excellent class of drug carrier modifiers. Antibodies have been paid more and more attention in various therapeutic fields [68].

Antibody-targeted liposomes are mainly used for modification of monoclonal antibody (MAB), fragment of antigen binding (Fab'), single-chain fragment variable (scFv). Monoclonal antibodies are more specific than polyclonal antibodies, so they are more widely used, but monoclonal antibodies are biological macromolecules with poor permeability. Compared with monoclonal antibodies, Fab' shows good penetration, and it can reduce immunogenicity of antibody and elimination rate [69].

In addition, a new therapeutic approach was discovered in the latest research, which is eliciting an immune response to modulate the inhibition of tumour growth by acting on the tumour microenvironment (TME). Liposomes are currently an indispensable tool in cancer therapy, introducing them into TME modulators and cancer-related immune responses, inducing immunogenic cell death (ICD). Liposomes can overcome formulation difficulties and reduce drug toxicity. The ability to enhance the distribution of antitumor drugs to tumour growth sites makes liposomes ideal candidates as a formulation strategy for modulating the TME. At the same time, the use of both antibodies and ligands to modify liposomes can produce synergistic effects and increase the amount of drugs reaching the tumour area [70].

1.3.3 Environment-responsive liposomes

1.3.3.1 Thermosensitive liposome

In view of the heat-labile physicochemical properties of liposomes, special release mechanism for liposomes have been developed, which is thermosensitive liposome. 37 °C is recognised as the optimal temperature for chemical reactions in cells of mammals while 43-45 °C is defined as hyperthermia [71]. The combination of hyperthermia and thermosensitive liposomes (TSLs) can enhance curative efficacy by increasing blood flow volume at heated area, rising permeability of tumour vessels for more liposome accumulation in tumour and remaining same cytotoxicity to cancer while reducing harm to healthy cells [72]. The blood circulation around human tumour tissue is not smooth, and sometimes there is tissue oedema around tumour, which result in its poor heat dissipation, so the temperature around human tumour tissue is slightly higher than that of other human tissues. Studies have shown that tumour cells will be damaged at 42-45 °C, while normal tissue will not be damaged even if the temperature reaches 45 °C because the heat dissipation of normal tissue is better than that of tumour tissue. Therefore, hyperthermia can also be used as a conventional tumour treatment method, which is also beneficial to targeted therapy for temperature-sensitive liposomes. Common hyperthermia approaches include near-infrared hyperthermia, radio-frequency hyperthermia, microwave hyperthermia, and hyperthermia by alternating magnetic fields [73]. Doxorubicin can play a better therapeutic effect in high temperature tissues or cells, which is one of the reasons why doxorubicin has a good effect on tumour cells and tissues [74]. TSLs can be categorised into five main types: traditional thermosensitive liposomes (TTSLs), lysolipid-containing thermosensitive liposomes (LTSLs), polymer-modified thermosensitive liposomes (PTSLs), Stealth thermosensitive liposome (Stealth TSL) and Hyperthermia-activated cytotoxic liposome (HaT).

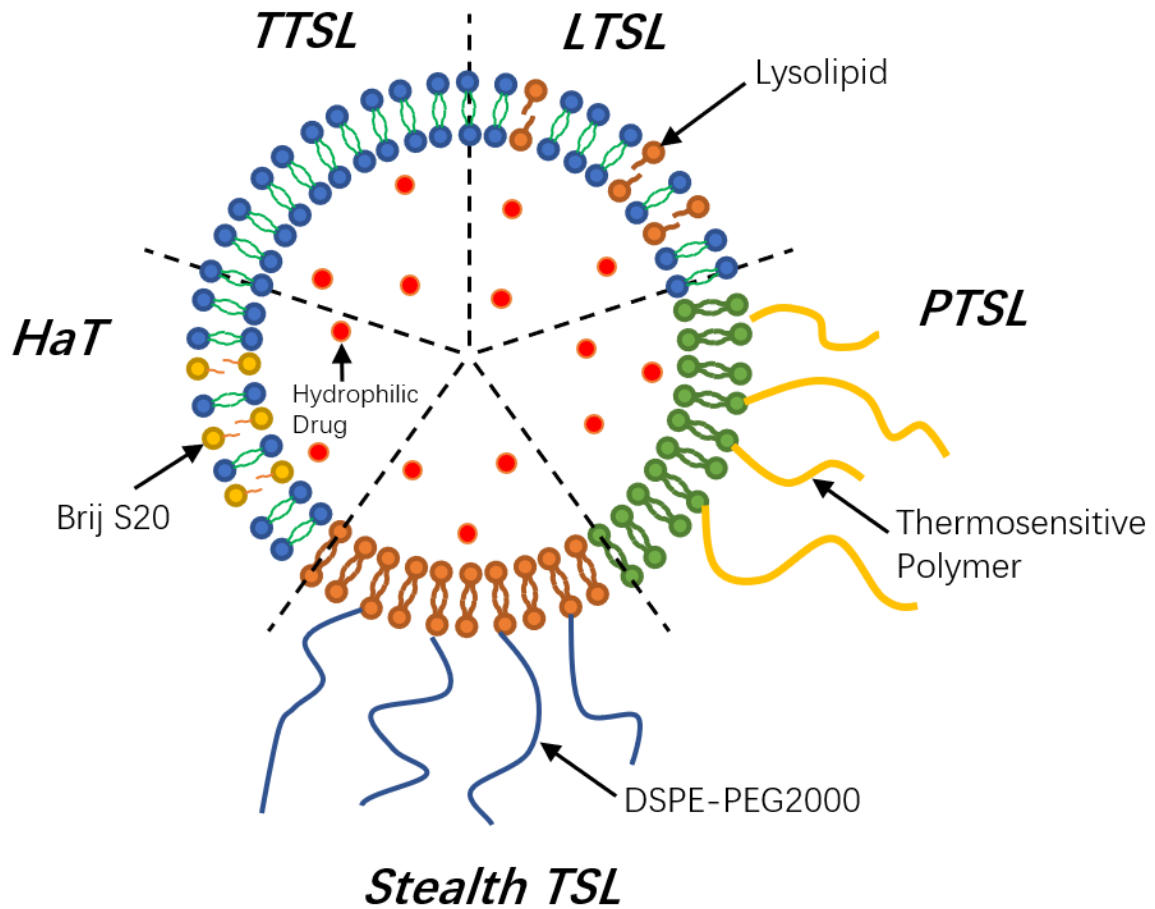


Figure. 1.7 Schematics of traditional thermosensitive liposomes (TTSLs), lysolipid-containing thermosensitive liposomes (LTSLs), polymer-modified thermosensitive liposomes (PTSLs), Stealth thermosensitive liposome (Stealth TSL) and Hyperthermia-activated cytotoxic liposome (HaT).

1.3.3.1.1 Traditional thermosensitive liposome (TTSL)

Yatvin et al firstly developed liposomes composed of DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) (16 carbon chain, $T_c = 41^\circ\text{C}$) and DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) (18 carbon chain, $T_c = 54^\circ\text{C}$) encapsulated with neomycin to restraint

bacteria reproduction *in vitro*, which was considered as the first TTSLs [75]. The liposomes experience a phase transition from gel to liquid at phase transition temperature (T_c) which varies depending on the lipid composition [76]. Below T_c , the liposomes exist in the gel phase, the lipid molecules are organised and completely extended, and the liposome is relatively impermeable. Above T_c , liposomes are in liquid phase and much more permeable, and the lipid molecules move randomly and rapidly [77]. DPPC was chosen as the primary lipid for TTSL because it has a T_c of 41 °C, making it ideal for mild hyperthermia. However, the drug release rate and released amount of liposomes composed of pure DPPC are relatively small [75]. To overcome this defect, it is necessary to introduce other lipids into the liposome formulation, such as DSPC and HSPC (L- α -phosphatidylcholine, hydrogenated (soybean)), the addition of these two phospholipids can accelerate the release rate and increase the release amount of encapsulated drugs from liposomes. The rationale here is that the mixing of different types of lipids increases the incompatibility of the liposome membrane structure, thereby making it easier for encapsulated drugs to leak out of the liposomes [78] [79]. Many preclinical studies in different tumour models have indicated that, after the application of hyperthermia, DPPC-based liposomes loaded with various kinds of drugs (such as bleomycin, methotrexate, doxorubicin, and cisplatin) demonstrated enhanced extravasation in the tumour capillary system and more drug accumulation in tumour tissues [80-83].

Nevertheless, the addition of lipids with longer carbon chains than DPPC in DPPC based liposome formulations resulted in an increase in the liposome phase transition temperature (T_c) [84]. The phase transition temperature of liposomes composed of DPPC and HSPC (18 carbon chains) in a molar ratio of 2:1, measured by differential scanning calorimetry (DSC), is around 46 °C, which is a too high T_c that can cause the risk of necrosis to healthy tissue [79]. Studies have shown that when pig muscle tissue is heated at different temperatures for 30 minutes, there will be different degrees of damage (mild damage at 40-43 °C, moderate

damage at 44-46 °C, and severe damage at above 46 °C) [85]. Therefore, it is necessary to develop a liposome with a phase transition temperature between 39 and 42 °C, and at the same time, it can also remain the characteristic of rapid release or burst release. The rapid release of the drug can ensure a sufficient dose of the drug to produce efficacy in the targeted area, while avoiding the development of drug resistance in cancer cells. There are mainly two approaches to achieve this goal, the first is to add lysolipids into the formulation of the liposome membrane, and the second is to modify the surface of the liposome with membrane-disrupting polymers [72].

1.3.3.1.2 Lysolipid-containing thermosensitive liposome (LTSL)

In 1999, Anyarambhatla and Needham first proposed adding lysolipids to liposome membranes to decrease the phase transition temperature for rapid drug release. Specifically, the addition of 10mol% lysolipid, MPPC (1 -palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine), to traditional thermosensitive liposomes, can make its T_c drop from 43 °C to roughly 40 °C, and 50% of the encapsulated drug was released in 20 seconds under heating at 42 °C [86]. Therefore, compared with traditional thermosensitive liposomes, LTSL can achieve rapid release of effective doses of drugs at lower temperatures and in less time, leading to a lower probability of necrosis of healthy cells due to the mild hyperthermia conditions required for release. Because lysolipids contain only one acyl chain, they form highly curved micelles at the phase transition temperature, giving rise to the formation of nanopores in the liposome membrane. Another explanation is that lysolipids would be desorbed directly from the bilayer at T_c , creating defects in the liposome membrane and allowing the drug to leak out from these defects. But the results of mass spectrometry analysis of highly dialyzed liposomes displayed that lysolipids remained in the liposomes, so the first explanation seems more probable [87].

One research has demonstrated that the administration of LTSL was able to make more doxorubicin bind to DNA in tumours cells of animals and reduce tumour growth more effectively than free doxorubicin, traditional TSL and non-thermosensitive liposomes [88]. In another study, mice were inoculated with five cancer cell lines (4T07, SKOV-3, HCT116, Fadu and PC3), and compared to untreated controls and controls that received only hyperthermia or LTSL, the group that received both LTSL and hyperthermia treatment exhibited the longest tumour growth time and tumour suppressive effect in all the mice inoculated with the above five types of cancer cell lines [89]. In addition, biomolecules in plasma, such as plasma proteins, can lead to desorption of lysolipids in LTSL. Studies have demonstrated that 70% of the lysolipids were desorbed at physiological temperature within one hour after liposomes were injected into mice [90]. This desorption behaviour would cause two significant consequences. First, it would substantially affect the phase transition temperature of liposomes and thus influence its thermosensitive characteristic. Second, it will contribute to the early leakage of encapsulated drugs before liposomes reach the targeted area and cause damage to healthy cells [91] [92] [72]. Therefore, the mild hyperthermia should be performed swiftly after the administration of LTSL, in order to guarantee that sufficient amount of drug is delivered to tumour cells and early leakage are prevented as much as possible.

The LTSL liposome containing encapsulated doxorubicin, ThermoDOX[®], has undergone further drug development by Celsion Corporation (Columbia, MD, USA). As of 2023, it has not yet been approved by the FDA nor EMA for marketing. Its specific clinical trial progress is as follows [93]:

Table 1.2 Summary of latest clinical progress of ThermoDOX[®]

Conditions or Diseases	Phase	Status	Actual Start Date	Completion Date or Estimated Completion Date
Primary And Metastatic Tumours of Liver	I	Completed	02/2007	12/2009
Livers Tumours	I	Completed	07/2014	04/2017
Pediatric Refractory Solid Tumours	I	Recruiting	10/2016	10/2022
Pancreatic Tumours	I	Recruiting	06/2021	12/2022
Breast Cancer	I And II	Completed	02/2013	06/2016
Hepatocellular Carcinoma (HCC)	III	Completed	06/2014	08/2018

1.3.3.1.3 Polymer-modified thermosensitive liposome (PTSL)

One of the existing problems of thermosensitive liposomes is that the membrane material must be a synthetic phospholipid with a phase transition temperature of around 41 °C, and its source is relatively restricted. Polymer-modified thermosensitive liposome is a newly developed thermosensitive liposome. Since its membrane formulation can use not only synthetic phospholipids but also natural phospholipids, it may become a more efficacious approach to solve this shortcoming. The principle is to attach a layer of polymers on the surface of liposomes, and these polymers have a lower critical solution temperature (LCST). When the temperature is below LCST, polymers can form hydrogen bonds with water molecules and they become hydrophilic. When the temperature rises to LCST, due to the

dramatic motion of molecules, the hydrogen bonds with water molecules are destroyed, and they essentially become hydrophobic. Based on this principle, when the temperature is adjusted higher than LCST, the bilayer membranes of the polymer-modified thermosensitive liposomes are destroyed, forming highly hydrophobic regions, from which the loaded drugs are rapidly released. These polymers can both endow non-thermosensitive liposomes with temperature-sensitive functionality and enhance the temperature responsiveness of thermosensitive liposomes [94]. The most widely used thermosensitive polymers are poly(N-substituted acrylamides) (e.g. p(NIPAAm)), poly(ethyleneglycols) (PEG), poly(N-alkyl oxazolines) and poly(N-vinylalkylamides)) [72].

1.3.3.1.4 Stealth thermosensitive liposome (Stealth TSL)

The long-circulating thermosensitive liposome (Stealth TSL) is a relatively common thermosensitive liposome. Hydrophilic macromolecules such as PEG are incorporated to the surface of the thermosensitive liposome. Thus, it can not only reduce the recognition and uptake of RES, prolonging the circulation time in the body, but also can release the drug rapidly at the heating site under heating conditions, playing the role of targeted drug release. However, the addition of macromolecules may compromise the thermosensitivity of liposomes. Therefore, the selection of suitable macromolecules has become the focus of research.

Li et al. incorporated DSPE-PEG2000 to 80 nm-sized liposomes composed of DPPC and DSPC to prepare stealth TSL. In order to determine the optimal DSPE-PEG2000 molar percentage, liposomes containing 1 to 10 mol% DSPE-PEG2000 were prepared and carboxyfluorescein (CF) were encapsulated as a drug model. The *in vitro* results showed that liposomes containing DSPE-PEG2000 greater than or equal to 6 mol% caused CF to

leak early at 37 °C, while liposomes containing 5 mol% DSPE-PEG2000 could remain stable at physiological temperature, while 60% of CF was released when heated to 42 °C for 1 minute, and almost 100% of CF was released in 1 hour. The release of CF in mice implanted with human BLM melanoma cell line was observed by intravital confocal microscopy. The results indicated that considerable CF was released rapidly at above 41 °C. Therefore, 5 mol% DSPE-PEG2000 is regarded as the most ideal molar percentage for this liposome formulation [95]. In another study, Li et al. encapsulated doxorubicin into stealth TSL and compared it with LTSL. Stealth TSL exhibited better stability in serum at physiological temperature. Stealth TSL started to release at 39 °C while LTSL had a slight release at 37 °C. Because there is no lysolipid component in the Stealth TSL membrane, it releases doxorubicin more slowly at 42 °C (75% in one minute), while LTSL releases 99% in one minute. Furthermore, in the experiment of mice inoculated with cancer cells, Stealth TSL exhibited better tumour suppressive effect than LTSL [96].

Apart from the most commonly used PEG2000 lipids, other substances have also been utilised as long-circulating materials. Kazuo et al. used the ganglioside GM1 as a long-circulating material to prepare GM1/DPPC/DSPC liposomes using a pH gradient method. The *in vitro* release test demonstrated that, in serum at 42 °C, the liposome released more than 45% of loaded doxorubicin within 5 minutes. The stealth TSL, traditional thermosensitive liposome and doxorubicin solution were injected into the tail vein of mice inoculated with colon cancer cells respectively. The tumour sites were heated by local hyperthermia at 42 °C for 20 minutes. The results displayed that the drug concentration of stealth TSL group in the tumour tissue of mice was 2.5 and 6 times higher than that of the traditional thermosensitive liposome group and the free drug group, respectively [97].

1.3.3.1.5 Hyperthermia-activated cytotoxic liposome (HaT)

In order to prepare a new type of liposome with better performance than LTSL, Tagami et al. invented HaT liposome for the first time, which is composed of DPPC and Brij78 (Brij S20). Because Brij surfactants consist of PEGylated acyl chains, they are considered to replace the functions of both the lysolipids and DSPE-PEG2000 in LTSL. By experimenting with different combinations of DPPC and various Brij, liposome composed of Brij S20 and DPPC was proved to be with the best characteristics. Brij S20 can be incorporated into the components of liposome membrane by the thin-film hydration method, and its optimal molar percentage should be 1-8 mol%. The phase transition temperature of HaT is less than 41 °C, which is slightly lower than that of LTSL (41.5 °C). According to the results of *in vitro* experiments, both were similar in stability at 37 °C in serum, but at 40 °C, HaT released more [98]. After local heating (43 °C) on tumour sites in mice, HaT delivered 1.4 times more doxorubicin to tumour tissues than LTSL. Compared with free DOX, HaT increased drug absorption in tumours by 5.2-fold and delivered 15-fold less doxorubicin to the heart. In contrast with LTSL and free DOX, HaT exhibited greater tumour regression and less cardiotoxicity [99].

HaT can not only be used to load doxorubicin, but also to transport non-membrane permeable drugs. Johnathon et al. loaded hydrophilic but poorly membrane-permeable drugs, gemcitabine (GEM) and oxaliplatin (OXA), into HaT for the first time, and discovered that HaT loaded with both drugs could successfully achieve temperature response and rapid release. But attempts to load both separately into LTSL have failed. Both leaked less than 5% of the encapsulated drugs within half an hour in serum at physiological temperature and released 80% of the drug within two minutes under hyperthermia of 42 °C. Compared with the free drug, the *in vivo* clearance of HaT-OXA was reduced by 3-fold, while that of HaT-

GEM was decreased by 50-fold. Compared to free GEM, 25 times more GEM was successfully delivered to heated tumour tissue by HaT-GEM, and complete regression of tumours in mice was observed [100].

1.3.3.2 pH-sensitive liposomes

The pH sensitivity comes from weakly acidic or weakly alkaline groups in the structure. Once these acid-sensitive materials reach the acidic region, they will undergo reversible protonation according to the pKa value of the functional group and the pH change in the microenvironment. Hence, the dissolution, disturbance or charge change of the material are triggered, resulting in the structural transformation of the nanocarrier and the release of the loaded drug. For example, the introduction of materials with protonatable groups into the surface of nanocarriers can make the carriers protonated to be positively charged in a low pH environment *in vivo* and can promote cellular uptake through electrostatic adsorption after contacting with negatively charged cell membranes [101].

The pH value of the tumour site is lower than that of normal tissue, roughly within 5.0 to 6.5, which is mainly because of the oedema, inflammation and local acidification of the tumour site, while the pH value of normal tissue fluid plasma is about 7.0 to 7.4 [102]. The pH-sensitive liposome has been developed based on this characteristic of the tumour site, which can target the tumour site, reduce systemic toxicity, and has the characteristics of simple production and high safety, and can be used as a gene carrier to improve gene transfection efficiency. Materials commonly used to make pH-sensitive liposomes are phosphatidylethanolamine (PE), dioleoyl phosphatidylethanolamine (DOPE), cholesterol succinate monoester (CHEMS), palmitoyl homocysteine (PHC) and acid sensitive

polymers. It is currently believed that the pH sensitivity of liposomes constructed from the above membrane materials is due to the fact that at neutral pH, fatty acid carboxyl ions can provide effective electrostatic repulsion to stabilize the liposomes in the lamellar state; The protonation of fatty acid carboxyl groups results in the formation of a hexagonal crystal state, which destabilizes the liposomes and facilitates aggregation, fusion and release of contents [103].

Another approach to construct pH-sensitive liposomes is to insert fusion proteins or fusion peptides into the phospholipid bilayer. In nature, a virus can enter the target cell in a fusion way because there is a special protein on the virus membrane, such as vesicular stomatitis virus G protein (VSV-G). It does not activate when the pH is neutral but activates when the pH is around 6. By inserting this protein into liposomes in an active form, "viroosomes" can be prepared, in the meantime, the mechanism of viral fusion is preserved [104].

A significant drawback limiting the application of pH-sensitive liposomes is that pH-sensitive liposomes are less stable than ordinary liposomes and can spontaneously transform into the hexagonal liquid crystalline state during storage, thereby inducing fusion, aggregation, and leakage of loaded drug. It is also easy to induce instability of liposomes and increased leakage of encapsulated contents due to the interference by plasma components after intravenous administration. The effect of plasma composition on pH-sensitive liposomes is complex, and it has been reported that plasma albumin can jeopardize its stability, while apolipoprotein A-1 (ApoA-1) can make it more stable. Besides, adding a certain amount of cholesterol during preparation increases its stability, however at the same time reduces its pH sensitivity [105].

1.3.3.3 Photosensitive liposomes

Like pH sensitive liposomes, light responsive liposomes can rapidly and accurately release encapsulated drugs at specific locations and time points. Nevertheless, pH sensitive liposomes require a specific chemical environment, which can be divided into two types. One is the environment *in vivo* in the organism with low controllability, and the other is the environment regulated by external means. These external invasion measures will inevitably bring about problems such as biological toxicity and biocompatibility. Unlike pH sensitive liposomes, light responsive liposomes do not require any changes in the chemical environment and are non-invasive, clean, and highly efficient. The preparation method of light responsive liposomes is mainly incorporating light sensitive units into the liposome structure [106].

Photosensitive liposomes mainly refer to liposomes loaded with photosensitizers and take advantage of the tumour targeting effect of liposomes to make photosensitizers reach the tumour site, overcoming the hydrophobicity of most photosensitizers. Appropriate light is then implemented, so that the photosensitizer can induce apoptosis of tumour cells. Since light cannot penetrate the deep tissues and organs in the body, photosensitive liposomes are only suitable for tumour areas in the superficial tissues, and thus have limitations in practical applications [107].

There are four types of photo-responsive groups, depending on their response to light. The first is the photoisomerization type represented by azobenzene (azo). Second, the photocleavage type represented by o-nitrobenzene derivatives. Third, the photodimerization type represented by coumarin (cou). Fourth, the intramolecular photo-induced bonding and cleavage type represented by spiropyran (spiro) [108-111].

azo is a typical representative of photoisomerized molecules, and it is also regarded as the most widely discussed photosensitive unit in the application and research field of photo responsive liposome. Other types of photosensitive drug delivery system such as hydrogels, micelles and vesicles also commonly apply azo for modification. Under the stimulation of 300-400 nm ultraviolet (UV) light, azo can change from the *trans* conformation to the *cis* conformation. While under the light stimulation of wavelength >435 nm, it can achieve the transition from the *cis* conformation to the *trans* conformation [112, 113].

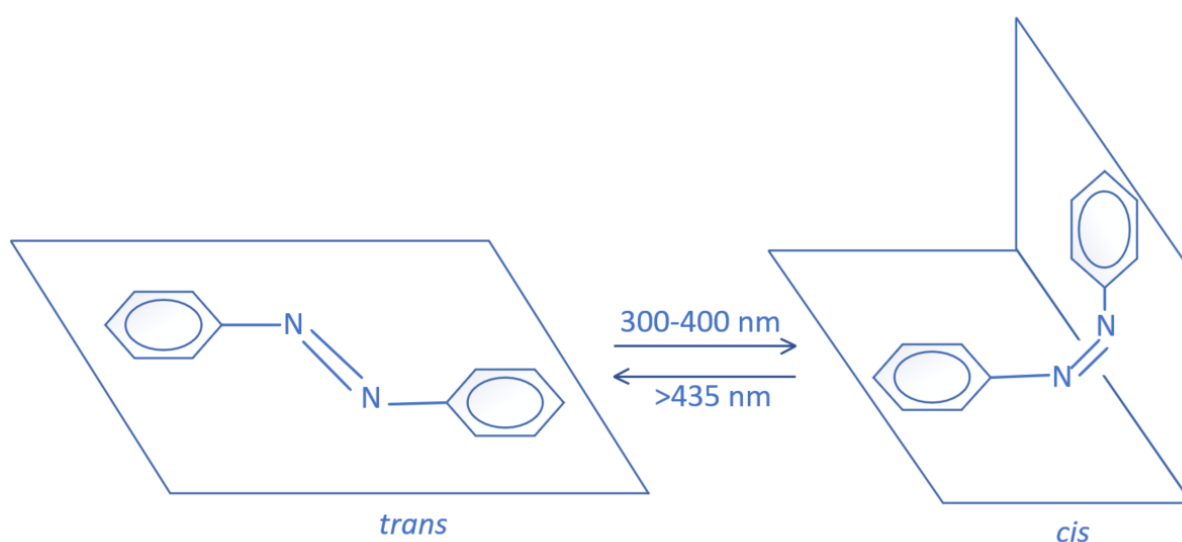


Figure 1.8 Illustration of the transition of azobenzene between the trans conformation and the cis conformation.

Liposomes containing azo structures are also usually prepared by incorporating azo at the lipophilic end of the polymer or amphiphilic molecules that constructs the liposome. Compared with the *cis* conformation, azo in the *trans* conformation can make the hydrophobic end structure more ordered, and the bilayer membrane structure of the liposome is more compact. Therefore, the thermodynamic stability of liposomes is higher when azo in the liposome is in the *trans* conformation than when it is in the *cis* conformation. Studies have demonstrated that

when azo is in the *trans* conformation, the liposome can be stored stably in the dark environment for several months, and after UV stimulation, azo is transformed from *trans* to *cis* state, and the presence of a large number of *cis* conformations greatly reduces the stability of the liposome structure and enables rapid drug release. Furthermore, after 10 mins of UV stimulation and then induced by visible light, the structure of azo undergoes a transition from *cis* to *trans* again, and the lipophilic end of the liposome becomes ordered. This structural rearrangement leads to the extrusion of the liposome structure, leading to further release of the drug [114]. Studies have indicated that the photosensitivity degree of azo-containing liposomes can be regulated by adjusting the composition and lipid ratio of liposomes. For instance, reducing the cholesterol percentage in the liposome composition can remarkably increase its photosensitivity [114].

1.3.3.4 Magnetic liposomes

Magnetic liposomes are also an effective drug delivery mechanism. Magnetic substances encapsulated liposomes are often guided by a magnetic field and delivered into tumour tissues and this procedure effectively increases the drug concentration in the target area. Hence, the magnetic carrier materials must have high magnetic permeability and magnetic induction. Magnetic liposomes are generally composed of magnetic carrier materials, lipids, cholesterol. The drug is released from liposomes in a controlled manner, and then exerts its pharmacological effect at the cellular or subcellular level of the tumour tissue, so it has little effect on normal tissue [115].

During the targeted transport of magnetic liposomes in blood vessels, the resistance of blood flow in blood vessels to liposomes competes with the magnetic force generated by external magnetic fields to liposomes. When the arterial blood velocity is less than 0.05cm/s, the

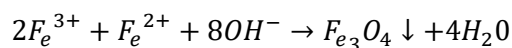
magnetic force is greater than the blood flow resistance, and the magnetic liposomes can be transported to the target area smoothly. However, when the blood flow rate is 0.55-0.1 cm/s, the blood flow resistance is greater than the magnetic force, and the magnetic liposomes will be completely retained in the blood vessels and cannot reach the target tissue [116].

To ensure the safety, effectiveness and targeting of magnetic liposomes, the magnetic carrier material should have the following features. First, the magnetic carrier material should be non-toxic, with maximum biocompatibility and minimum antigenicity, degradable and can be excreted from the body. In addition, it is worth noting that the iron content of the magnetic liposomes used in the whole course of treatment should not exceed the total amount of iron supplements routinely used in patients with anaemia. Second, the magnetic particles should have excellent magnetic induction intensity and magnetic conductivity, and under a suitable external magnetic field intensity, the magnetic liposomes can be transported to the desired position. Third, the particle size of the magnetic carrier material should be less than 20nm, because the maximum particle size of the magnetic liposome cannot exceed 3 μ m, otherwise it will sediment in the blood vessels, unable to maintain sufficient repulsion between each other to prevent aggregation, and easily block capillaries, furthermore, there is no guarantee that the magnetic liposomes will be evenly distributed at the target sites [116].

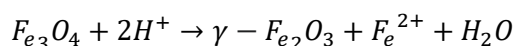
According to chemical composition, universally used magnetic carrier materials can be divided into four kinds. First, elementary substance such as iron, nickel, samarium, cobalt. Second, oxides like Fe₂O₃, Fe₃O₄, Mn₂O₃, CoO, and CoMnP. Third, alloys such as iron-aluminium alloys and iron-nickel alloys. Fourth, mixed magnetic materials [117].

Ferrite is generally used as a magnetic carrier material for the preparation of magnetic liposomes, and the most commonly used ones are nanoscale Fe₃O₄ and γ -Fe₂O₃. There are many approaches for synthesizing magnetic iron oxide particles, such as microemulsion

method, sol-gel method, co-precipitation method. Among them, co-precipitation method is the simplest and most effective method, whose essence is that Fe^{3+} and Fe^{2+} undergo hydrolysis reaction under alkaline conditions to obtain precipitation [118-121].



Fe_3O_4 is unstable and easily being oxidized into $\gamma\text{-Fe}_2\text{O}_3$ in the presence of oxidants.



In addition, magnetic fluid hyperthermia (MFH) is currently a commonly used method for tumour treatment. Since the alternating magnetic field generates heat, the temperature of the tumour area increases during the process of hyperthermia. Because the tumour cells are not heat-resistant, temperature changes will play a role in tumour elimination [122].

1.3.4 Dual drug-loaded liposomes

Owing to the different physicochemical properties of drugs, pharmacokinetics and tissue distribution *in vivo*, direct co-administration in clinical practice is difficult to ensure synergistic effects. If the combination of two drugs are co-loaded in the same carrier to achieve the same drug distribution in the body, it is beneficial to exert the synergistic effect of the drugs. In addition, by using the complementary characteristics between the two drugs to exert a synergistic or superimposed effect, therapeutic efficacy is ameliorated and toxicity to healthy tissues is reduced [123]. The advantages of dual drug-loaded liposomes in drug delivery have paramount implications for the treatment of some kinds of cancers. In the combination therapy of anti-tumour, the following conditions usually need to be satisfied, namely the effectiveness of a single drug, the difference of the mechanism of action of the two drugs,

the synergistic effect, and the absence of overlapping toxicity [124]. When preparing double drug-loaded liposomes, it is usually suggested that drugs with different anti-tumour mechanisms are selected, which can not only attack different biochemical targets of cancer cells, but also act on tumour cells with different growth cycles [125].

Due to the diversification of tumour cells, co-administration is particularly critical.

Chemotherapeutic drugs often have high cytotoxicity, so the selection of safe and effective delivery vehicles and reasonable combined administration methods has become a difficult problem in current cancer treatment. At present, dual drug-loaded liposomes often adopt a drug-loading method in which hydrophobic drugs are loaded in the phospholipid bilayer, and hydrophilic drugs are loaded in the aqueous phase of the liposome. This design not only ensures that there will be no excessive contact between different drugs during drug delivery, resulting in reaction denaturation, but also facilitates the combined administration of drugs of different properties, reducing the limitations of combined application of anti-tumour drugs [126].

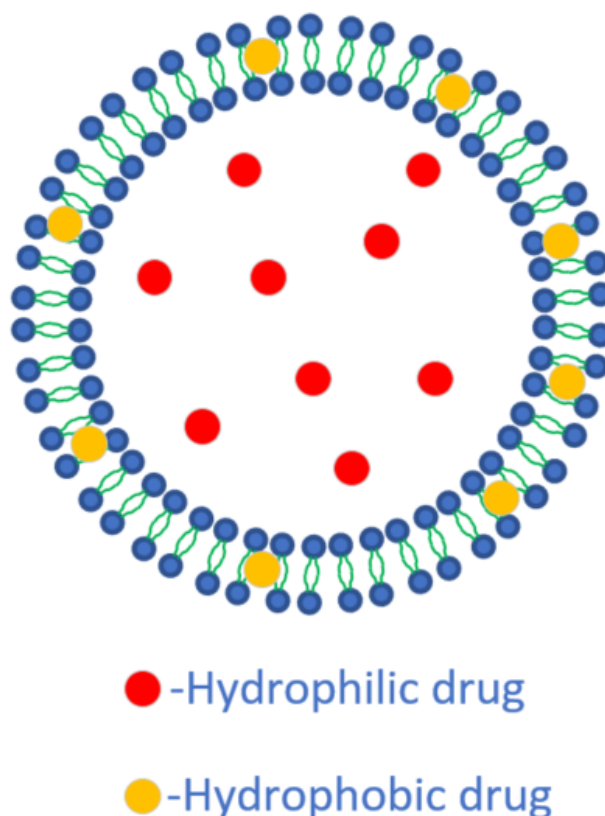


Figure 1.9 Schematic of dual drug-loaded liposomes

5-Fluorouracil has been clinically approved but has limited efficacy in curbing rectal cancer. A study has revealed that the combination of 5-fluorouracil and apigenin can produce an outstanding synergistic effect and substantially ameliorate the treatment of hepatocellular cancer, ascitic tumour and colorectal cancer [127] [128]. Apigenin is a natural flavonoid that is abundant in fruits and vegetables and can be easily obtained. Based on a research, liposome encapsulation with apigenin has been demonstrated to be effective in constraining the growth of colorectal cancer in both *in vitro* and *in vivo* experiments [129]. Sen et al., using a modified film hydration method, further studied the double drug-loaded liposomes encapsulating with both 5-fluorouracil and apigenin at the same time, and found the best molar ratio of 5-fluorouracil and apigenin loading in the liposome for the treatment of colorectal cancer. This combination regimen is able to enhance the bioavailability of

apigenin, effectively reduce the proliferation of cancer cells, stimulate the apoptosis of cancer cells, and inhibit angiogenesis dramatically. Compared with free 5-fluorouracil and apigenin, and their single-drug-loaded liposomes, dual-drug-loaded liposomes were more cytotoxic to two types of human colon cancer cells. It was also indicated in preclinical experiments in nude mouse tumour xenograft models that this preparation can exhibit better anti-tumour effects. This novel dual drug-loaded liposome provides a promising solution for potential clinical applications in the future [130].

In another study, a novel carfilzomib and doxorubicin dual drug-loaded liposome was developed to treat multiple myeloma tumours and improve patient outcomes. First of all, the researchers screened out the best drug-loading molar ratio (1:1) of doxorubicin and carfilzomib that could produce the best synergistic therapeutic effect in multiple myeloma cell line experiments. Then, double drug-loaded liposomes with a diameter of 115 nm were successfully prepared. *In vitro* experiments have shown that this formulation can produce a good synergistic inhibitory effect on tumour cells compared to the free forms of the two drugs. In mice, it can effectively suppress tumour growth while minimizing side effects on healthy cells [131].

Combination therapy has made eminent clinical progress in the field of anti-tumour liposome. Celator (Canada) has developed 3 compound liposomes: cytarabine-daunorubicin liposome compound (CPX-351), irinotecan-fluorouracil liposome compound (CPX-1), and irinotecan-cis-platinum liposome compound (CPX-571), of which CPX-351 is used for the treatment of acute myeloid leukemia (AML), CPX-1 is used for the treatment of colorectal cancer and CPX-571 is used to treat small cell lung cancer, the first two drugs have entered the clinical stage, and CPX-571 is in preclinical research [132-134]. It shows that the compound liposome has begun to enter the clinical application of anti-tumour drugs.

1.4 Heating approaches for thermosensitive liposomes (TSLs)

The primary purpose of heating approaches for thermosensitive liposomes is to raise the temperature merely in targeted areas safely and efficiently while reducing the heating areas within healthy tissues [72]. Many heating approaches have been developed.

Firstly, the simplest approach is the regional hyperthermia, which has been widely applied in most of animal tests [80] [82]. The targeted area on animals was heated by water baths. The drawback of this method is its restriction to superficial tumour and large area of healthy tissues was heated simultaneously [135].

The second approach is the external electromagnetic applicators which emit microwaves. Commercially available applicators have a maximum heating depth of 3 cm and a restricted heating area. The uneven surfaces including axilla, head and neck however can impair its efficiency [136].

The third approach is the localised interstitial hyperthermia which inserts antennas into the tumour to heat it deeply with minimal invasion. Many antennas have been investigated for preclinical research including laser fibers, electrodes and microwave antennas [137]. A novel electrode with several expandable probes has been invented for large tumour areas which could maintain the whole tumour area at uniform temperatures [138]. Localised interstitial hyperthermia is restricted by the area that is viable to insert antennas and the maximum tumour diameter is 5 cm [139].

The fourth approach is the high intensity focused ultrasound (FUS) which is specialised in heating deeply in a focal zone in tumours with an adjustable temperature control by regulating the intensity and duration of the ultrasound [140]. FUS is operated without inserting probes into human body, thus provide a non-invasive method for localised hyperthermia [141]. Nevertheless, in order to monitor real-time temperature variation in tumour, invasive thermocouples are introduced for the measurements [142].

The fifth approach is the magnetic resonance imaging which provides a non-invasive approach to monitor temperature change with combination of the FUS due to its outstanding tissue differentiation [143]. This combination was regarded as magnetic resonance-guided focused ultrasound (MRgFUS) as demonstrated in Fig 1.3. Experiments on rabbits and rats demonstrated higher drug uptake in tumours treated by MRgFUS and liposome compared to liposome or free drug [144]. In research of doxorubicin encapsulated liposome, higher doxorubicin fluorescence intensity was observed in deep locations of tumours treated with both MRgFUS and liposome [145]. Whereas, doxorubicin fluorescence intensity was merely observed in the tumour perimeter in control group (liposome only or free doxorubicin) [146].

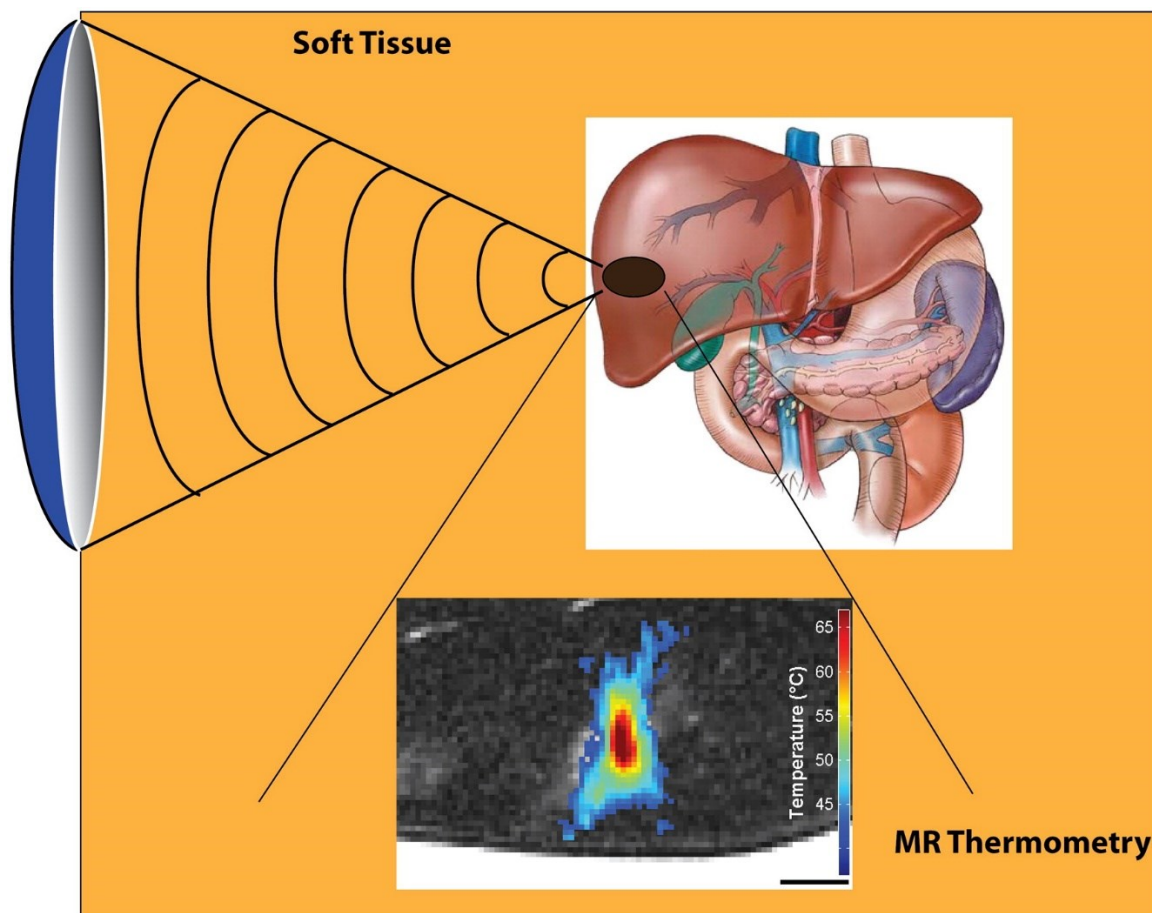


Figure 1.10 Schematic of MRgFUS. Focused ultrasound can heat tumours rapidly transmitted from skin while protecting neighbouring tissues from damage. Two-dimensional maps of ultrasonic heating are displayed by the application of MR thermometry, providing real-time feedback for focused ultrasonic system. MRgFUS can be used for sustained and controlled heating on tumours to trigger drug release from thermosensitive liposomes [12].

1.5 Chitosan coated liposomes

The chemical name of chitosan is (1,4)-2-amino-2-deoxy- β -D-glucan, which is white translucent solid, insoluble in water, alkali solutions, and most organic solvents, but soluble in water under acidic conditions. Chitosan is the deacetylation product of chitin, as demonstrated in the Figure 1.11. Chitin is the second most abundant natural

macromolecular substance in nature after cellulose, and widely exists in lower arthropods, lower crustaceans (such as crab, shrimp, other crustacean, and insects) and lower plants (such as fungi and algae). Therefore, chitosan has a wide range of sources, and it is the only natural alkaline cationic polymer known so far [147].

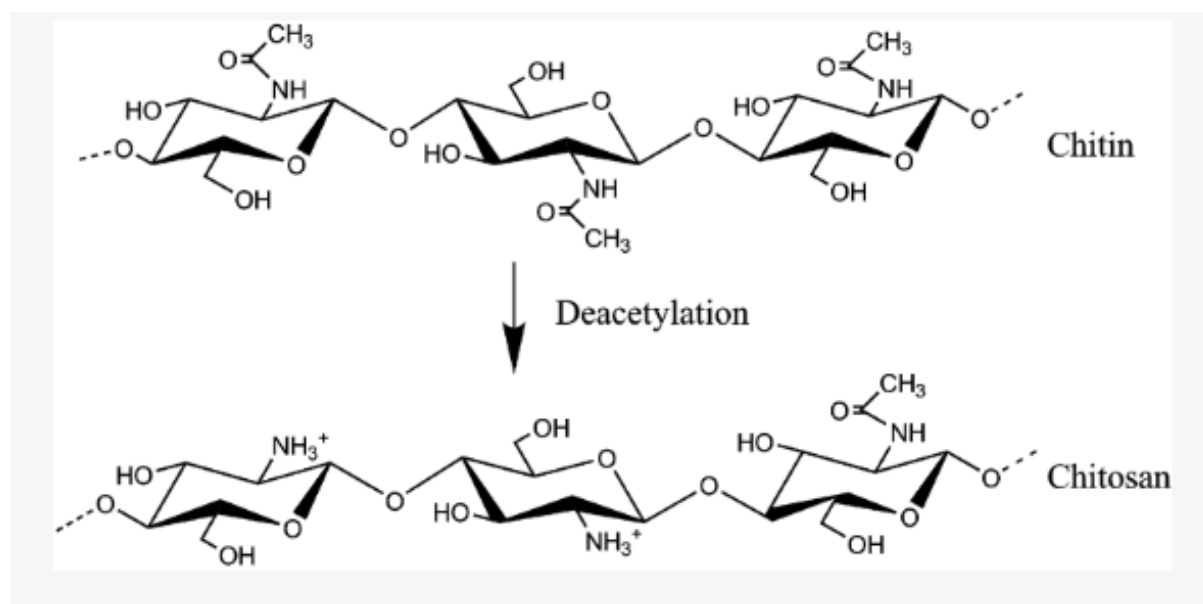


Figure 1.11 Structure of chitin and chitosan [148]

Chitosan has been utilised in many areas, including ophthalmology, DDS, hydrogels, microcapsules, antibacterial agents, blood anti-coagulants, anti-thrombogenic, artificial skin and haemostatic materials, because of its excellent characteristics (e.g., non-toxicity, biodegradability, biocompatibility, adsorption.) [149] [150]. There are a large number of amino, hydroxyl and other groups on the chitosan molecular chain that can form hydrogen bonds, which can chelate with metal ions or form network molecules, so that some heavy metal ions can be adsorbed. Chitosan is positively charged under acidic conditions and can combine with negatively charged molecules on the bacterial cell membrane to damage the cell membrane. The material inside cell leaks out due to the destruction of the cell membrane, which makes the shape of the bacteria and the interior of the cell undergo

significant variations, thereby bacteria are killed, and the bacteriostatic effect of chitosan is achieved. The metabolite of chitosan in the human body is glucosamine, thus chitosan is degradable and has no toxic side effects. Nevertheless, due to the cationic charge chitosan, it is easy to interact with the negatively charged red blood cells, contributing to the rupture of the cell membrane of red blood cells, which is termed haemolysis. For this reason, anionic groups, hydrophilic groups, or materials with better blood compatibility are usually introduced to make up for this deficiency [151, 152].

1.5.1 Chitosan coated liposome for oral delivery

Berberine hydrochloride (BH) has excellent anticancer properties, but its oral bioavailability is low. To overcome this shortcoming, researchers chose nanoscale liposomes to improve its oral availability. By coating a layer of positively charged chitosan on the surface of negatively charged liposomes, Nguyen et al. developed chitosan-coated nanoliposomes for the delivery of encapsulated BH. Under the storage conditions of 4 °C and 25 °C, the chitosan-coated liposomes leaked less drugs than the uncoated liposomes and had better storage stability. Furthermore, liposomes coated with 0.3% (w/v) chitosan were more stable than 0.1% (w/v) chitosan. In addition, chitosan-coated liposomes are also more stable in simulated gastrointestinal (GI) fluid with less leakage than plain liposomes. 0.1% (w/v) chitosan-coated liposomes had higher percentage of drug released than 0.3% (w/v) chitosan-coated liposomes in simulated GI fluid. Besides, all the pharmacokinetic data of chitosan-coated liposomes in rabbits were better than those of uncoated liposomes. Therefore, chitosan-coated liposomes can be used as an effective and promising approach to transport BH orally [153].

Chitosan-coated and polyplex-loaded liposomes (PLLs) for DNA vaccine delivery were developed by Channarong et al. Plain liposomes composed of phosphatidylcholine and cholesterol were prepared by reverse-phase evaporation method. The zeta potential of the liposome surface increased significantly after coating with chitosan. By using green fluorescence protein (GFP) as an indicator, *in vivo* experiment showed that the signal of GFP was detected in all regions of intestine, which means the chitosan coated PLLs have a great potential for DNA delivery to the distal intestine [154].

In order to increase the stability of progesterone in gastro-intestinal (GI) fluid and the adsorption of drug to enhance the patient compliance, progesterone loaded liposome was prepared by ethanol injection approach, and chitosan was coated onto its surface. After coating with chitosan, the half-life of liposomes increased by 1.12 times in simulated gastric fluid and 76.3 times in simulated intestinal fluid compared to uncoated liposome *in vitro*. The progesterone adsorption rose by nearly 2 times in contrast with traditional formulation *in ex-vivo* GI fluid. Besides, the drug release was followed the Higuchi model kinetics [155].

1.5.2 Chitosan coated liposome for ocular delivery

Li et al. developed low molecular weight chitosan-coated liposomes (LCHL) encapsulating cyclosporine A (CsA) were developed. *In vitro* release experiments demonstrated enhanced stability and delayed release of LCHL compared to uncoated liposomes. In a rabbit conjunctival epithelium (RCE) cell line, LCHL can significantly promote the internalization of CsA in cells without affecting cell viability. The results obtained from *in vivo* experiments in rabbits displayed low toxicity to RCE and a significant increase of CsA concentration in rabbit sclera, cornea, and conjunctiva after ocular administration of LCHL. Therefore, because LCHL has excellent biocompatibility, prolonged drug retention time in the target

area, and enhanced drug penetration, it can be considered as a potential ocular drug carrier, worthy of further development [156].

Another study conducted by Li et al. had similar results by using LCHL encapsulating diclofenac sodium. Low molecular weight chitosan (LCH) altered the zeta potential of the liposome surface and resulted in a marginal rise on the liposome diameter without changing its encapsulation efficiency. *In vitro* experiments with LCHL indicated a prolonged drug release profile compared to uncoated liposomes. After 30 days of storage at room temperature, LCHL was more physiochemically stable and leaked less than uncoated liposomes. In addition, LCHL also exhibited prolonged precorneal retention and enhanced transcorneal drug penetration rate in rabbit experiments. At the same time, it also has excellent ocular tolerability, after 7 days of continuous use of LCHL, it did not cause significant irritation or toxicity. Therefore, LCHL has great potential for ocular drug delivery due to its outstanding stability and biocompatibility [157].

1.5.3 Chitosan coated liposome for pulmonary delivery

Chitosan coated liposome encapsulated with rifampicin (RIF) was developed for pulmonary administration by nebulisation. Chitosan-coated liposome was prepared by addition of chitosan solution dropwise into liposome dispersion. Charged liposomes are more easily to be coated with chitosan compared to non-charged liposomes. Chitosan-coated liposome displayed a slightly higher encapsulation efficiency (EE) and significantly elevated nebulisation stability (NER%), in contrast with uncoated liposomes. Furthermore, enhanced mucoadhesive characteristic and lower toxicity to epithelial cells were discovered as well for chitosan coated liposome in contrast with plain liposome or free RIF [158].

Another study on chitosan-coated liposomes encapsulating curcumin has been investigated. Liposomes are prepared from PC, PE, LysoPC, fatty acids and triglycerides. The diameter of the curcumin-loaded uncoated liposomes was roughly 100 nm, and an increase of roughly 130 nm on the liposome diameter has been observed after chitosan coating. The coating of chitosan transferred the zeta potential on the liposome surface from negative to positive successfully. During the nebulisation process, chitosan coating dramatically enhanced the stability of liposomes, promoting more accumulation of curcumin in the farthest region of lungs. Furthermore, the antioxidant ability of curcumin was ameliorated after being entrapped in chitosan coated liposomes [159].

Kudsiova et al. prepared vesicles composed of DSPC or DMPC lipids and coated it with chitosan to verify whether chitosan could improve the permeabilities of membranes of human colonic cancer cells and human bronchial epithelial cells. Results showed that both chitosan-coated vesicles and chitosan solutions of the same concentration can significantly reduce the transepithelial electrical resistance (TER) of both cell membranes. After removing the chitosan or chitosan-coated vesicles from the solution, the TER value of colonic cells could recover to half of their initial value, while that of bronchial cells could recover completely. Hydrophilic markers with different molecular weights were used to test their ability to penetrate bronchial cell membranes, and the results demonstrated that their enhanced transport capabilities were equivalent in chitosan solution and chitosan-coated vesicle solution at the same concentration. Most essentially, neither free chitosan nor chitosan coated vesicles presented significant toxicity to both kinds of cells [160].

1.5.4 Chitosan coated liposome for intravenous injection

Very little research has been reported on the application of chitosan coated liposome for intravenous injection. Zhuang et al. developed a liposome composed of phosphatidylcholines (PC) and cholesterol with a molar ratio of 5 :1 to encapsulate mitoxantrone (MTO). The variation of particle size, zeta potential after chitosan coating and the desorption of chitosan verified the successful coating of chitosan on the liposome surfaces. in vitro release experiments, 54% of MTO was released from uncoated liposomes, while chitosan-coated liposomes released only 31% of MTO. Besides, MTO saline solution, uncoated liposome and chitosan-coated liposome were injected intravenously in rats, respectively. The pharmacokinetic results indicated that chitosan-coated liposome had longer retention time and plasma concentration than the first two [161].

Kudsiova et al. developed chitosan coated vesicles composed of DSPC, DPPC or DMPC using the thin film approach for gene delivery. The results from small angle neutron scattering (SANS) demonstrated that DNA was trapped in the interlamellar aqueous space between the two lipid bilayers rather than in the space inside vesicles. Besides, the circular dichroism (CD) measurements also justified this hypothesis, discovering the DNA conformation is C form, a less hydrated and more compressed structure, rather than the more common B form, which is a more hydrated one and usually existed inside the vesicles. SANS and zeta potential measurements indicated that the chitosan was successfully coated on the surface of vesicles and the amount of chitosan coated on the vesicles surface depends on the initial added chitosan concentration in the coating process. Last but not least, after 7 and 14 days of storage, chitosan coated vesicles leaked less DNA than uncoated vesicles, and hence exhibited better stability [162].

In another study, chitosan coated liposome with the encapsulation of docetaxel was prepared for the drug delivery to laryngeal cancer cells. The increase of both liposome size and zeta potential verified the success of chitosan coating process. The chitosan coating

formation was also confirmed by the fourier transform infrared spectroscopy (FTIR) spectroscopy. Blank liposome and chitosan coated liposome were both not cytotoxic to human laryngeal cancer cells and stromal cells. Nevertheless, the docetaxel encapsulated chitosan coated liposome demonstrated greater cytotoxicity to human laryngeal cancer cells than stromal cells. In addition, after 3 hours of exposure in human blood cells, haemolytic effect was not discovered, suggesting the feasibility of the intravenous administration [163].

1.6 Objectives of this work

1. Preparation of HaT liposome composed of DPPC and BrijS20 with a molar ratio of 96:4 and chitosan coated HaT liposome. Investigating the influence of ultrasonic time on the particle size of empty and drug encapsulated liposomes, the influence of pH on the zeta potentials of DPPC, Brij S20, low, medium, and high MW chitosan and the influence of different molar ratio of DPPC and Brij S20 in liposome composition on the liposome size and its zeta potential.
2. Comparison of TEM images of plain HaT liposomes and low, medium, and high MW chitosan coated liposomes (freshly made ones and stored ones). Determining the most suitable molecular weight chitosan (low, medium, and high) and chitosan concentration for the drug encapsulated liposomes characterizations.
3. Comparison of calcein encapsulated plain liposome and chitosan coated liposomes by investigating their characterizations (e.g., calcein encapsulation efficiency, release property at 37 °C, 40 °C, 42 °C, shelf life). Determining the best kinetic model to fit release curves of calcein encapsulated liposomes based on the analysis of fitting parameters such as R^2 , BIC, and RSS.

4. Investigating the influence of chitosan coating on the characteristics of doxorubicin encapsulated liposomes such as controlled release behaviour triggered by the hyperthermia treatment, and their shelf life. Finding out the best kinetic model among four models (Higuchi, Korsmeyer-Peppas, Weibull and First-order models) to fit release curves of doxorubicin encapsulated liposomes by comparing the fitting parameters.

1.7 Thesis outline

Chapter 1 contains the review of (i) the commonly used ingredients and basic methods of liposome preparation, (ii) introduction of four main types of liposomes such as stealth liposome, active targeted liposome, environment-responsive liposome, and dual drug-loaded liposome, (iii) the advantages and disadvantages of several types of thermosensitive liposome (TSL) (e.g. TTSL, LTSL, PTSL, Stealth TSL and HaT), (iv) the merits and demerits of heating approaches for TSLs, and (v) the applications of chitosan coated liposomes for oral, pulmonary and ocular deliveries and intravenous injection.

Chapter 2 (i) introduces the preparation of drug encapsulated HaT liposomes coated with and without chitosan, (ii) discusses the influence ultrasonic time on the particle size of empty and drug loaded liposomes, (iii) investigates the zeta potentials of DPPC, Brij S20, low, medium, and high MW chitosan at different pH and their IP points, (iv) discusses the influence of different molar ratio of DPPC and Brij S20 in the liposomal composition on the liposomal size and its zeta potential, (iv) compares the TEM images of plain HaT liposome and three MW chitosan coated liposomes, (v) compares the particle size and zeta potential of low, medium, and high molecular weight chitosan coated liposomes (fresh samples and samples stored at 4 °C after one month) with various chitosan concentration (0.1%, 0.2%,

0.3% (w/w)) in order to determine the most suitable molecular weight and concentration of chitosan for the following experiments.

Chapter 3 (i) plots the standard curve of calcein fluorescence intensity and discusses the influence of pH on the calcein fluorescence intensity standard curve, (ii) investigates the effect of initial added calcein in the liposome preparation process on the calcein encapsulation efficiency, (iii) compares the release property and shelf life of plain liposome and chitosan coated liposome, (iv) determines the best kinetic model to stimulate the calcein release curves based on the analysis of fitting parameters.

Chapter 4 (i) discusses the influence of pH on the doxorubicin fluorescence intensity standard curve and plots its standard curve, (ii) the influence of initial added doxorubicin on the doxorubicin encapsulation efficiency, (iii) compares its release property and shelf life, (iv) compares the fitting results of various kinetic models and chooses the optimum kinetic model for fitting the doxorubicin release curves according to the fitting parameters calculated by the Origin software.

Chapter 5 exhibits the conclusions of this thesis and suggestions for the future work.

Chapter 2. Development of chitosan-coated thermosensitive liposome

2.1 Introduction

Liposomes are considered to be a promising drug delivery system. They have extensive applications, such as anti-cancer drug carrier for chemotherapy [164], vaccine [165], gene therapy [166] and imaging by loading radioactive tracers [167]. In contrast with original chemotherapeutic drugs, liposomes encapsulated with anti-cancer drugs have a higher therapeutic effect and lower toxicity [168]. Specifically, liposomes can accumulate passively and massively inside tumour tissues and spread little to healthy regions of the body due to the special morphology and distribution of tumour vessels [169].

Nevertheless, one of the most problematic drawbacks of liposomes is its stability *in vitro* and *in vivo*. Firstly, liposomes frequently have relatively short circulation half-life in human body [170]. Besides, small liposomes are likely to degrade, aggregate and merge into large vesicles, which will result in varying degrees of encapsulated drug leakage [171]. Therefore, considerable effort has been undertaken to improve their stability such as modification on the liposome surface by polyethylene glycol chains. The characteristics on the liposome surface play a paramount role on the stability of liposomes, such as fluidity, hydrophobicity and electrostatic charge [172] [173]. Coating of polymer with specific properties is considered as a promising and easy approach to improve the liposome surface characteristics because the coating process merely requires a simple mixture of liposome solution and polymer solution rather than a complicated chemical reaction to bond polymer and lipid molecules [174]. Owing to the repulsion between the same charged polymers

coated on liposomes, the possibility of degradation, aggregation, and fusion decreases, increasing the stability of liposomes.

Approaches has been undertaken to modify the surface of the liposomes with polymers, including natural polymer and artificial polymer like polyacrylamide, polyethylene oxide and polyvinyl alcohol [175] [176]. Nevertheless, among these efforts, chitosan was considered to be a promising polymer to modify liposome characteristics for its excellent performance [177]. Chitosan (pK_a value= 6.5-6.6) [178] has many advantages such as biodegradability, biocompatibility and nontoxicity [147]. Besides, it can be obtained easily and massively from natural sources as it is derived from the shells of crustaceans and insects [179]. In addition, chitosan has been proved that it can bind with biological membranes and disrupt the tight junctions between the cells to increase the permeability of an epithelium [180]. Most importantly, chitosan has been approved for use by the FDA, thus it can be safely utilised in many areas such as biomedical, agriculture, pharmaceutical and food industries [181] [182].

Recently it has been shown that the coating of chitosan on liposomes can enhance the stability of liposome [183]. Nevertheless, most liposomes containing chitosan are used for oral, lungs and ocular drug delivery [184] [158] [185] [186]. Little research has been undertaken on chitosan-coated liposomes for intravenous injection. In this research, HaT (Hyperthermia-activated cytotoxic) liposome composed of DPPC lipid and Brij S20 (96:4 molar ratio) was selected as the plain liposome. HaT liposomes have superior characteristics than LTSL (lyso-lipid temperature-sensitive liposomes) which is in clinical trials at present. For example, doxorubicin encapsulated HaT liposomes demonstrated faster release at 40-41 °C, an increased amount of drugs was delivered to locally heated tumours of mice together with reduced amount delivered to the mice heart compared to LTSL. However, a relatively high amount of doxorubicin (10%-20%) was released at 37 °C in 30 mins [99]. Therefore, this research will discuss the influence of chitosan coating on the stability of HaT

liposome at three temperatures (37 °C, 40 °C and 42 °C). But firstly, the characteristics on liposomes surfaces coated with low, medium, and high molecular weight chitosan of different concentrations need to be evaluated and the most appropriate concentration and molecular weight chitosan will be selected from this chapter for further experiments on stability property analysis. To the best of our knowledge, studies on the comparison of low, medium, and high chitosan coated thermosensitive liposome for intravenous injection have not yet been reported.

Consequently, in this chapter, the two most essential parameters, zeta potential and particle size of low, medium, and high molecular weight chitosan coated liposomes and plain HaT liposomes will be determined and discussed thoroughly. Before that, firstly, the optimum ultrasonic time need to be determined in the liposome preparation process to make sure the liposome size is decreased and well uniformed. Secondly, all isoelectric points of essential molecules such as DPPC, Brij S20 and three MW chitosan were measured. Thirdly, the influence of different molar ratio of lipid composition on zeta potential and liposome size were discussed and compared. Most importantly, clear TEM images of both plain and stored HaT liposomes and different MW chitosan coated liposomes were taken to provide more details in terms of liposomal morphology.

2.2 Materials and Methods

2.2.1 Materials

1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was purchased from Avanti Polar lipid, Alabaster, AL, USA.

Calcein Disodium Salt was purchased from Honeywell Fluka.

Brij S20, low molecular weight Chitosan (50-190 kDa), medium molecular weight Chitosan (190–310 kDa), high molecular weight Chitosan (310-375 kDa), Triton X-100, doxorubicin hydrochloride, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and phosphotungstic acid hydrate were purchased from Sigma-Aldrich.

Sephadex G-50 (medium) was purchased from GE healthcare.

All water used was de-ionised and had a conductivity less than 80 mS.

2.2.2 Preparation of plain thermosensitive liposome

STEP 1. The liposomes were prepared by the thin film hydration method, 10 mg of DPPC and Brij S20 lipids in a molar ratio of 96:4 were dissolved in chloroform and dried under a stream of nitrogen gas to form thin lipid films.

STEP 2. The lipid film was placed in the vacuum environment for 4 hours to remove the remaining organic solvent.

STEP 3. The lipid film was hydrated with 1 ml deionised water at 60 °C to form multilamellar vesicles (MLVs).

STEP 4. The MLVs were sonicated for at least 8 min (Sonicator, 120 watts, Fisher Scientific) and placed at room temperature for at least 4 hours to form large uni-lamellar vesicles (LUVs)

2.2.3 Preparation of chitosan-coated thermosensitive liposome

STEP 1. 0.1% chitosan solution was prepared by adding 1 g of low or medium molecular weight chitosan powder into 990 mL distilled deionised water and 10 mL glacial acetic acid. The solution was left to stir for 12 hours (overnight).

STEP 2. The chitosan solution was neutralised by the addition of 0.1 M NaOH solution.

STEP 3. 1 mL of the chitosan solution was added dropwise to 1 ml of the liposome dispersion under magnetic stirring at room temperature (20 °C) for at least 4 hours.

2.2.4 Preparation of doxorubicin encapsulated thermosensitive liposome

Steps 1,2 and 4 were the same as the preparation of plain thermosensitive liposomes (section 2.2.2).

However, in Step 3, 1 ml deionised water is replaced by the 1 ml of 3 mg/ml doxorubicin water solution.

The unencapsulated doxorubicin was then separated from doxorubicin encapsulated liposome by using a self-assembled gel filtration column (diameter 0.8 cm* length 25 cm) containing Sephadex G-50.

The purified liposome solution was collected from the tap at the bottom of the column every 1 ml (see section 2.2.6).

2.2.5 Preparation of calcein disodium salt encapsulated thermosensitive liposome

Steps 1,2 and 4 are again the same as the preparation of plain thermosensitive liposome.

Step 3 is again modified, such that 1 ml of deionised water is replaced by 1 ml of 7 mg/ml calcein disodium salt water solution.

Then the unencapsulated calcein was separated from calcein encapsulated liposome in a self-assembled gel filtration column (diameter 0.8 cm* length 25 cm) containing Sephadex G-50.

The purified liposome solution was collected from the tap at the bottom of the column every 1 ml. (see section 2.2.6).

2.2.6 Separation of liposomes from the unencapsulated drug by gel filtration

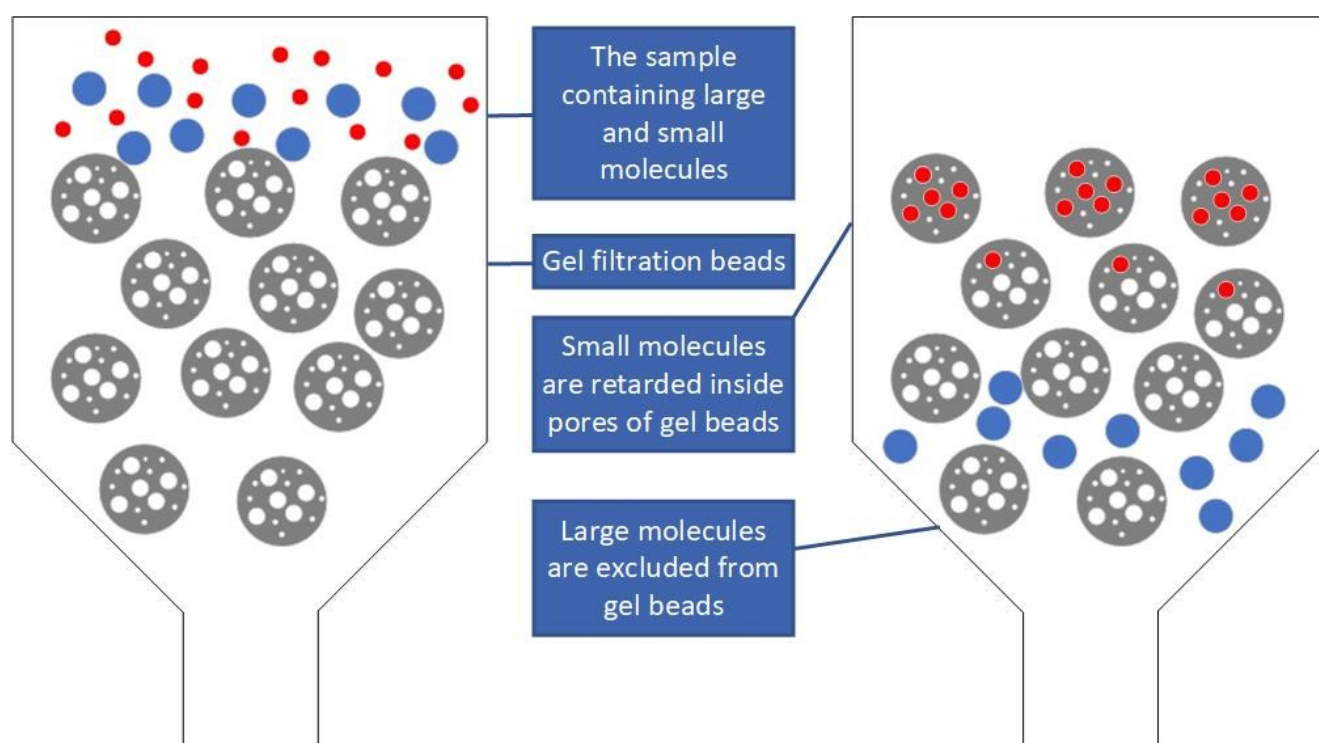


Figure 2.1 The principle of gel filtration

Gel filtration is based on a solid phase which is consisted of spherical gel pellets with a specific size and porosity. Physical property, which is the size of the material being separated, rather than the chemical property is the only factor that functions in the gel filtration. When the mobile phase flows through the solid phase, different materials will be

separated depends on their physical size (in the case of liposomes) or molecular weight (in the case of polymers) and shapes. Smaller entities have a longer path because they are retarded inside the pores of gel pellets. Therefore, materials with varying molecular weight can be separated in the gel chromatography column. Larger molecules elute first and smaller ones elute later as demonstrated in Figure 2.1 [187].

A longer gel chromatography column generates higher separation efficiency. Whereas excessive sample induces overlap of excluded and included molecules and lower separation efficiency. Thus, the volume of loading sample is recommended to be lower than 5% of the total volume of the gel chromatography column [188].

A gel filtration column was assembled in this research using Sephadex G-50 as the media. Sephadex G-50 was hydrated with HEPES buffer solution and stirred for 5 minutes, before being stored overnight at 4 °C to remove any bubbles in the suspension completely. Glass wool and quartz sand were placed at the bottom of 25 ml burette as foundation bed to support the gel beads. After that, the Sephadex G-50 water suspension was loaded dropwise into the burette and then left for 4 hours to enable the bed to settle. The liposome dispersion was then added dropwise into the gel filtration column. Then, HEPES buffer solution was added continuously to the top of the burette to let liposome solution flow downwards constantly. Finally, the purified liposome solution was collected from the bottom of the burette in 1 ml vials.

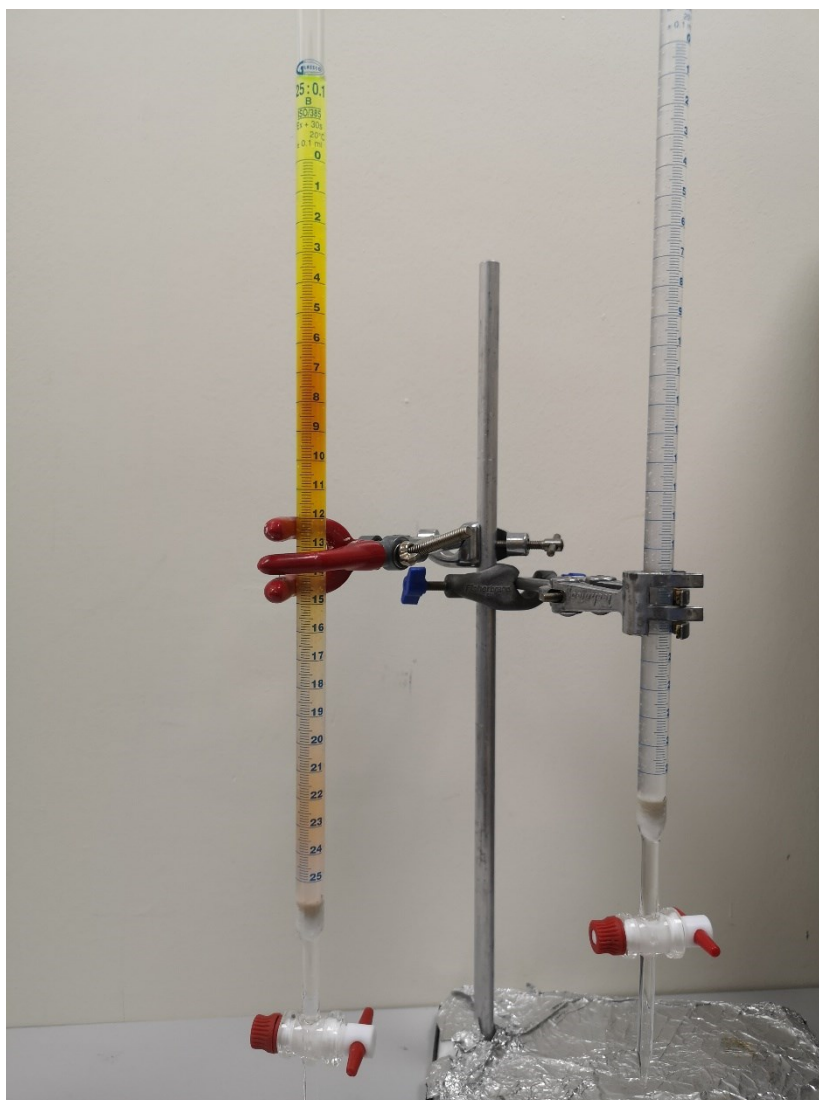


Figure 2.2 Photograph of the calcein disodium salt encapsulated liposome gel filtration process

As can be seen from Figure 2.2, the bottom layer with a light orange colour was filled with calcein encapsulated liposome solution. This is because the diameter of the liposome was much larger than the pore diameter of Sephadex G-50 beads and liposomes were completely excluded from the pores of gel beads. In that case, the calcein encapsulated liposome eluted first and reached the burette bottom faster than any remaining unencapsulated calcein. The upper layer with dark orange colour was filled with unencapsulated calcein because calcein was retarded inside the pores of gel beads and

eluted last. The bright green colour of the upmost layer was the diluted unencapsulated calcein. This is because the fluorescence intensity of calcein disodium salt increases as the concentration of calcein decreases until a limit, which will be discussed extensively in the following chapter. A clear gap between the upper and bottom layers can be observed in this column, which means the separation of unencapsulated calcein disodium salt from liposomes was successfully achieved.

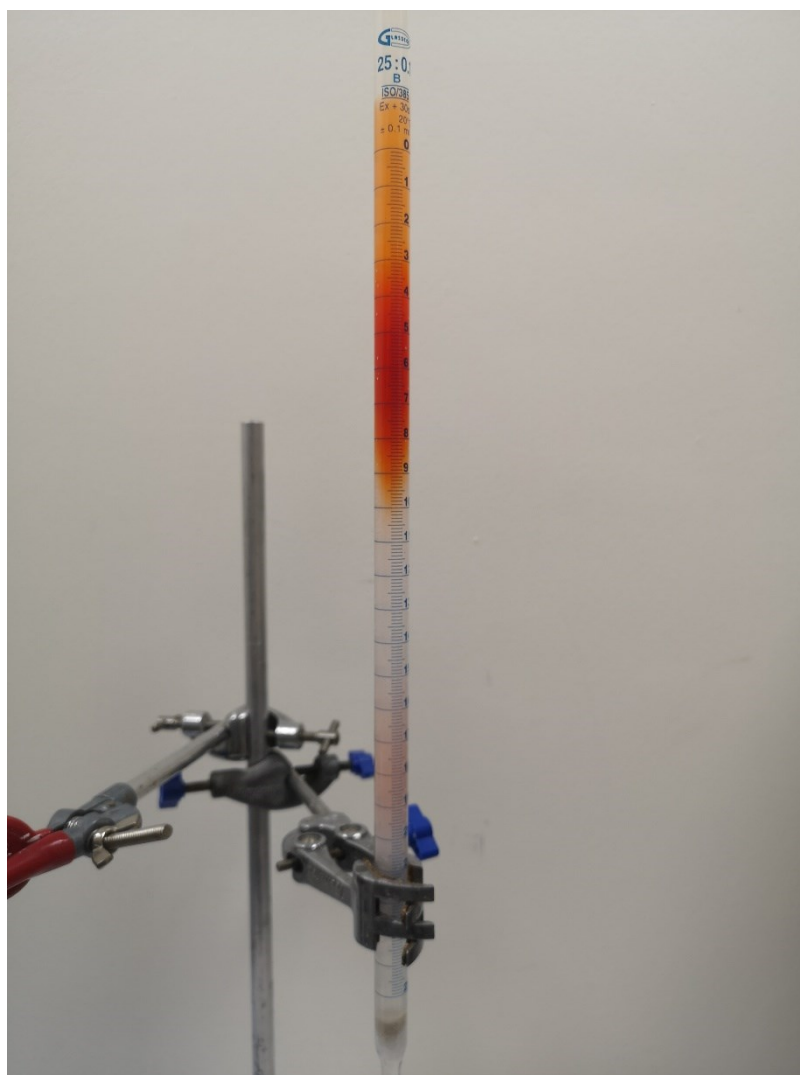


Figure 2.3 Photograph of the doxorubicin encapsulated liposome gel filtration process

For doxorubicin, the colours of the two layers in Figure 2.3 were different from those in Figure 2.2. The bottom layer with a light pink colour was composed of doxorubicin encapsulated liposomes. The unencapsulated doxorubicin formed the upper layer with dark red colour. A clear gap can also be observed in this column, which indicates the separation of doxorubicin encapsulated liposome from unencapsulated doxorubicin was achieved as well. As can be observed in this column, no bright colour in the upmost layer can be found. The dilution of doxorubicin merely induced a lighter red colour in the upmost layer rather than a strong enhancement on the fluorescence intensity just like calcein disodium salt. This is due to the differences of fluorescence characterisation of two materials.

2.2.7 Selection of gel filtration media

Molecular Weight Ranges for Gel Filtration Media

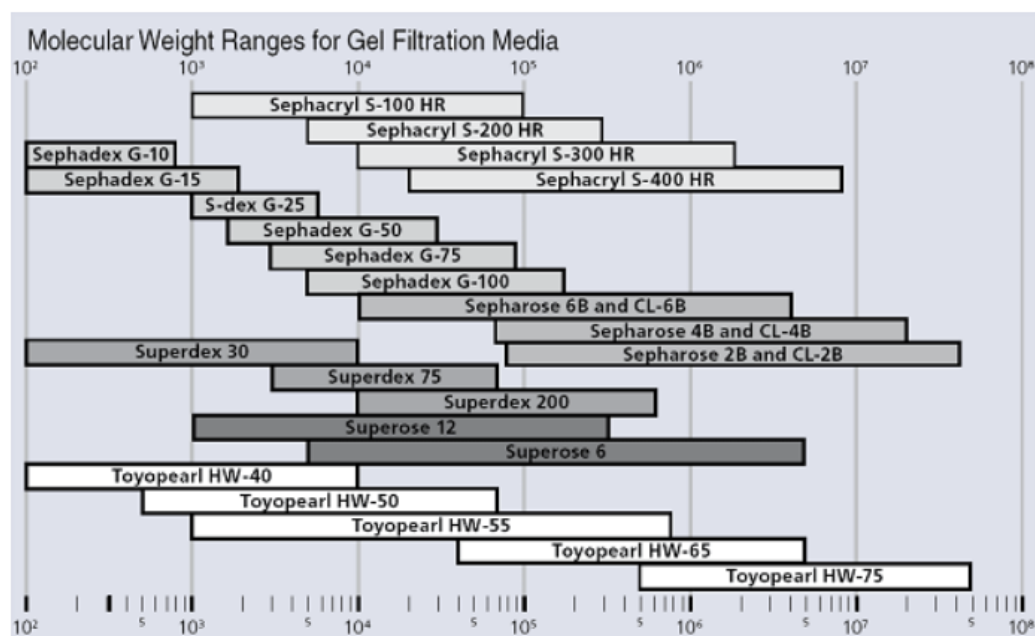


Figure 2.4 Molecular weight ranges for gel filtration media [189]

Figure 2.4 plots the appropriate molecular weight ranges which can be separated for a variety of different commercially available gels. Molecules whose molecular weight and hence size is located at the left side of the rectangle of a gel filtration media can completely enter inside the pores of gel beads and elute last. Nevertheless, molecules whose molecular weight is located at the right side of the rectangle of a gel filtration media can completely flow around gel beads without entering inside and elute first. Solutes whose molecular weight is located inside the range of the rectangle of the media will elute between the excluded and included fractions and its separation result will not be distinct.

The molecular weight of calcein disodium salt is 666.50 g/mol and that of doxorubicin is 543.52 g/mol. Both of them are located at the left side of the rectangle of Sephadex G-50. Since the Sephadex range is cheaper and more readily available than some of the other gels, Sephadex G-50 was therefore selected in the current experiments.

2.2.8 Liposome Characterisation: Zeta potential

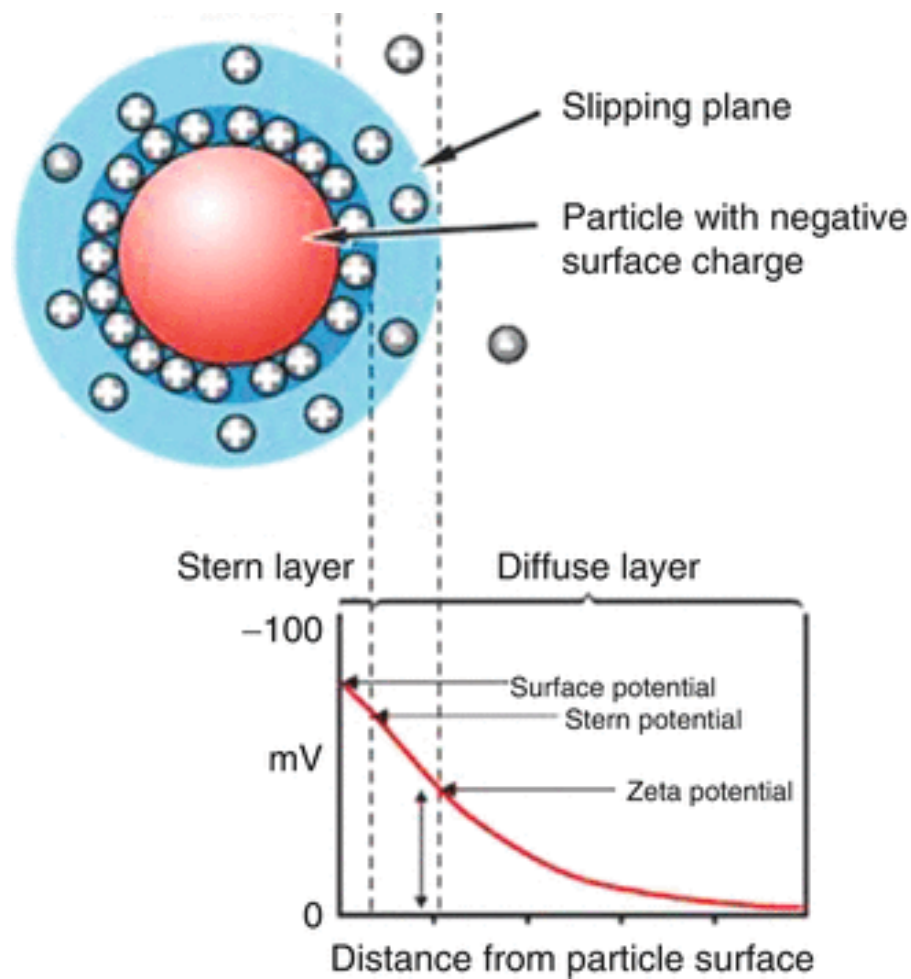


Figure 2.5 Figure demonstrating the surface potential, stern potential, and zeta potential as a function of distance of a spherical particle [190].

Two layers of counterions surround charged particles dispersed in a liquid: The inner (first) layer is called the “Stern Layer” which is formed by oppositely charged ions to the particle which are strongly bound to the particle surface through electrostatic forces. The second layer consists of weakly attracted ions outside Stern Layer which is defined as the “Diffuse Layer”. Zeta potential is the electrostatic potential at the boundary between the Diffuse Layer and the particle surface when an electric potential is applied [191].

The charge on the colloidal particle dispersed in the aqueous system is determined by the particle itself and the surrounding medium. The detachment of hydrogen ions on the particle surface results in a negative charge on it, and vice versa. Zeta potential has been regarded as an essential indicator of interaction between dispersed particles and the stability of the particle. The increase of zeta potential induces an increase of repulsive force between vesicles suspended in the solution and if high enough prevents aggregation of the vesicles [192]. A system with an extremely high or low zeta potential (above 30 mV or below -30 mV) is generally reckoned to be stabilised. The zeta potential is calculated by determining the velocity of a particle in a known electric field. Nowadays this velocity is determined by the fluctuation of light scattered through the motion of particles in an electrical field [193].

In the current work, the zeta-potential was determined using a Zetasizer Nano ZS (Malvern Panalytical, UK). The Smoluchowski model for estimating the zeta potential was selected as it is more appropriate than the Huckel model due to the larger size of the liposomes. All measurements were conducted at room temperature and were diluted to 0.5 mg/mL with de-ionised water. 1.6 mL solution was loaded in macro polystyrene cuvettes and was averaged over at least 6 runs.

2.2.9 Liposome Characterisation: Particle size

Transmission electron micrograph (TEM), size-exclusion chromatography (SEC) and dynamic light scattering (DLS) are commonly utilised to acquire particle size and size distribution.

Transmission electron micrograph (TEM) can provide intuitive images and morphology information of vesicles. Nevertheless, the structure of phospholipid bilayer of liposomes is

formed in an aqueous liquid solution and TEM technique requires a series of complicated preliminary treatments on liposomes before measurement, which could result in an undesired reduction of liposome size and shape deformation [194].

The size distribution of liposomes can be obtained using HPLC-SEC (High Performance Liquid Chromatography- size-exclusion chromatography), whereas lipids of liposomes may be spoiled during the process of adsorption in the HPLC column [193].

Dynamic light scattering (DLS) technique requires no preliminary treatment before measurement requires little sample preparation and the measuring process is simple and fast. Vesicles dispersed in sample solution are irradiated by a laser light source from the DLS instrument. The signal is collected after the light hits on vesicles and scatters. The Brownian movements of vesicles result in the alteration of scattering intensity with time, which correlates to the particle size. By analysing these fluctuations, the diffusivity and hence particle size can be calculated [193].

The particle size and size distribution were explored based on a dynamic light scattering platform Zetasizer Nano ZS (Malvern Panalytical, UK). All measurements were conducted at room temperature and were diluted to 0.5 mg/mL with de-ionised water. 1.6 mL solution was loaded in macro polystyrene cuvettes and each sample was repeated at least 5 times.

2.2.10 Liposome Characterisation: TEM

The research on advanced materials at the nanometre scale has reached the atomic scale in recent decades. To observe such atomic-scale materials, optical microscopes obviously

cannot meet the requirements, so more advanced research equipment is needed. Because fine structures smaller than 0.2 microns, which are called sub microscopic structures or ultrafine structures, cannot be seen clearly under an optical microscope. To see these structures clearly, it is necessary to choose a light source with a shorter wavelength to improve the resolution of the microscope. The wavelength of the electron beam is much shorter than that of visible light and ultraviolet light, and the wavelength of the electron beam is inversely proportional to the square root of the voltage at which the electron beam is emitted, In other word, the higher the voltage, the shorter the wavelength. Based on this principle, Ernst Ruska and Max Knolls invented the first electron microscope in 1931, which was converted into a transmission electron microscope (TEM) in 1933 by the company of Ernst Ruska and Sieman. With the continuous advancement of transmission electron microscope technology, its resolution has been improved from 2-3 nm in 1938 to the current 0.2 nm. TEM has a wide range of applications such as biology, microbiology, nanotechnology, forensic research. Table 2.1 compares the resolution and magnification of several commonly used microscopes.

Table 2.1 The summary of the resolution and magnification of several microscopes and the naked eye

	Resolution	Magnification
Naked eye	0.2 mm	1:1
Optical microscope	0.2 μm	1000:1
Scanning electron microscope	3.5-10 nm	2000:1
Transmission electron microscope	0.2 nm	1000000:1

The working principle of a transmission electron microscope (TEM) is to transmit an accelerated and focused electron beam onto a very thin sample, and the electrons collide with atoms in the sample to change their direction, resulting in solid angle scattering. The size of the scattering angle is related to the density and thickness of the sample, so images with different brightness and darkness can be formed, and the images will be displayed on the imaging device after zooming in and focusing. The current transmission electron microscopes on the market are basically composed of three parts: electron optical system, vacuum system, and power supply system. The electron optical system is inside the lens barrel, which is arranged with illumination system, sample room, imaging system and image recording system from top to bottom, as demonstrated in Figure 2.6.

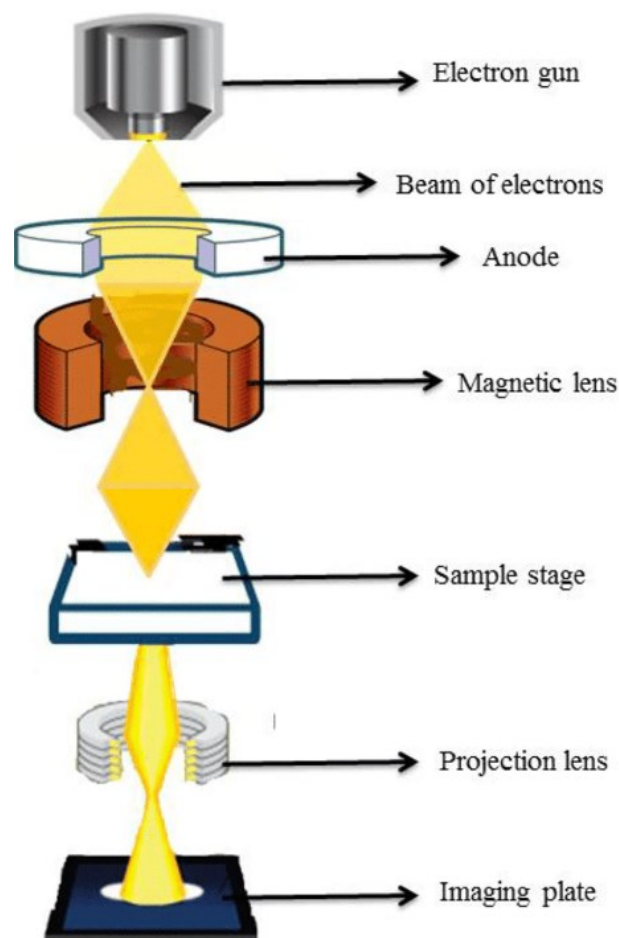


Figure 2.6 Working principle of TEM [195]

The advantages and limitations of transmission electron microscopy are listed in the table 2.2.

Table 2.2 The summary of merits and restrictions of TEM

Merits	Restrictions
It can produce very efficient, high-quality images with high definition	It is very sensitive to vibration and electromagnetic movement, so it can only be operated in isolated areas
It can be used to obtain a wealth of information about compounds and their structures	Its purchasing expense and maintenance charge are very expensive
Powerful magnification, about 1000 times that of an optical microscope	It can only produce monochrome and 2D images
It can produce permanent images	The electron radiation it produces can cause damage to the sample
The operation procedure is relatively simple and easy to train	Samples need to be thin and electron transparent
It has a wide range of applications, from basic biology to nanotechnology, from educational to industrial applications	Its resolution is so high that only a small portion of the sample can be observed

All TEM micrographs were taken using JEM-1400 Flash (JEOL, Japan) microscope. All samples needed to be negatively stained firstly for a better imaging of TEM. To be specific, Plain liposome or chitosan coated liposome solution were diluted to 0.1 mg/ml. 3 μ L of liposome solution was dropped on the ultrathin carbon film of copper grid. After waiting 60 seconds, the excess of the sample on the grid was blotted by a wet blotting paper. Instantly after the blotting, 3 μ L of ultra-pure water was placed on the grid. After 5 seconds, the excess of ultra-pure water was blotted as well. Then, 3 μ L of 2% (w/w) solution of phosphotungstic acid hydrate was dropped on the same grid for 10 seconds. The excess of solution was blotted too. Finally, the negatively stained sample was placed until it is completely dry at the room temperature. The grid with the sample then placed in the TEM for imaging.

2.3 Results and discussion

2.3.1 The influence of ultrasonic time on the liposome size

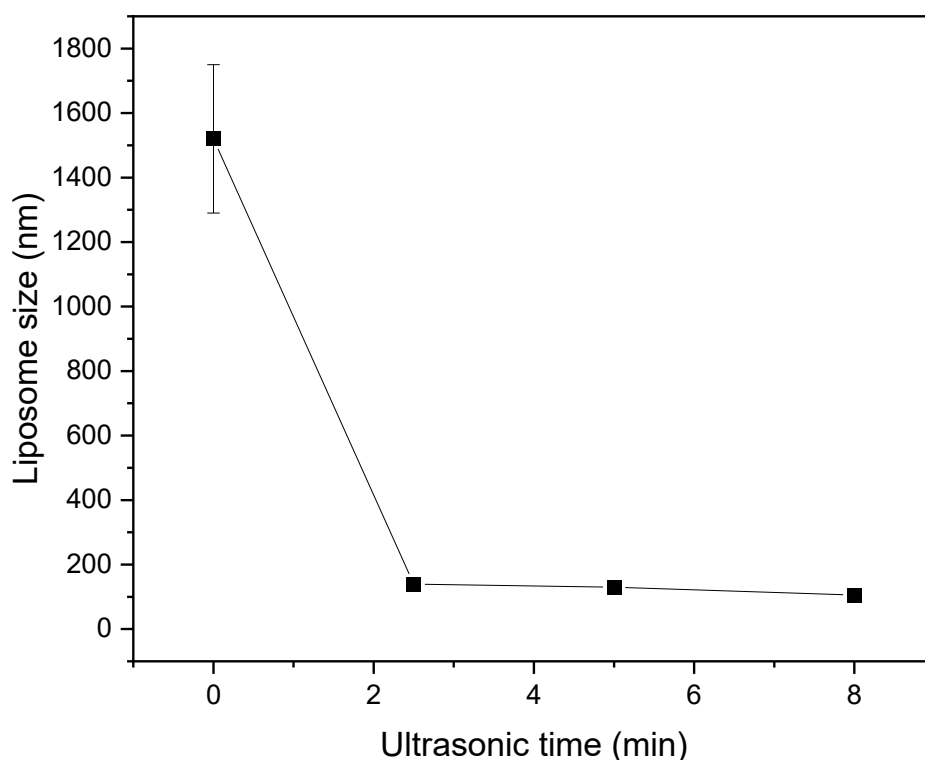


Figure 2.7 The particle size of plain liposome after ultrasonication. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.7 depicts the variation of particle size of plain liposome after a specific time of ultrasonication. Before ultrasonication, the liposomes were very large with a diameter of 1520 ± 230 nm. After 2 and a half minutes of ultrasonication, the particle size was reduced substantially to 139 ± 3 nm. Then the number declined slightly to 129 ± 18 nm after 5 minutes and 105 ± 4 nm after 8 minutes. The extremely high standard deviation (230 nm) of plain liposome before ultrasonication means the difference between each liposome was relatively large and low standard deviation (less than 4 nm after 8 minutes) after ultrasonication means that the liposome suspension achieved a high level of homogeneity. The dramatic decline on the liposome size strongly suggests the successful transformation of liposome structure from multi-lamellar vesicles (MLV) to uni-lamellar vesicles (UV).

Therefore, it can be verified that the ultrasonication procedure cannot only decrease the size of liposome dramatically but also increase the uniformity of liposomes.

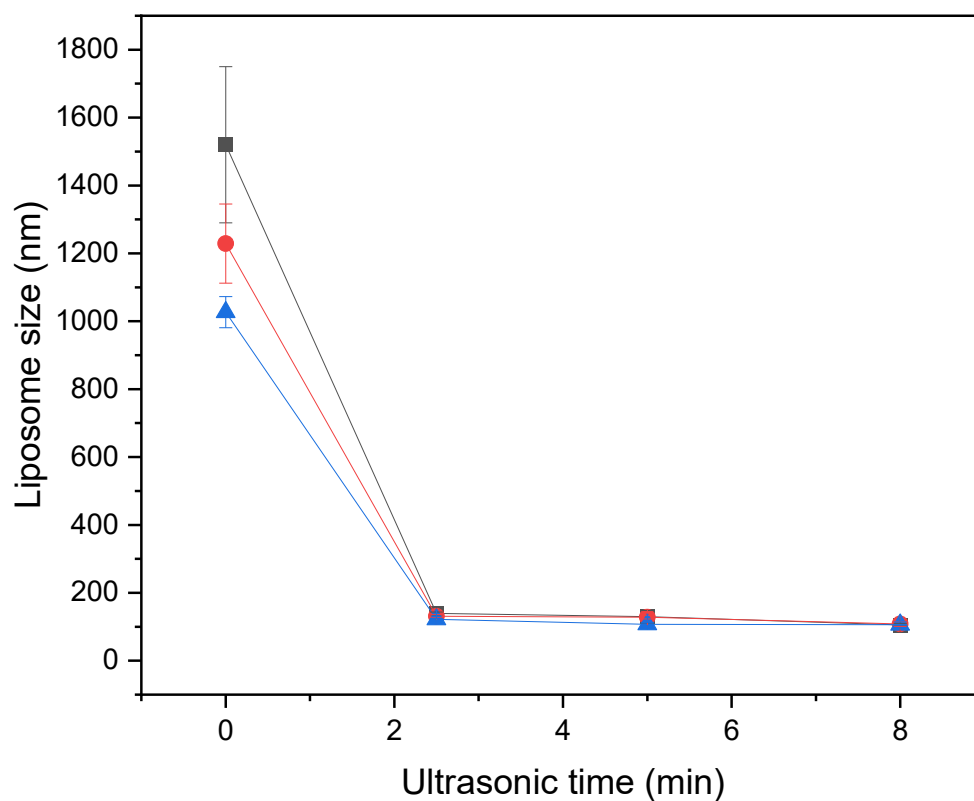


Figure 2.8 The particle size of plain liposomes (■), calcein encapsulated liposomes (●) and doxorubicin encapsulated liposome (▲) after various time of ultrasonication. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.8 compares the particle sizes of three kinds of liposomes, namely plain liposomes, and liposomes containing encapsulated calcein or doxorubicin. It is clear that after 2.5 minutes of ultrasonication, all three kinds of liposomes with or without drugs encapsulation had similar diameters. Nevertheless, before ultrasonication, liposome size of three kinds varied a lot. This is because the majority of structures of liposomes before ultrasonication were MLVs which had more random diameters and forms. The similar diameters of three

kinds of liposomes after 2.5 minutes ultrasonication proved that the encapsulation of calcein or doxorubicin using passive loading method did not alter the particle size of the liposomes significantly.

2.3.2 Isoelectric point of lipids and different MW chitosan

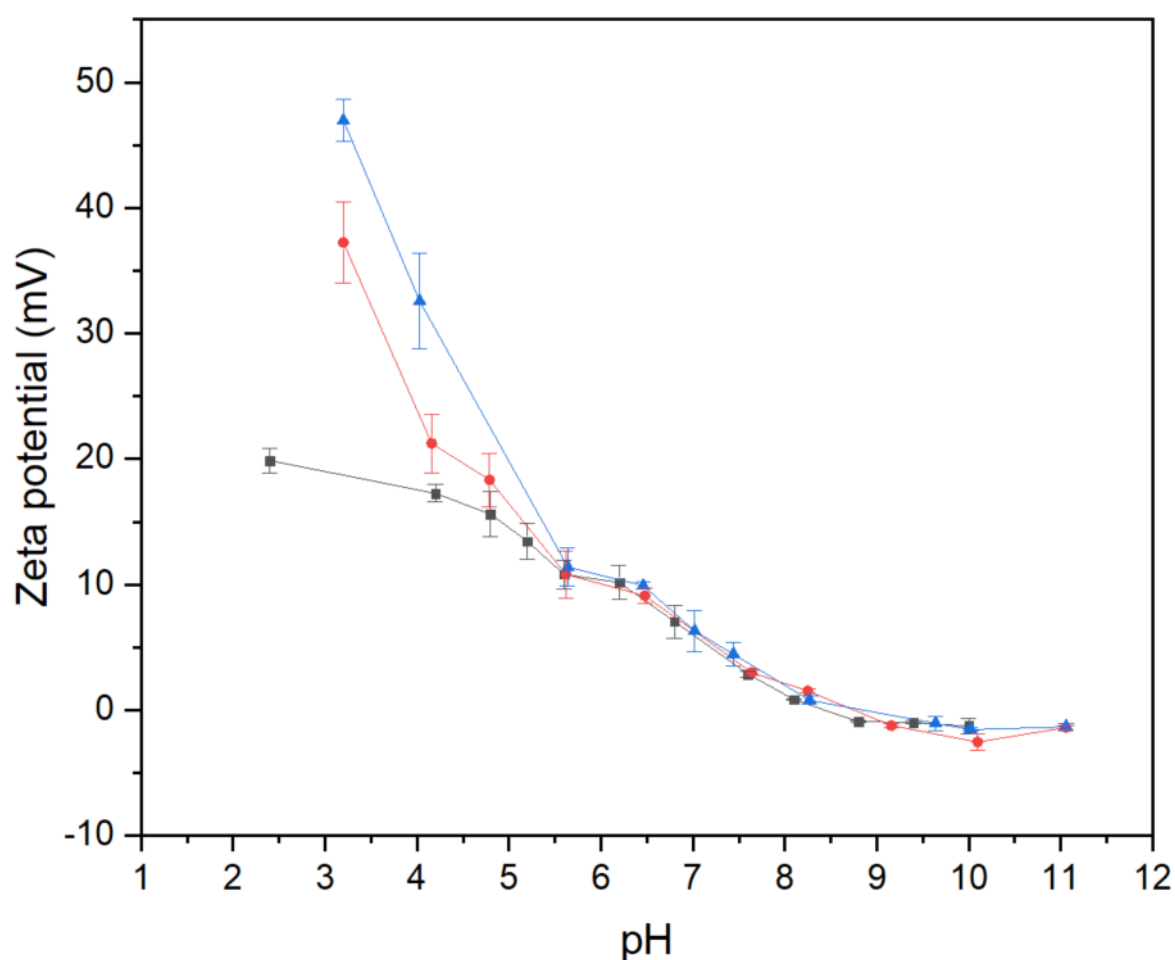


Figure 2.9 The effect of pH on the zeta potential of 0.1% (w/w) low MW chitosan (■), 0.1% (w/w) medium MW chitosan (●) and 0.1% (w/w) high MW chitosan (▲). Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 2.9 compares the change of zeta potential of three MW chitosan solution depends on pH. As can be observed from this graph, all three curves experience declines as pH rises. Besides, these curves are almost the same when the pH is above 5.5, dropping steadily from around 10.8 mV at pH 5.5 to almost zero at pH 11. Below pH 5.5, high MW chitosan keeps the highest zeta potential with a decrease from 47.0 mV at pH 3.2 to 11.4 mV at pH 5.6 while low MW chitosan remains with the lowest value of zeta potential, with the smallest decline of approximately 10 mV. In addition, tiny deviations are found in all three curves above pH 5.5 while, below that, zeta potential of the three have relatively high deviations.

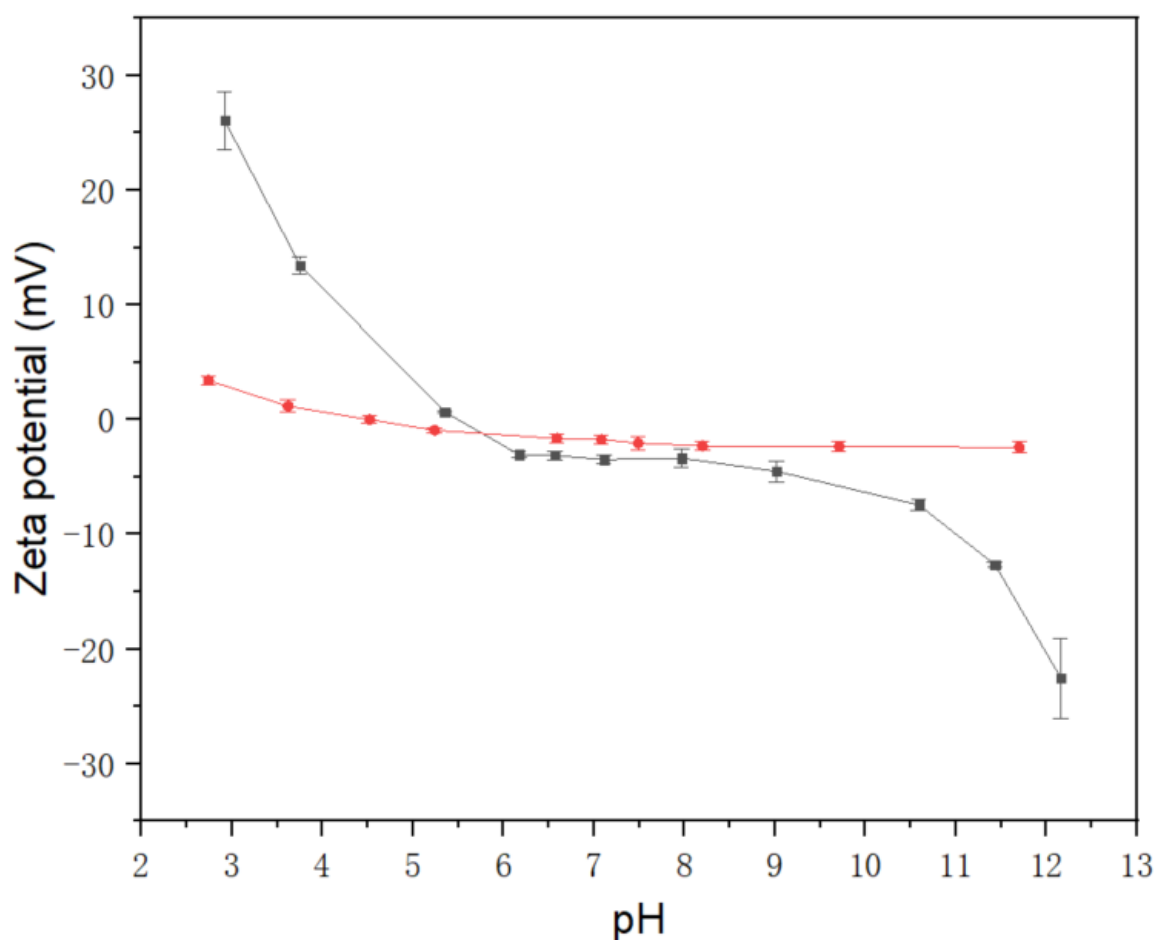


Figure 2.10 The effect of pH on the zeta potential of DPPC (■) liposomes and Brij S20 (●) micelles. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 2.10 reveals how the zeta potential of DPPC liposomes and Brij S20 micelles vary with the pH. It is manifest that zeta potential values of both curves descend as the pH value ascends, which form an inverse relationship. To be specific, from extreme acidic environment (pH 2.7) to strongly alkaline solution (pH 11.7), the zeta potential of Brij S20 witness a slight variation of roughly 5.0 mV, decreasing from 3.4 mV to -2.4 mV. At pH 2.9, the zeta potential value of DPPC is 26.1 mV, which is 9 times more than that of Brij S20. Then, the value of DPPC decreases substantially to -3.1 mV at pH 6.2, before dropping marginally to -4.5 mV at pH 9.0. Finally, it plummets to -22.6 mV at pH 12.1 with a high deviation of 3.5 mV. Overall, it can be concluded that DPPC is more sensitive than Brij S20 to the change of pH, due to the zwitterionic nature of DPPC and the non-ionic nature of Brij S20.

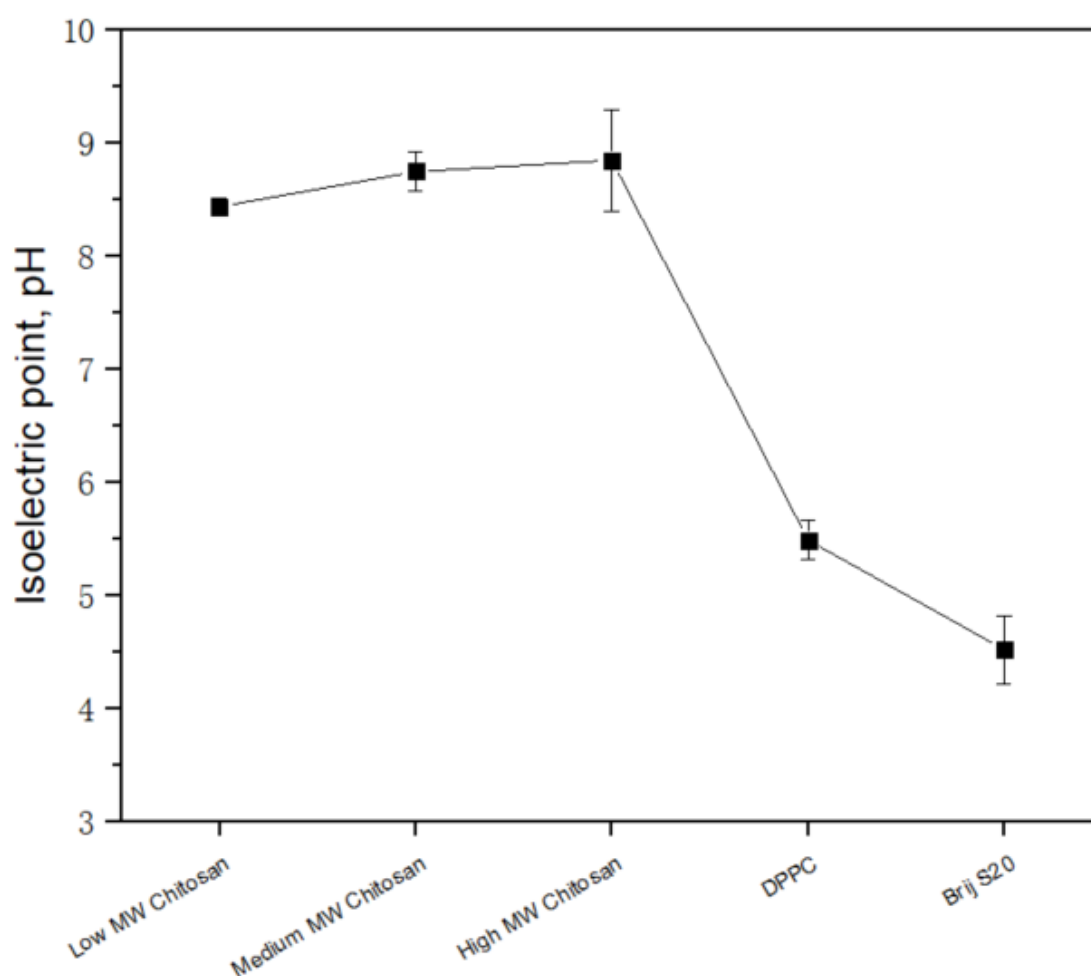


Figure 2.11 The isoelectric point (IP) of 0.1% (w/w) low, medium, high MW chitosan, DPPC and Brij S20. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.11 compares the IP value of 0.1% (w/w) low, medium, high MW chitosan, DPPC and Brij S20. The isoelectric point (IP) means the pH when the zeta potential of selected chemical compound reaches 0 mV. It is apparent that all three MW chitosan need weakly alkaline environment to make their zeta potential equals zero. By contrast, two kinds of lipids, DPPC and Brij S20, need acidic environment. To be specific, IP values of three MW chitosan are fairly similar. As the molecular weight of chitosan increases, its IP value rises marginally, from 8.4 of low MW chitosan to 8.9 of high MW chitosan. Nevertheless, the difference between IP values of DPPC and Brij S20 are relatively significant. The IP of DPPC is 5.5 while that of Brij S20 is 4.5.

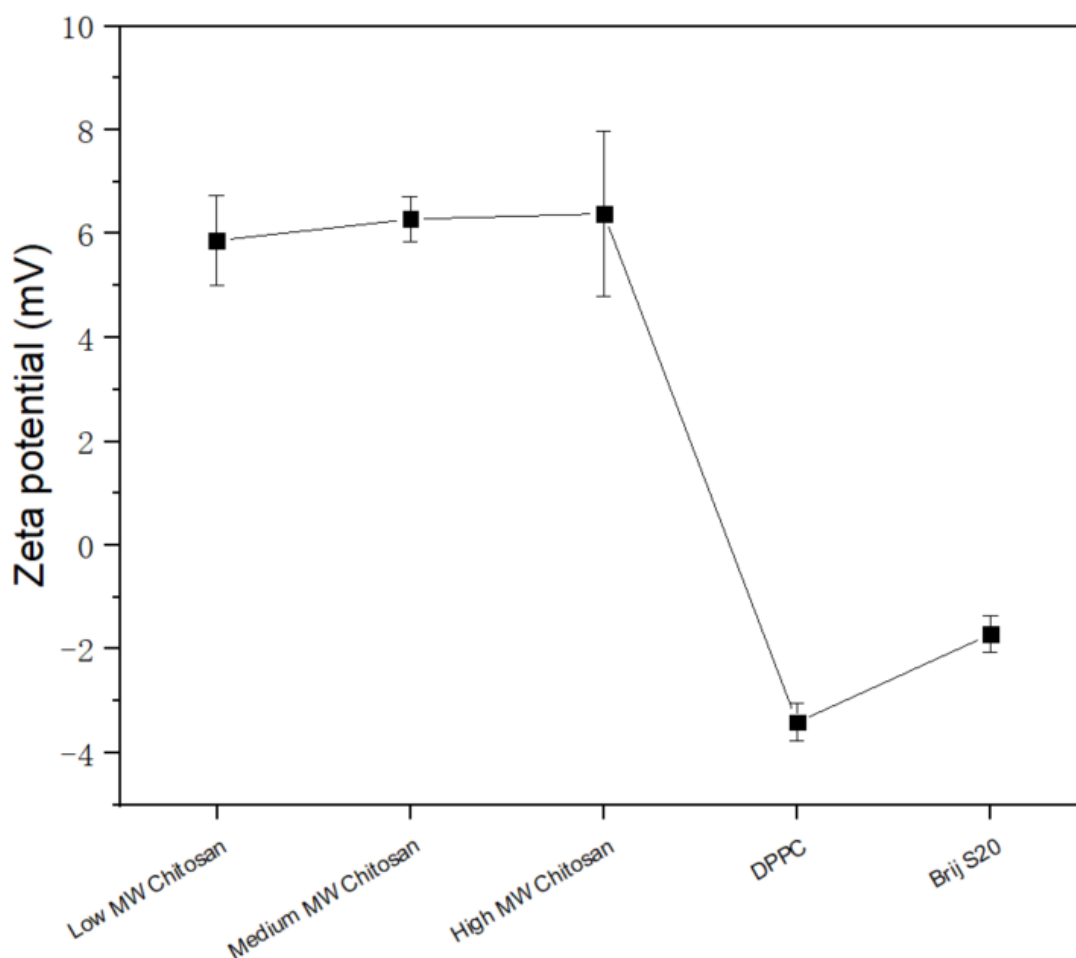


Figure 2.12 The zeta potential of 0.1% (w/w) low, medium, high MW chitosan, DPPC and Brij S20 when the pH value is 7. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.12 displays the zeta potential of three MW chitosan and two lipids in the neutral environment. It can be observed from this graph that all three chitosan are positively charged when pH is 7 with a tiny difference. To be specific, high MW chitosan has the largest zeta potential of 6.4 mV and the smallest zeta potential of 5.9 mV is discovered in low MW chitosan. In contrast, DPPC and Brij S20 have negative charges in the neutral solution, among which DPPC has a smaller zeta potential of -3.4 mV while the zeta potential of Brij S20 is -1.7 mV. This result indicates that all three MW of chitosan are able to attach on the liposome composed of DPPC and Brij S20 theoretically by electrostatic force in a neutral environment, which will be further proved by TEM images in the following section.

2.3.3 The influence of different molar ratio of lipids on particle size and zeta potential of liposomes

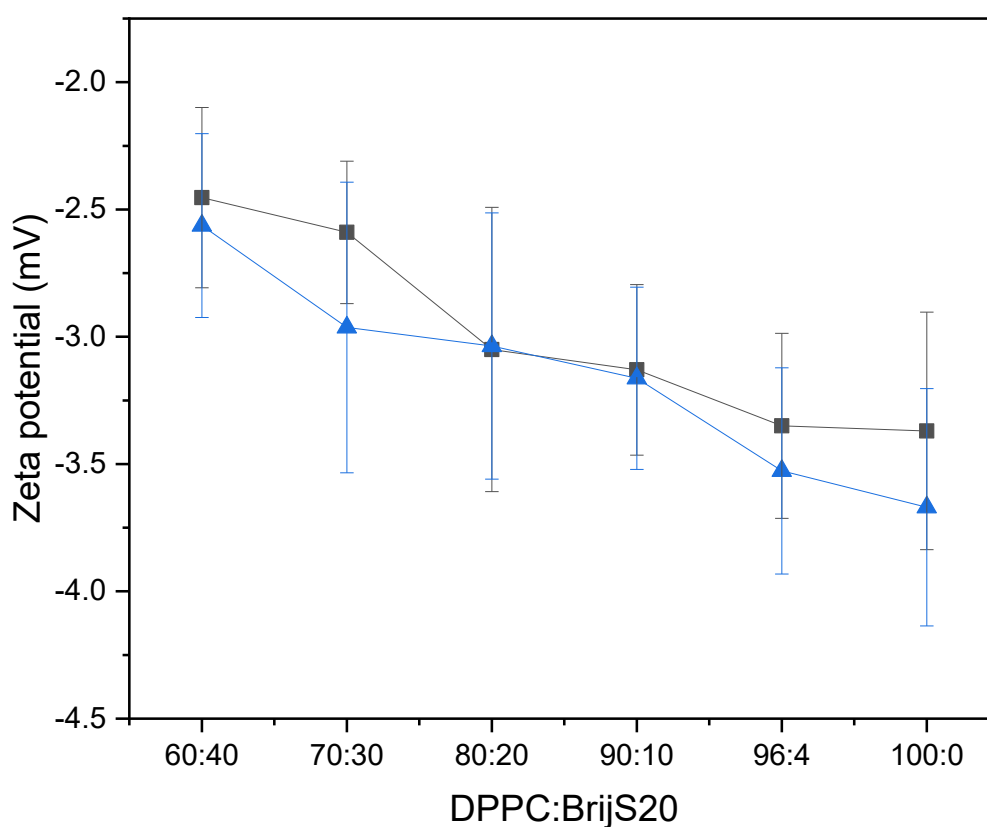


Figure 2.13 The influence of various molar ratio of lipids on zeta potential of freshly made liposomes (■), liposomes after one month storage at 4 °C (▲) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.13 makes comparison of liposomes with different molar ratio of DPPC and Brij S20 and their variations after one month storage at 4 °C. Apparently, as the molar percentage of DPPC rises, the zeta potential of liposome descends, which is because the zeta potential of

DPPC is lower than that of Brij S20 in neutral environment, as demonstrated in Figure 2.12. To be specific, the zeta potentia of freshly made liposomes decreases gradually from -2.5 mV at '60:40' to -3.4 mV at '100:0'. In addition, the zeta potential of plain HaT liposome, whose molar ratio is 96:4, rises marginally from -3.4 mV to -3.5 mV after storage. Also, it can be observed that the changes between all fresh samples and stored samples are not significant (less than 0.4 mV), especially in the group of '80:20' and '90:10', which indicates that the one month storage does not has an essential influence on their zeta potential.

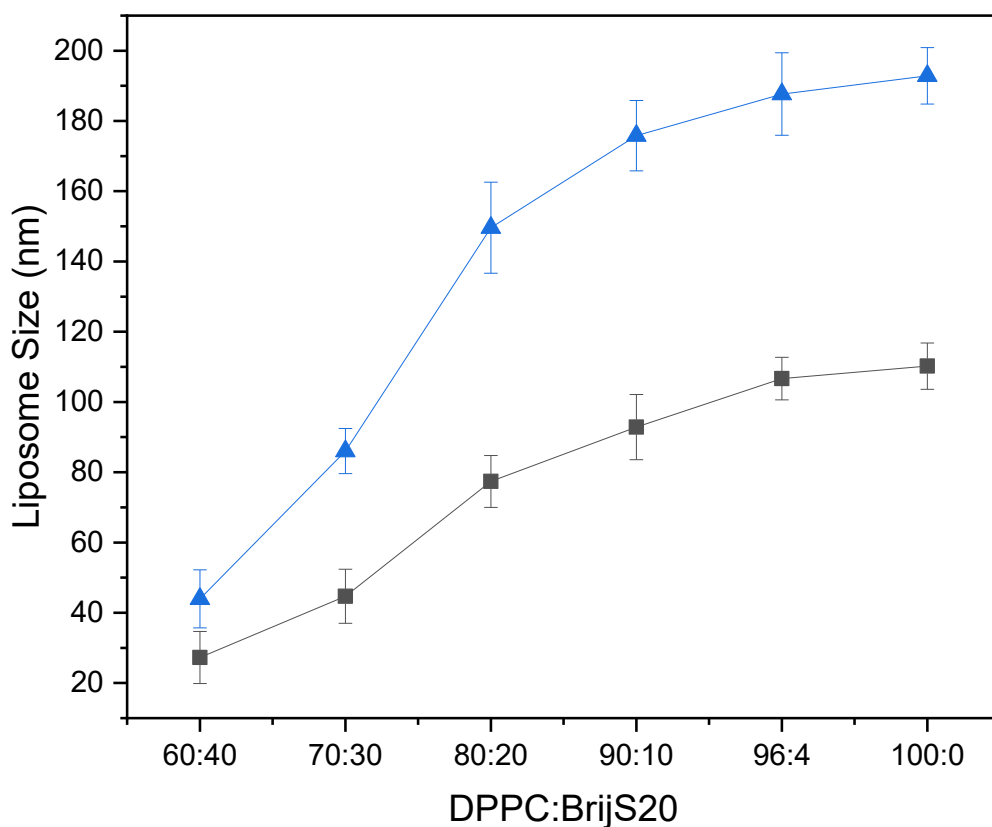


Figure 2.14 The influence of molar ratio of lipids on liposome size of freshly made liposomes (■) and liposomes after one month storage at 4 °C (▲) in deionised water. Data represents mean ± SD. n=3 independent batches.

Figure 2.14 shows the size variation of liposomes with different molar ratio of lipids. It can be discovered that, as the molar ratio of DPPC ascends, the sizes of both fresh and stored liposomes increase gradually. Specifically, the fresh '100:0' liposome is around 4 time larger than '60:40' liposome. Besides, after storage, the size of each group increase by a factor of approximately 1.5-2 times. The results indicates that the higher percentage of lysolipid such as Brij S20 in liposome composition decreases the liposome size drastically. This is probably because lysolipid contains only one fatty acid chain as lipid tail, thus Brij S20 molecule is smaller than DPPC molecule sterically.

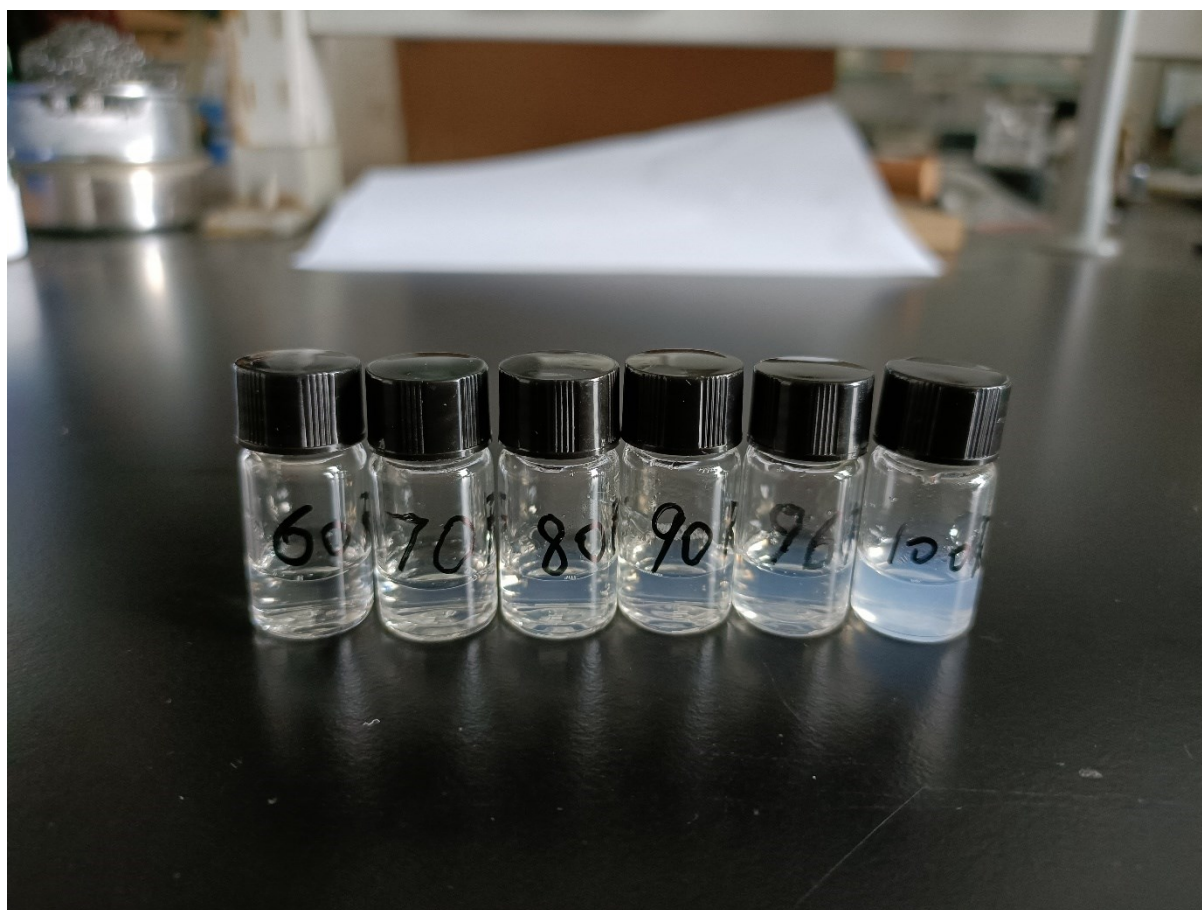


Figure 2.15 Photograph of liposomes solution consisted of different molar ratio of DPPC and Brij S20 in deionised water. Numbers on the vials indicate the molar ratio of DPPC in the liposome composition.

Figure 2.15 depicts the fresh liposomes solution with various molar ratio of lipids. It is manifest that the higher DPPC molar percentage makes the liposome solution more turbid. That is due to the rise of liposome diameter, which is in accordance with the above argument of Figure 2.14.

2.3.4 The TEM graphs of different MW chitosan coated liposomes

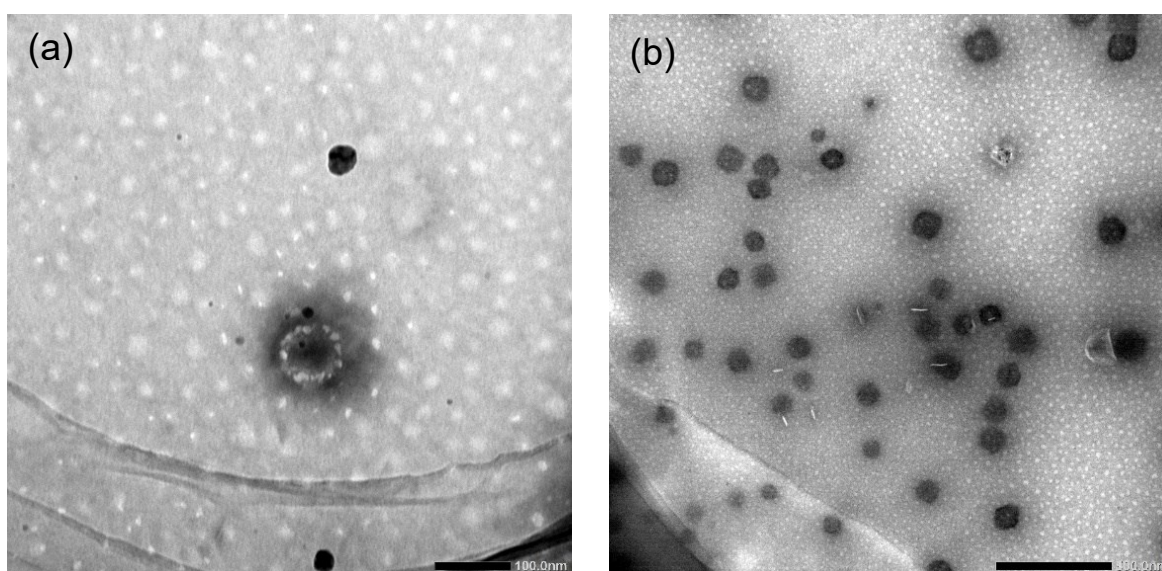


Figure 2.16 Close view (a) and distant view (b) of TEM images of freshly made plain HaT liposome. The scale bar of TEM image (a) is 100 nm and that of TEM image (b) is 500 nm.

It can be observed from Figure 2.16 (b) that most plain HaT liposomes which are freshly prepared have a near-spherical shape. Nevertheless, some oval liposome can also be found in Figure 2.16 (b). It is apparent that most liposomes are separated and not aggregated as shown in Figure 2.16 (b). Still, both the close shot and distant view of TEM images indicate that most freshly made plain HaT liposomes have uniform sizes of roughly 80-110 nm.

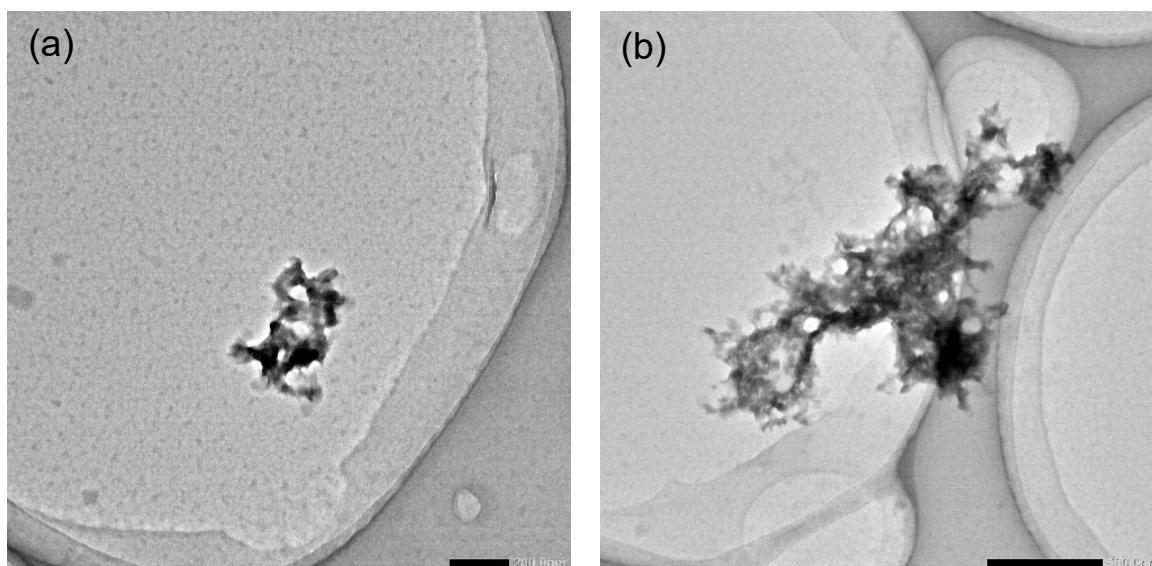


Figure 2.17 Close view (a) and distant view (b) of TEM images of plain HaT liposome after one month storage at 4 °C. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 500 nm.

As demonstrated in Figure 2.17 (a) and (b), after one month storage at 4 °C, plain HaT liposomes experienced significant variation both in their size and morphology, changing from unified near-spherical shape into various forms with a remarkable increase on their size. Figure 2.17 (a) presents the aggregation and fusion of a small amount of liposomes with a compact structure. Besides, in a few cases, a large number of liposomes aggregate and fuse together forming massive clots, which are presented in Figure 2.17 (b). These liposomes clots are easily becoming sediments and obviously not suitable for intravenous injection due to their tremendous diameters (approximately 600nm to 1000nm). Therefore, it is demonstrated that, after one month of storage at 4 °C, plain HaT liposomes without chitosan coating tend to aggregate and fuse easily.

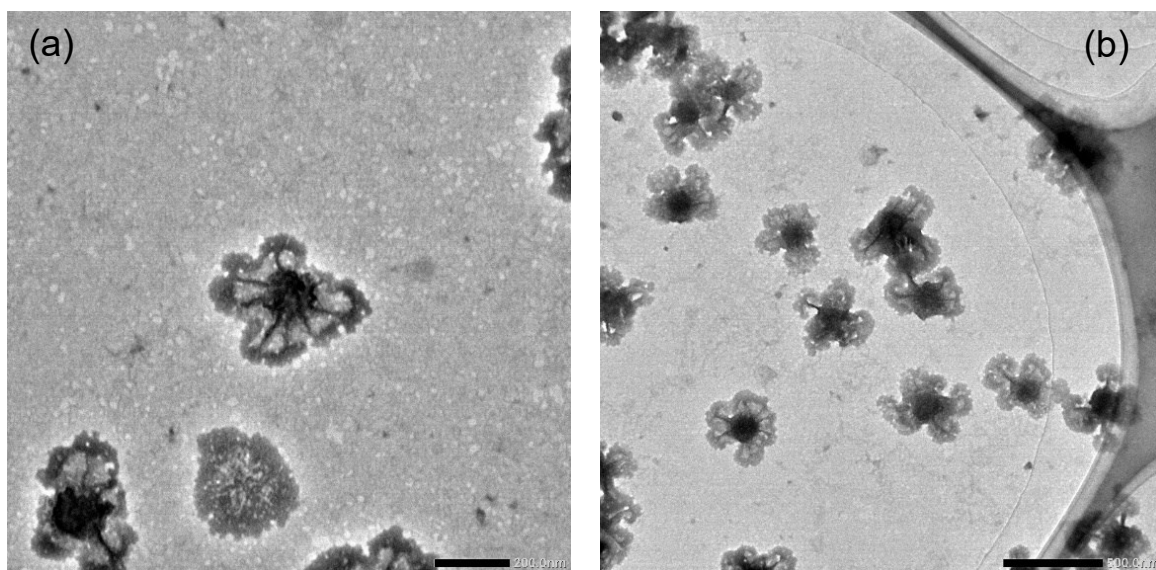


Figure 2.18 Close view (a) and distant view (b) of TEM images of freshly made 0.1% (w/w) low MW chitosan coated HaT liposome. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 500 nm.

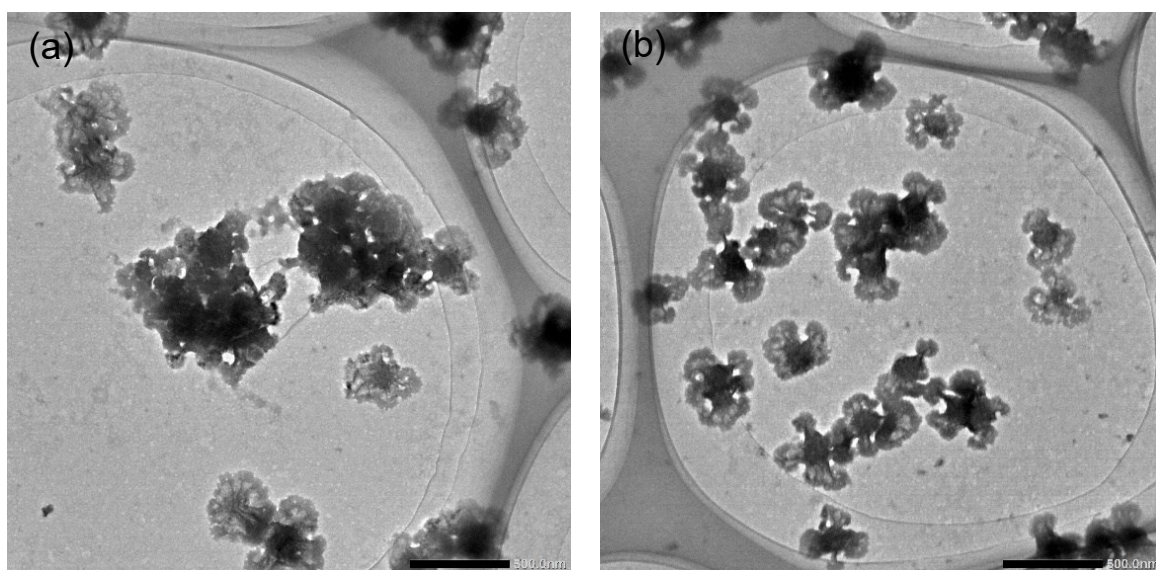


Figure 2.19 The distant view (a,b) of TEM images of 0.1% (w/w) low MW chitosan coated HaT liposome after one month storage at 4 °C. The scale bars of TEM images (a,b) are both 500 nm.

It can be observed from Figure 2.18 that the average overall size of 0.1% (w/w) low MW chitosan coated HaT liposome are around 200-350 nm and chitosan coatings are about 100-150 nm thick. What is noteworthy is that they have 'flower' shapes, whose 'petals' are their chitosan coatings and 'hearts' are liposomes. This special structure shows that positive charged chitosan successfully attaches on the negatively charged surface of liposomes. Nevertheless, part of liposome surfaces is not well covered with chitosan. Thus, slight aggregations can be discovered even in freshly made 0.1% (w/w) low MW chitosan coated liposome, as shown in Figure 2.18 (b). Furthermore, as demonstrated in Figure 2.19, aggregations become more widespread and greater after one month storage, which indicates the 0.1% (w/w) low MW chitosan coating is not efficient enough to prolong the shelf life of liposome. However, the phenomenon of aggregation and fusion among 0.1% (w/w) low MW chitosan coated liposomes are improved compared to those of plain liposomes, which indicates the effectiveness of chitosan coating on preventing liposomes from aggregation and fusion. This is probably because the coating of chitosan on liposome forms a spatial shield and electrostatic forces among liposomes due to positive charge of chitosan.

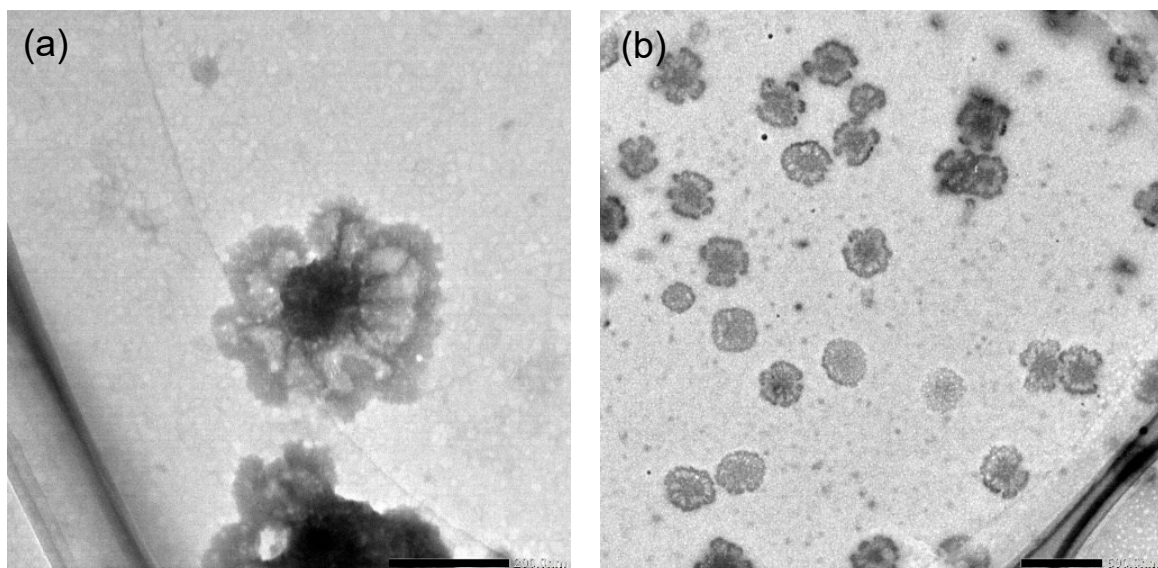


Figure 2.20 Close view (a) and distant view (b) of TEM images of freshly made 0.1% (w/w) medium MW chitosan coated HaT liposome. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 500 nm.

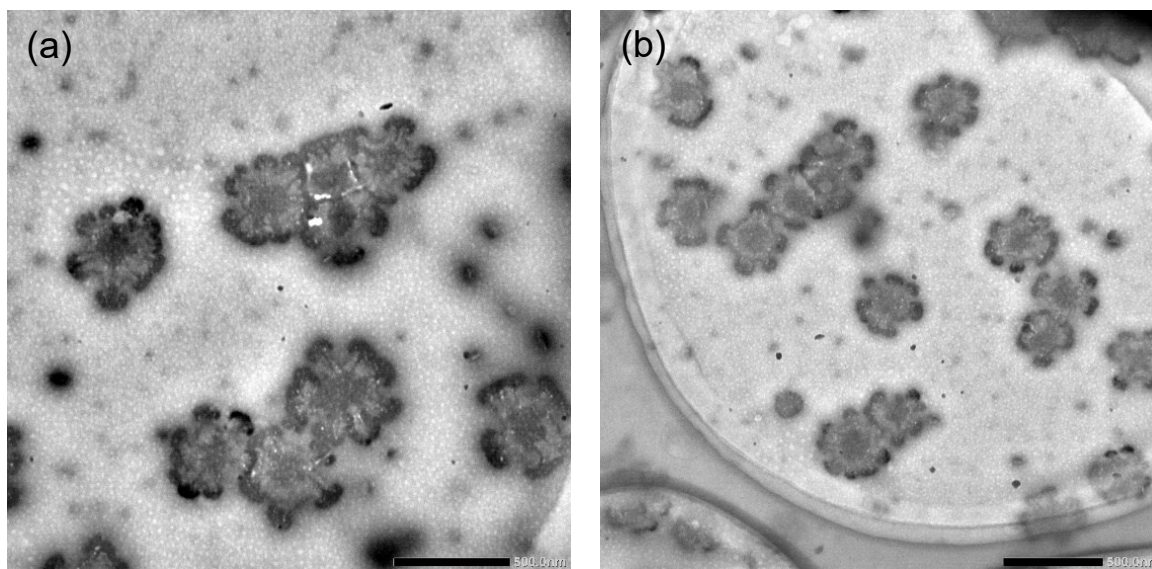


Figure 2.21 The distant view (a, b) of TEM images of 0.1% (w/w) medium MW chitosan coated HaT liposome after one month storage at 4 °C. The scale bars of TEM images (a,b) are both 500 nm.

As illustrated in Figure 2.20, the average diameters of 0.1% (w/w) medium MW chitosan coated HaT liposomes are approximately 250-350nm and their chitosan coatings have a thickness of around 150-250nm, which is slightly longer than that of low MW chitosan. The structure remains 'flower' shape but most of liposome surfaces are covered with chitosan, making the structure more spherical. This could be because the longer chain of chitosan polymer molecules makes the chitosan coating layer more compact and thicker. It can be also observed that there are rare cases of freshly made medium MW chitosan coated liposomes aggregating together. Furthermore, after one month storage, as shown in Figure 2.21, most liposomes remain separated, and the level of aggregation are much slighter than that of low MW chitosan coated liposomes because the boundaries of each liposome in the

aggregated clot are clear and not twisted together. Thus, medium MW chitosan demonstrates an enhanced ability to make liposomes more stable during the storage.

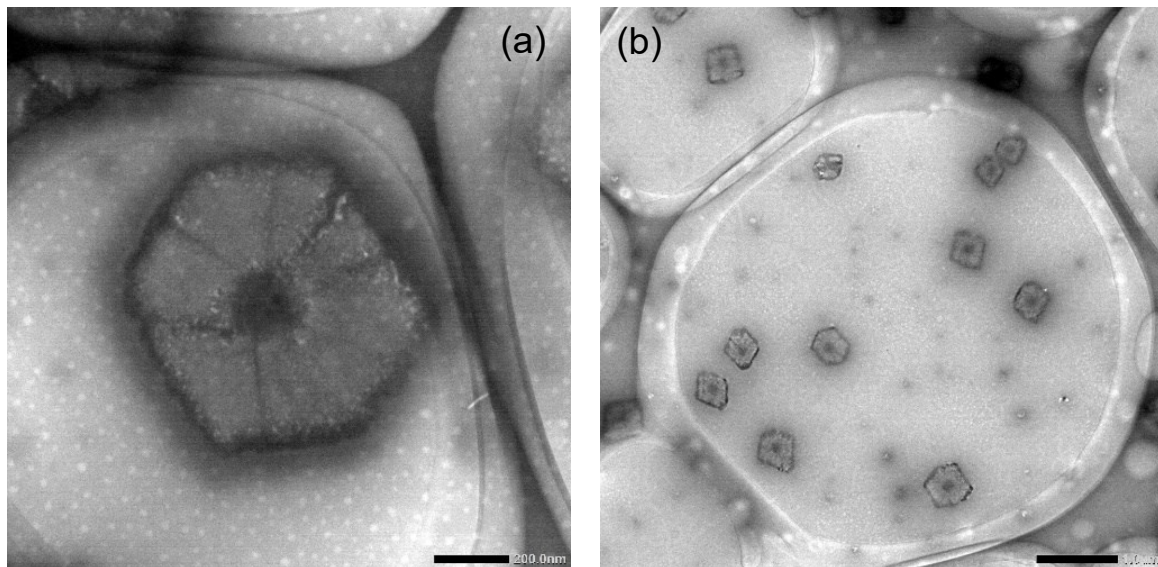


Figure 2.22 Close view (a) and distant view (b) of TEM images of freshly made 0.1% (w/w) high MW chitosan coated HaT liposome. The scale bar of TEM image (a) is 200 nm and that of TEM image (b) is 1 μ m.

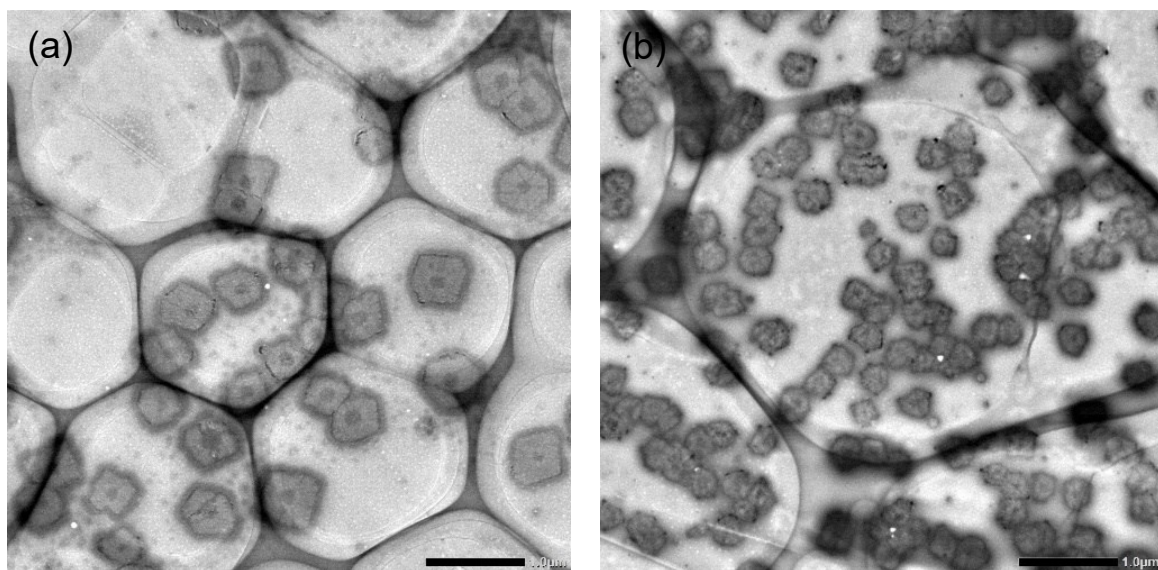


Figure 2.23 The distant view (a, b) of TEM images of 0.1% (w/w) high MW chitosan coated HaT liposome after one month storage at 4 °C. The scale bars of TEM images (a,b) are both 1 μ m.

Figures 2.22 and 2.23 depict the TEM images of 0.1% (w/w) high MW chitosan coated HaT liposomes. It is manifest that their diameters increased dramatically to around 400-600nm with a 300-500nm thick chitosan coatings. Most importantly, their shapes turn into regular hexagon or quadrangle. That is because the polymer chain of high MW chitosan is longer than that of medium MW chitosan, inducing the chitosan coating become more compact and thicker. The thick chitosan coating on the liposomes provides the most effective protection from aggregation and fusion, which can be proved in Figure 2.22. It is difficult to discover any aggregated or fused liposomes in the freshly made liposomes samples. Nevertheless, many deformed and aggregated liposomes were found in Figure 2.23 after one month storage, which may because the high MW chitosan coating is too thick that makes it more elastic or because the detachment of part of chitosan from liposomes leads to the non-uniformity of chitosan coating. Besides, it is apparent that the 400-600nm diameter of 0.1% (w/w) high MW chitosan coated liposomes is too large for intravenous injection, because it will be easily recognized by human immune system and eliminated by endocytic cells. Additionally, it has been proved by other researchers that the high MW chitosan and chitosan with high degree of deacetylation presented the highest toxicity [196] [197]. Overall, it is verified that all three MW chitosan coating can enhance the stability of HaT liposomes after long time storage in contrast with that of plain liposomes.

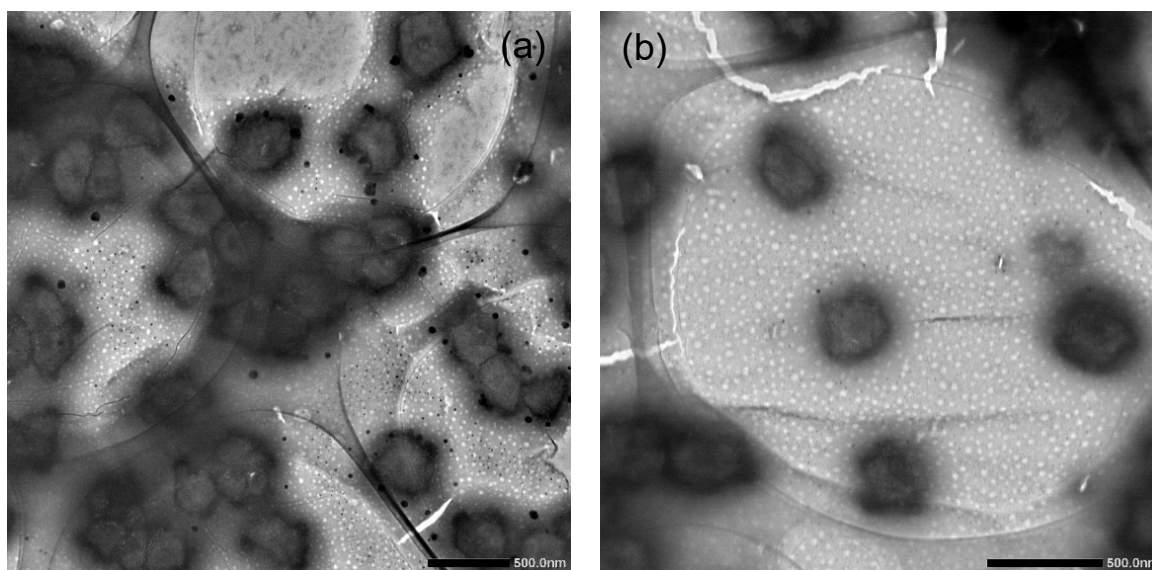


Figure 2.24 The distant view (a, b) of TEM images of liposomes samples pre-treated by wrong procedure of negative staining. (a) Liposome sample was over stained, and it is difficult to observe details of these kinds of samples. (b) The carbon layer on the copper grid for TEM was fractured due to the wrong procedure. The scale bars of TEM images (a,b) are both 500 nm.

Figure 2.24 (a,b) feature the negative examples of using the wrong procedure of negative staining before TEM imaging. To be specific, 3 μL of diluted 0.1 mg/ml liposomes solution was dropped on the carbon layer and air dried without blotting. After 10 minutes of waiting, 3 μL of 2% (w/w) solution of phosphotungstic acid hydrate was dropped on the same copper grid and air dried again without blotting. The greatest problem of this procedure is the lack of blotting step, which induces some serious consequences. For instance, as illustrated in Figure 2.24 (a), the over rich phosphotungstic acid hydrate makes liposomes over stained, and it is not easy to acquire any details of liposomes such as their sizes and forms from these images. Furthermore, Figure 2.24 (b) indicates that, in some cases, the carbon layer on the copper grid can even be damaged which results in the failure of imaging process. Therefore, this wrong procedure of negative staining should be avoided in future research.

2.3.5 The comparison of particle size and zeta potential of different MW chitosan coated liposomes

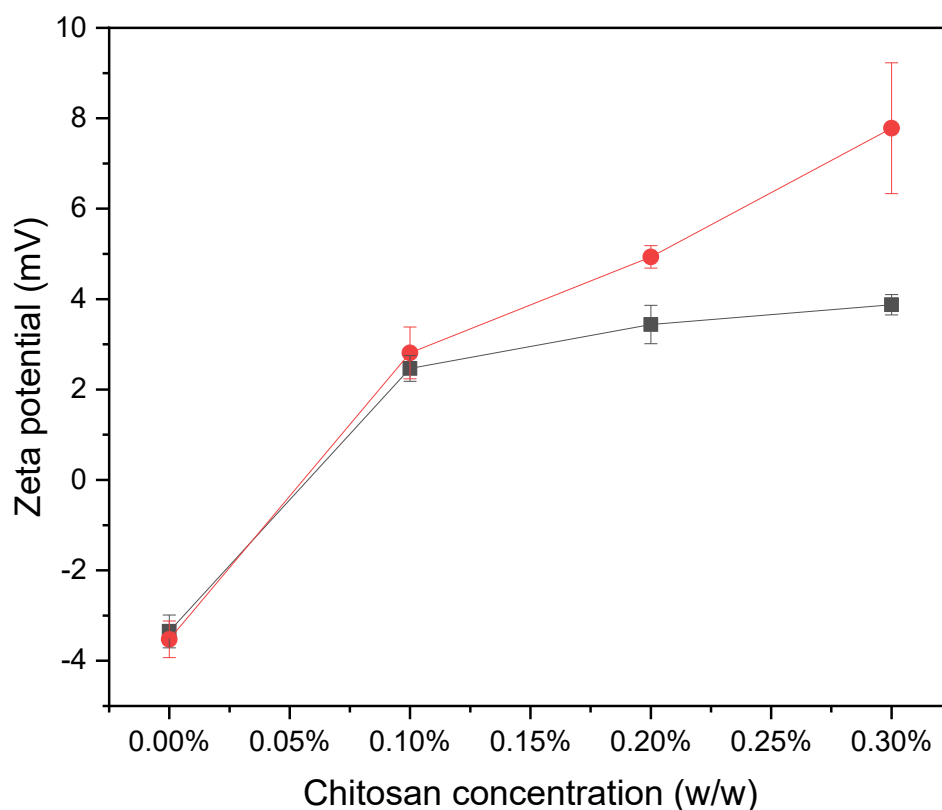


Figure 2.25 The zeta potential of freshly made low MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.25 outlines the comparison of zeta potential of fresh liposome and liposome stored in the fridge for one month. It is clear that both new and old plain liposome without chitosan coating had a negative charge of zeta potential. As the concentration of low molecular weight chitosan rose from 0.1% to 0.3%, the zeta potential of fresh liposome increased gradually from 2.5 to 3.9 mV. After one month stored in the fridge at 4 °C, the zeta potential of plain liposome decreased slightly from -3.4 to -3.5 mV. In addition, the zeta potential of all

three low molecular chitosan-coated liposome samples increased marginally after one-month storage. The higher the chitosan concentration was, the more increase the zeta potential experienced. The zeta potential of liposome coated with 0.3% (w/w) chitosan increased by around 3.9 mV after one-month storage in the fridge whilst 0.4 mV increase was found in the 0.1% (w/w) chitosan-coated liposome and 1.5 mV in 0.2% (w/w) group. Therefore, the relatively low zeta potential (less than 5 mV) of both fresh and stored low molecular weight chitosan-coated liposomes results in a great tendency for liposomes to aggregate and fusion into large ones, which can be proved in TEM images of Figure 2.18 and 2.19. Besides, the shift in zeta potential from the negative charge to the positive charge in the freshly made sample is due to the successful chitosan adsorption on the liposome surface, which is consistent with TEM results as well.

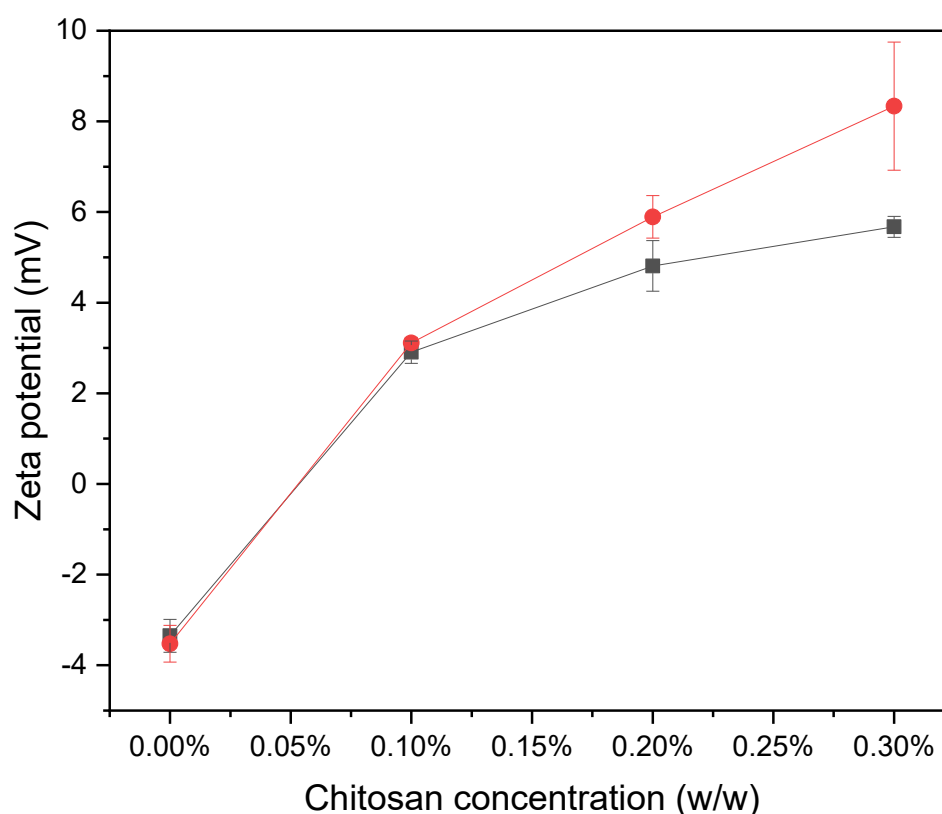


Figure 2.26 The zeta potential of freshly made medium MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.26 features the change of zeta potential of the medium molecular weight chitosan-coated liposome after one month of storage in the fridge. Before coating, the zeta potential of the plain liposomes had a similar number with plain liposomes in Figure 2.25. A sharp rise can be observed after the coating of chitosan, which demonstrates that the medium molecular weight chitosan is successfully coated on the liposome surface. Besides, the 0.10% (w/w) medium molecular chitosan had the slightest influence on ascending the zeta potential after storage, with an almost 0.2 mV increase. While around 1.1 mV and 2.7 mV increase were observed in 0.20% (w/w) and 0.30% (w/w) group. All zeta potentials of fresh and stored medium MW weight chitosan coated liposomes are higher than those in Figure 2.25, which is consistent with the results in Figure 2.12 that indicates medium MW chitosan has a greater zeta potential than low MW chitosan.

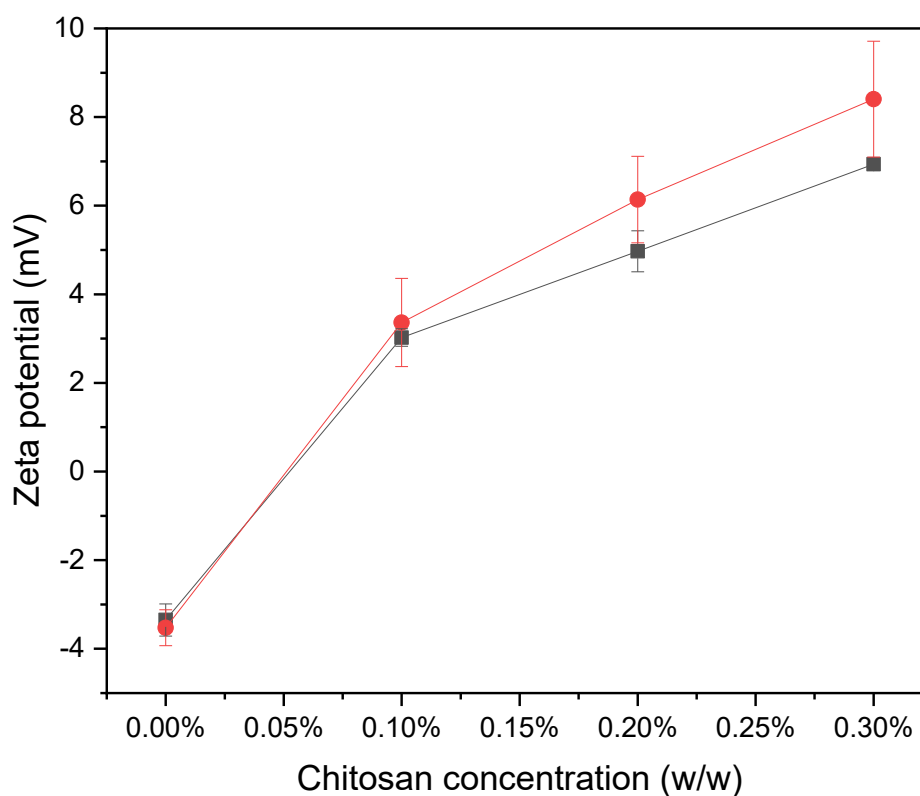


Figure 2.27 The zeta potential of freshly made high MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.27 compares the zeta potential of fresh and stored high MW chitosan coated liposomes. It is manifest that all high MW chitosan coated liposomes have slightly higher zeta potentials than low and medium ones. Similarly, the zeta potential of 0.1% high MW chitosan coated liposome experienced the smallest rise after storage from 3.0 mV to 3.4 mV. Besides, zeta potentials of 0.2% and 0.3% high MW chitosan coated liposome increased around 1.1 mV and 1.5 mV, respectively.

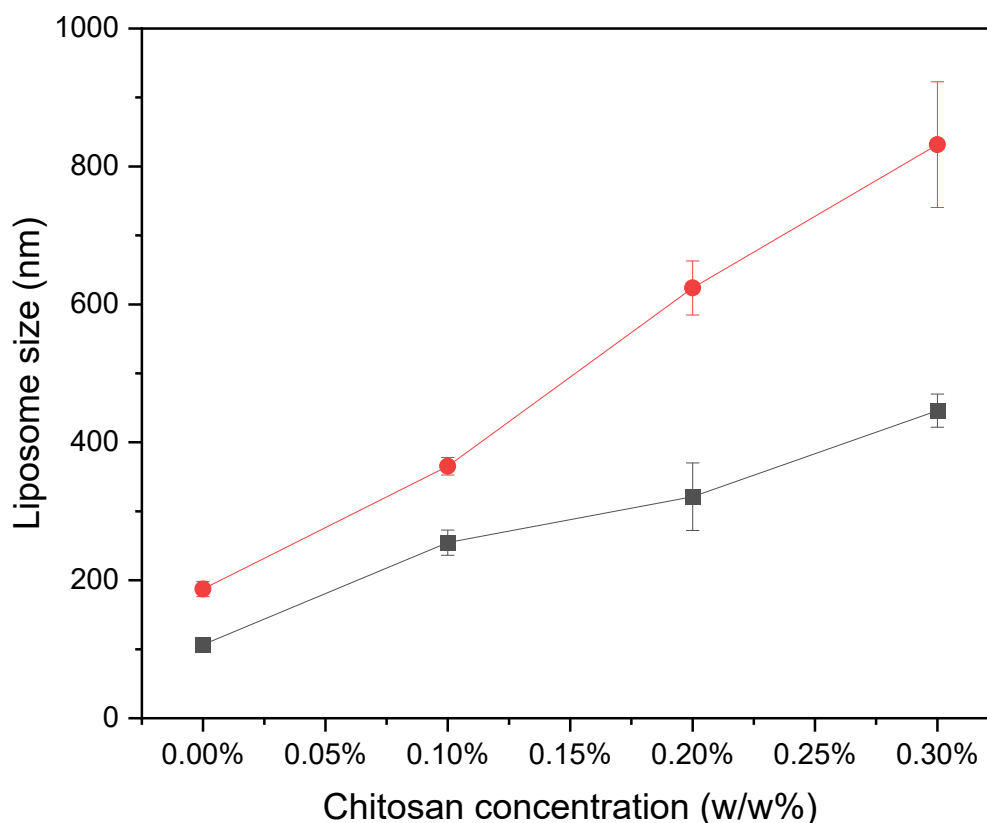


Figure 2.28 The particle sizes of freshly made low MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.28 contrasts the size of freshly prepared and stored liposomes. As the chitosan concentration rises, the particle size increases dramatically in both curves. For the freshly made liposomes, the size approximately doubles to 254 nm after 0.10% (w/w) low molecular weight chitosan coating. This increase on the liposome diameter indicates the formation of a thin layer on the liposome surface, which is attributed to the strong molecular electrostatic attraction between negatively charged phosphate groups in the liposome lipid head groups and NH_3^+ functional groups of chitosan [198]. Then, the particle size rises slightly to 321 nm when chitosan concentration reaches 0.20% (w/w) and to 446 nm in the 0.30% (w/w) group,

which means the increase of the concentration of low molecular weight chitosan can result in the significant rise on the particle size of chitosan-coated liposomes.

The particle size of stored liposomes experiences a gradual growth from 187.3 nm of plain liposome to 831.7 nm of 0.30% (w/w) chitosan-coated liposome, with an approximately 200 nm growth as chitosan concentration rises 0.10% (w/w). The size of plain liposome roughly doubles after one-month storage from 106.8 nm to 187.3 nm while only 111 nm growth is found in 0.10% (w/w) group which is the lowest rise among three chitosan-coated liposomes. Whereas the substantial increase of roughly 300 nm is observed in both 0.20% (w/w) and 400 nm in 0.30% (w/w) groups. This is because the aggregation and fusion behaviour of liposomes with thin and partly covered chitosan coating, as shown in TEM images.

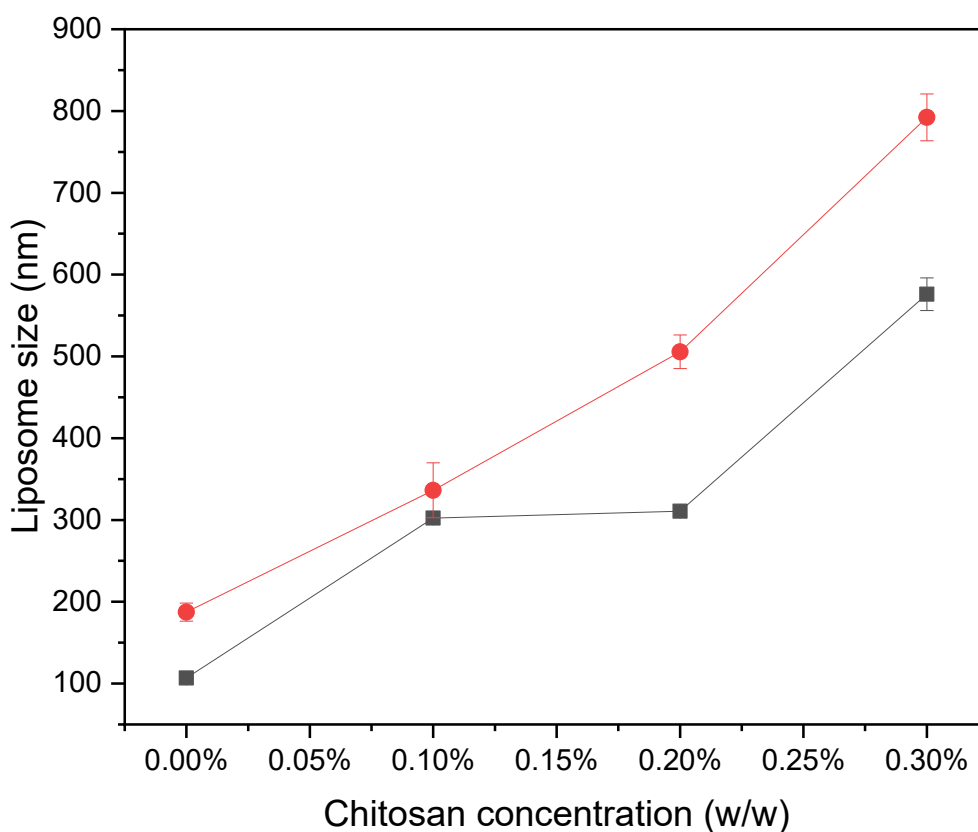


Figure 2.29 The particle sizes of freshly made medium MW chitosan-coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.29 describes the variation of particle size of new and stored medium molecular weight chitosan coated liposomes. In this sample, the particle size of plain liposome experiences similar change with the numbers in Figure 2.28. The differences between new and stored liposomes in this figure are significantly smaller than the gaps in Figure 2.28 overall. This is because medium MW chitosan coated liposome has thicker chitosan coating than low MW chitosan coated liposome, which are illustrated in TEM images. Thicker chitosan coating provides stronger repulsions between each liposome and lower tendencies to aggregate and fuse. Therefore, an only 34 nm amplification on the particle size is discovered in 0.10% (w/w) medium molecular chitosan coated liposome while a 111 nm increase in the 0.10% low molecular one.

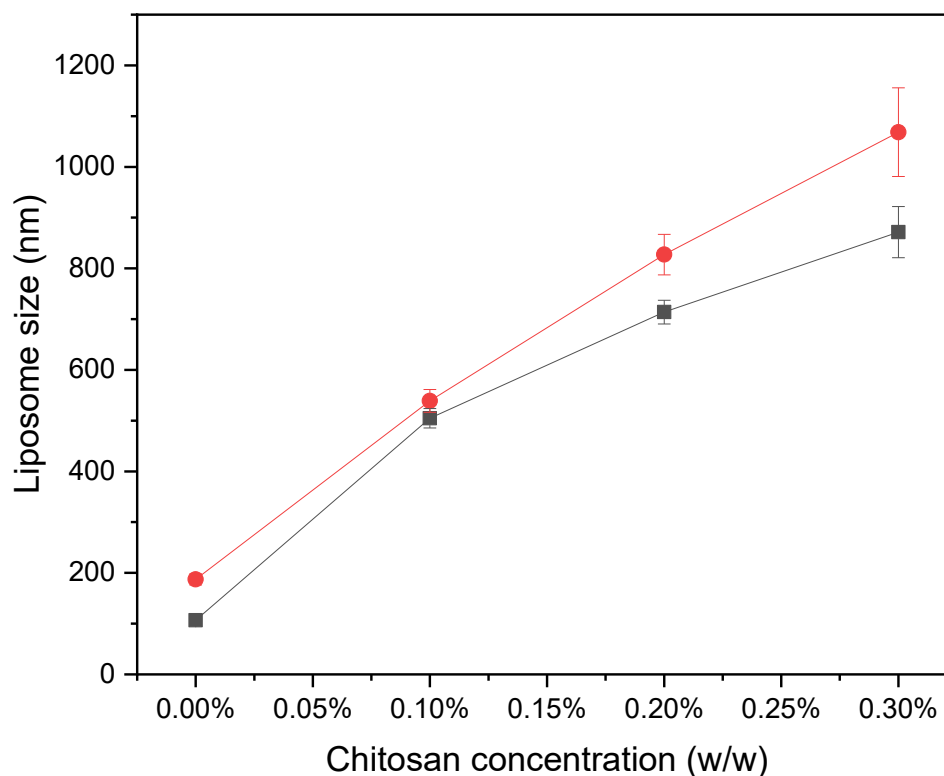


Figure 2.30 The particle sizes of freshly made high MW chitosan coated liposome (■) and liposome stored at 4 °C after one month (●) in deionised water. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 2.30 makes comparison of fresh and stored high MW chitosan coated liposome. As can be seen from this graph, all three high MW chitosan coated liposome have larger particle sizes than low and medium MW ones, which is due to the longest chain of high MW chitosan polymer molecules. Remarkably large size of 504 nm is observed in 0.1% (w/w) chitosan coated liposome, 713 nm in 0.2% group and 871 nm in 0.3% group, respectively. The significantly thick chitosan coating also results in the relatively smallest increases on liposome sizes after one month storage compared to other two kinds of liposomes, providing excellent protection for liposomes from aggregation and fusion. The results are in consistent with those in TEM images.

2.4 Conclusion

Firstly, this chapter revealed that the application of 8 minutes of ultrasonication in the preparation process of liposomes can reduce both plain liposome and drug-loaded liposome size to around 100 nm and achieve homogenisation. Secondly, zeta potentials of DPPC, Brij S20, low, medium, and high MW chitosan at various pH and their IP points were measured. At pH=7, DPPC and Brij S20 are negatively charged while all three MW chitosan are cationic, which indicates that chitosan could possibly absorb on the surface of liposomes composed of DPPC and Brij S20 due to the electrostatic force. Thirdly, the influence of different percentages of liposome composition on their size and zeta potential are discussed. It is indicated that the higher molar ratio of Brij S20 results in the decrease of liposome size and rise of liposomal zeta potential. Fourthly, TEM images of plain liposomes and three MW chitosan coated liposomes are taken. We discover that plain liposomes are extremely easy to aggregate and fuse without the protection of polymer coating, especially after long time storage. After coating of chitosan, all three MW chitosan can enhance liposome stability and aggregation and fusion are improved significantly. Most importantly, different MW chitosan coated liposome exhibits different forms in TEM. Low MW chitosan coated liposome has a shape of 'flower' with partly covered chitosan. Besides, more spherical forms of 'flower' are observed in medium MW chitosan coated liposomes because their surfaces are better covered. In contrast, the forms of high MW chitosan coated liposomes become hexagon or quadrangle with extremely large size of 400-600 nm which offers the best protection on liposomes but is not appropriate for intravenous injection.

Finally, comparisons of three various concentration (0.1%, 0.2% and 0.3% (w/w)) of low, medium, and high molecular weight chitosan coated liposomes on their zeta potential and

size were made to select the most appropriate one overall for further evaluation. To be specific, the shift of zeta potential and the augmentation of particle size verified the successful coating of low, medium, or high MW chitosan on the liposome surface, which can be illustrated in the TEM images as well. In addition, all three MW chitosan coated liposomes (fresh or stored ones) have similar zeta potentials but have remarkably distinct sizes. The diameter of low MW chitosan coated liposomes increased dramatically after storage because its thin and partly covered chitosan coating, as illustrated in TEM images. Nevertheless, the variation of all stored medium MW chitosan coated liposomal sizes are relatively less, among which 0.1% (w/w) medium MW chitosan has the best performance overall. Its particle size ascended by the minimal number of 34 nm and remained under 350 nm even after one-month storage. Lastly, the high MW chitosan apparently prevents liposomes from aggregation and fusion most effectively because the minimal increases in their size are observed compared to sizes of fresh ones. Whereas its potential application of intravenous administration is limited because liposomes with large particle size are more easily to be recognised and removed by the human immune system and thus led to the substantial reduction of circulation time *in vivo*. Therefore, the 0.1% (w/w) medium MW chitosan with the smallest diameters among three different concentrations was chosen and will be evaluated further on its release property and drug encapsulation efficiency in the later chapters.

Chapter 3 Characterisation of chitosan-coated liposome with calcein disodium salt encapsulation

3.1 Introduction

Calcein is a water-soluble fluorescent dye with a self-quenching effect. It is also a polyanionic derivate of fluorescein and can be well retained in cells. which has been extensively applied in cell viability, angiography, mitochondrial microscopy imaging, cell volume changes, endocytosis, membrane permeability and liposomes leakage studies [199-202].

Maherani et al. encapsulated calcein into liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and used calcein as a model of hydrophilic drugs to study its ability to permeate across liposomal membranes by using the spectrofluorometer. In addition, the effects of different lipid ratios, temperatures, and pH during preparation on their osmotic capacity were also investigated. The results indicated that the release of calcein does not have any influence on the membrane structure, and its release rate depends on the physical properties of the liposomes, such as the species and proportion of the lipids, the fluidity and the bending elasticity of the membrane and the number of layers. Specifically, membranes composed of unsaturated lipids with higher fluidity and lower T_m values could allow calcein to permeate more easily, and a decrease in membrane curvature could lead to an increase in membrane permeability. In addition, the chemical properties of the drug such as hydrogen bonding and polar surface area (PSA) also affect the release rate. Finally, this study also simulated the environment of the human

digestive tract to investigate the effect of pH and enzymes on liposomes characteristics. The results demonstrated that duodenal digestive juices and bile salts accelerated the release of calcein [203].

In another study, a novel pH-sensitive egg yolk phosphatidylcholine (EYPC) liposome encapsulating calcein was developed and succinylated polyglycidol (SucPG) was attached to its surface. SucPG has the ability to enhance membrane fusion in an acidic environment. In the African green monkey kidney CV-1 cells, both EYPC liposomes and SucPG-modified EYPC liposomes were incubated at 37 degrees. By using the fluorescence microscope, the results revealed that only weak fluorescence of calcein was observed in cells incubated with plain EYPC liposomes, while the group of SucPG-modified (EYPC) liposomes demonstrated strong and diffuse fluorescence. In addition, as the amount of polymer, SucPG, attached to the liposome surfaces increased, the uptake capacity of the cells decreased marginally, yet the fluorescence intensity observed in the cells increased, indicating that higher amounts of polymer can more efficaciously deliver the encapsulated contents of liposomes to the cytoplasm of cells. In addition, the outcome of fusion assay by resonance energy transfer demonstrated that polymer-modified liposomes fused to intracellular lysosomal membranes, and more polymers resulted in a higher percentage of fusion [204].

In order to investigate the characteristics of chitosan coated liposomes, calcein was initially selected to be encapsulated, due to its extremely strong fluorescence intensity and comprehensive usages in biological studies. The standard fluorescence curve of calcein and encapsulation efficiency, release property and shelf life of calcein encapsulated liposomes with and without a chitosan coating will be reported in this chapter. Besides, multiple fitting models will be selected to fit liposomes release curves and the most fitted model will be determined. According to our knowledge, studies regarding to the comparison of calcein encapsulated HaT liposomes with and without chitosan coating have not been previously

reported. Therefore, the results obtained from this chapter can be regarded as starting point and a reference for the further investigations on chitosan coated liposome loaded with drugs both *in vitro* and *in vivo*. Hence, in Chapter 4, doxorubicin, a chemotherapy drug, will be loaded into chitosan coated liposome and its characteristics will be studied. In addition, in this chapter, 0.1% (w/w) medium molecular weight chitosan was selected to coat on the liposome surface following the preliminary experiments presented in in Chapter 2.

3.2 Materials and Methods

3.2.1 Materials

1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was purchased from Avanti Polar lipid, Alabaster, AL, USA.

Calcein disodium salt was purchased from Honeywell Fluka.

Brij S20, low molecular weight Chitosan (50-190 kDa), medium molecular weight Chitosan (190–310 kDa), high molecular weight Chitosan (310-375 kDa), Triton X-100, and 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich.

Sephadex G-50 (medium) was purchased from GE healthcare.

Spectrum™ Spectra/Por™ Float-A-lyzer™ G2 dialysis devices was purchased from Fisher Scientific

All water used was de-ionised and had a conductivity less than 80 mS.

3.2.2 The determination of calcein fluorescence intensity standard curve

A Calcein fluorescence intensity standard curve was plotted using Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) with excitation and emission wavelength of 494 and 517 nm, respectively. The standard curve was obtained by measuring the fluorescence intensity of each sample with specific calcein concentration. The first sample was prepared by dissolving 90 mg calcein disodium salt in 3 ml solution with a concentration of 30 g/l in a 4.5 ml plastic standard cuvette. Each following sample was made by diluting the previous one with same volume of solution. In that case, the calcein concentration of the following sample was the half of the previous one. A total of 29 samples were measured with a minimum concentration of 2.24×10^{-7} mg/ml and maximum concentration of 30 mg/ml. Calcein disodium salt was dissolved in HEPES buffer solution (pH 7.4) and acetic acid solution (pH 3.0) separately for obtaining two calcein fluorescence intensity standard curves at these pH's. The pure solution (HEPES buffer solution (pH 7.4) or acetic acid solution (pH 3.0)) without calcein was measured as well for contrast.

3.2.3 Liposome characterisation: Encapsulation efficiency

The encapsulation efficiency was calculated by the percentage of calcein encapsulated in liposomes relative to the total amount of calcein added in the liposome preparation process. Pure calcein encapsulated liposome suspension was collected after gel permeation chromatography through a Sephadex G 50 column (0.8 cm*25 cm) equilibrated with HEPES buffer (pH 7.4). To determine the total amount of calcein encapsulated in liposomes, firstly, 1 v/v % Triton X-100 surfactant was added to the calcein encapsulated liposome suspension to disrupt the liposomal membrane and release encapsulated calcein thoroughly. Then, 500 μ l of this solution was extracted and added into 4.5 ml plastic standard cuvette loaded with 2.5 ml HEPES buffer solution to measure its fluorescence intensity in the spectrofluorometer (HORIBA, USA). The concentration of encapsulated calcein released in this solution can then be obtained by referring to the calcein fluorescence standard curves in HEPES buffer

solution (pH=7.4) in Figure 3.3. Finally, the amount of encapsulated calcein inside the liposomes (M_{ce}) was the concentration of calcein obtained from the spectrofluorometer times the volume of liposome suspension collected from gel permeation chromatography.

The encapsulation efficiency was calculated using the following equation (3-1):

$$EE (\%) = \frac{M_{ce}}{M_{ct}} \times 100\% \quad (3 - 1)$$

Where $EE (\%)$ is the encapsulation efficiency, M_{ce} is the mass of calcein encapsulated in liposomes and M_{ct} is the mass of initial calcein added in the liposome preparation procedure.

3.2.4 Determination of the measurement assay and calculating equation of release property

Many researchers, including the inventors of HaT liposome, utilised the approach adopted by Mills and Needham [87] to measure the release property of drug encapsulated liposome [99]. To be specific, 5 μ l of drug encapsulated liposome suspension was extracted from the original liposome sample and loaded into a small glass vial. Then, dozens of these small glass vials containing drug loaded liposome in HEPES buffer solution were submerged into water bath at certain temperature. After various periods of time, one glass vial was removed from the water bath for the measurement of its fluorescence intensity. In this way, every test vial gave a fluorescence intensity value (FI_t) at single time point in time throughout the

whole incubation period. Six random samples were lysed with 10% TX-100 and their fluorescence intensity were measured and averaged to get a 100% released value (FI_{TX}).

Finally, the percentage of drug release was calculated using the following equation:

$$PR = \frac{FI_t - FI_{t0}}{FI_{TX} - FI_{t0}} \times 100\% \quad (3 - 2)$$

Where PR is the percentage release, FI_t is the fluorescence intensity of one sample in a glass vial removed from water bath at time point t , FI_{t0} is the fluorescence intensity of sample at 0 time point. FI_{TX} is the average fluorescence intensity of six sample after addition of TX-100.

This approach had a significant drawback when a small volume of drug encapsulated liposome suspension such as 5 μ l or 20 μ l was extracted from the original liposome sample, it cannot be guaranteed that each sample contained exactly same concentration or quality of liposome and various degrees of deviation existed among these extracted samples.

Because the amount of drug released from liposomes was extremely small, the deviation of concentration or quality of liposome in each extracted sample impaired the accuracy of fluorescence intensity measurements. Therefore, this approach was not used here. Instead, the approach described in section 3.2.5 was selected in this research. In this approach, only one single sample at certain temperature was monitored and the variation of its fluorescence intensity was recorded constantly after dialysis.

3.2.5 Liposome characterisation: Release Property



Figure 3.1 The photograph of self-assembly dialysis unit consisting of a Spectrum™ Spectra/Por™ Float-A-lyzer™ G2 dialysis device and a 50 ml plastic centrifugal tube

The release property of liposomes with and without coating chitosan was investigated using a dialysis tube diffusion technique. 2 ml of liposome suspension was placed in Spectrum™ Spectra/Por™ Float-A-lyzer™ G2 dialysis device with a molecular weight cut-off (MWCO) of 300 kDa and immersed in 15 ml HEPES buffer solution in a 50 ml centrifugal tube as demonstrated in Figure 3.1. The whole unit was then incubated in a water bath at the desired temperature (37 °C, 40 °C and 42 °C). Aliquots of 500 µl dissolution mediums were extracted and same volume of fresh medium was replaced every half an hour. Then, the 500 µl extracted medium was added into 4.5 ml plastic standard cuvette loaded with 2.5 ml HEPES buffer solution and the cuvette was inserted into Horiba FluoroMax-4

spectrofluorometer (HORIBA, USA) for the measurement of fluorescence intensity. By referring to the calcein fluorescence standard curves in HEPES buffer solution (pH=7.4) in Figure 3.3, the released calcein concentration (C_{ci}) after dialysis can be obtained. In addition, 1 v/v % Triton X-100 surfactant was added into original liposome solution sample in order to disrupt the liposomal membrane and release encapsulated calcein completely. Then, the calcein concentration (C_{ct}) in this solution was analysed by the same approach.

The release percentage of calcein from liposomes was calculated using the following equation (3-3):

$$RR (\%) = \frac{C_{ci} \times V_1 + \sum_{i=1}^{n-1} C_{ci} \times V_2}{C_{ct} \times V_3} \times 100\% \quad (3-3)$$

Where RR is the release ratio, C_{ci} is the concentration of total calcein released at moment i , V_1 is the volume of buffer solution in centrifugal tube (15 ml), n is the number of samples removed from the release medium, V_2 is the volume of removed release medium (500 μ l), C_{ct} is the concentration of initial liposome solution after the addition of 1 v/v % Triton X-100 and V_3 is the volume of initial liposome suspension loaded inside dialysis tube (2 ml).

3.2.6 Liposome characterisation: Shelf life

2 ml of plain liposome and 0.1% (w/w) medium molecular weight chitosan coated liposome suspensions were loaded separately in the same self-assembly dialysis unit as demonstrated in Figure 3.1. Both samples were stored at 4 °C and room temperature (25 °C) in a dark environment so as to exclude any light. Aliquots of 500 μ l dissolution medium were extracted and same volume of fresh medium was replaced every 5 days for one month. The 500 μ l of extracted medium was loaded into 4.5 ml cuvette loaded with 2.5

ml HEPES buffer solution and was inserted into Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) for the measurement of fluorescence intensity just like stated in the section 3.2.5. Finally, the leakage ratio (release percentage) was calculated with the equation (3-3) as well and each experiment was repeated in triplicate.

3.2.7 Determination of fitting models

The calcein release curves were fitted with various kinetic models by using Origin (9.65 Version). To be specific, because these kinetic models are not included in the initial database of Origin, all these models were imported to the database manually using the 'fitting function builder' function of Origin. Then, all calcein release curves were fitted using 'nonlinear curve fit' function. The coefficient of determination (R^2) and residual sum of squares (RSS) were selected from the fitting statistics and recorded. Bayesian information criterion (BIC) was obtained by using 'compare models' function. The goodness-of-fit of each kinetic model were evaluated based on these parameters. Table 3.1 gives a summary of the models which have been used to treat drug release data.

Table 3.1 The summary of frequently used kinetic models in pharmaceutical research [205], where Q represents the amount of released drug at time t , Q_0 is the initial amount of drug in the formulation or delivery system, k is the release constant.

Frequently used kinetic models	Mathematical models
Zero-order	$Q/Q_0 = k * t$
First-order	$Q/Q_0 = 1 - e^{(-k*t)}$
Higuchi	$Q/Q_0 = k * t^{0.5}$
Korsmeyer-Peppas	$Q/Q_0 = k * t^n$
Weibull	$Q/Q_0 = 1 - e^{(-b*t^a)}$
Hixson-Crowell	$Q^{1/3} - Q_0^{1/3} = k * t$
Hopfenberg	$Q/Q_\infty = 1 - (1 - k * t)^3$
Gompertz	$Q/Q_0 = e^{(a * e^{\beta * \log t})}$
Baker-Lonsdale	$(1 - (1 - Q/Q_0)^{2/3}) * Q/Q_0 = 2/3 * k * t$

The origins of some of these models will now be discussed.

● Zero-order kinetics

Zero-order kinetics are only a function of time, the drug is always released from the delivery system at a constant rate and the drug concentration in the blood remains constant regardless of drug concentration. Zero-order kinetics are suitable for the delivery of drugs of low water solubility, either ocular or transdermal administration, and may also be applicable to pain relief, cancer treatment, and long-term administration of antibiotics and antidepressants [206] [207].

For oral administration, the drug release profile from the coated tablet core can conform to zero-order kinetics. When the core drug of the tablet becomes hydrated, it dissolves to a saturation concentration and reaches the gastrointestinal fluid by means of membrane diffusion. Saturated drug concentration in the tablet core maintains a fixed concentration gradient across the membrane, resulting in a constant release rate. When the core drug concentration is below the saturation concentration, the release rate will decrease. To achieve zero-order kinetic release in transdermal drug delivery systems, drug reservoirs need to be made of specific materials such as hydrogels and mineral oil. Suspension of a drug is a common approach to ensure that the drug concentration remains saturated during administration [208].

Zero-order model is derived from reaction kinetics of chemistry and experimental conditions [209]. A simplified equation can be acquired:

$$\frac{dQ}{dt} = k$$

Where Q is the amount of released drug at time t , k is the zero-order kinetic release constant. After application of calculations,

$$dQ = k * dt$$

↓

$$\int_{Q_0'}^Q dQ = k \int dt$$

↓

$$Q - Q_0' = k * t$$

Where Q_0' is the initial drug amount in the solution, which is normally 0. Then we can get,

$$Q = k * t$$

Dividing this equation on both sides by Q_0 which is a constant. Where Q_0 represents the initial amount of drug in the formulation or delivery system. Hence, k remains a constant after dividing by Q_0 . We can get,

$$Q/Q_0 = k * t \quad (3 - 4)$$

As can be observed from the above equation, the released amount of the accumulated drug has a linear relationship with time, and the slope of the straight line is the value of k in the formula. This is an ideal pharmacokinetic, which keeps the drug concentration in the blood constant all the time. When the drug concentration in the blood fluctuates because the drug is rapidly eliminated or absorbed, frequent and repeated dosing is required to keep the drug concentration saturated and effective. However, this can lead to reduced patient compliance and complicated procedures [208].

● First-order kinetics

The first-order release rate depends only on the drug concentration gradient between the concentration in the static liquid layer next to the solid surface and that in the bulk liquid, and is proportional to the concentration gradient, the greater the drug concentration difference, the greater the release rate. This model can be used for the absorption and elimination of a variety of drugs and dosage forms. The release rate of water-soluble drugs incorporated into a porous matrix depends on the amount of drug remaining in the matrix, therefore this release rate decreases with time because the amount of drug in the matrix continues dropping [210].

In the first-order kinetics, variation in concentration with respect to variation on time is merely dependent on concentration. Therefore, the drug release complying with the first order model can be demonstrated by this equation:

$$\frac{dC_t}{dt} = -k * C_t$$

Then, applying some mathematics,

$$\frac{dC_t}{C_t} = -k dt$$

↓

$$\int_{C_0}^{C_t} \frac{1}{C_t} dC = -k \int dt \rightarrow \ln(C_t) \Big|_{C_0}^{C_t} = -k * t$$

↓

$$\ln(C_t) - \ln(C_0) = -k * t$$

↓

$$\ln\left(\frac{C_t}{C_0}\right) = -k * t \quad (3 - 5)$$

By substituting this equation: $\ln(10) \log(C_t) = \ln(C_t)$, one can get,

$$\ln(10) \log\left(\frac{C_t}{C_0}\right) = -k * t$$

Where $\ln(10) = 2.303$, then one can get,

$$\log\left(\frac{C_t}{C_0}\right) = -\frac{k * t}{2.303} \rightarrow \log(C_t) - \log(C_0) = -\frac{k * t}{2.303}$$

↓

$$\log(C_t) = \log(C_0) - \frac{k * t}{2.303} \quad (3 - 6)$$

Eqn. (3-6) is the most widely used and classical final representation of first-order kinetics [209]. Where C_t is the remaining drug concentration at time t , C_0 is the initial drug concentration, k is the first-order kinetic release constant.

In the following sections, using first-order model, release curves of drug encapsulated liposomes are fitted, which depicts the relationship between the percentage of released drug amount and time. Therefore, the classical Eqn. (2) need to be expressed in other form.

The new calculation starts from Eqn. (3-5),

$$\ln\left(\frac{C_t}{C_0}\right) = -k * t \quad (3-5)$$

↓

$$\frac{C_t}{C_0} = e^{-(k*t)}$$

Since $C_t = C_0 - C$, where C is the released drug concentration. Then, one can get,

$$\frac{C_0 - C}{C_0} = e^{-(k*t)} \rightarrow 1 - \frac{C}{C_0} = e^{-(k*t)}$$

↓

$$\frac{C}{C_0} = 1 - e^{-(k*t)}$$

Since $Q = V * C$, $Q_0 = V * C_0$, where V represents the volume of solution, which is a constant. Then, one can get,

$$\frac{Q}{Q_0} = 1 - e^{-(k*t)} \quad (3-6)$$

Eqn. (3-6) is the same as the formula in the table 3.1, which will be used for the fitting of release curves using Origin software.

- Higuchi model

From 1961 to 1963, Higuchi developed one of the most famous and commonly used mathematical models to describe the dissolution and release rates of drugs from matrix systems. Higuchi first selected the ointment matrix as the research object, then analysed its drug release rate, and finally proposed a mathematical model to describe the sustained drug release [211] [212] [213]. The Higuchi model can be used to simulate the release of various sustained-release drug systems, such as certain transdermal drugs and water-soluble tablets. The research of Higuchi's model has laid the groundwork for the development of many drug release models in the future.

To use the Higuchi model, the following assumptions need to be met [209]:

- 1) the solubility of the drug is much lower than the initial drug concentration in the matrix
- 2) Drug diffusivity remains constant
- 3) The dissolution and swelling of the matrix are negligible
- 4) The size of the drug particles is much smaller than the thickness of the system
- 5) The edge effect of the drug is negligible (drug diffusion is one-dimensional)
- 6) Perfect sink conditions (released drug concentration never exceeds 10% of drug saturation in the medium)

Higuchi proposed three different mathematical model expressions based on three kinds of systems [208]:

- a) For the dissolution of drug in a homogeneous, lipophilic, planar matrix, the mathematical expression derived by Higuchi is as follows:

$$Q = \sqrt{D(2C - C_s)C_s * t}$$

Where Q is the amount of released drug at time t , C is the initial drug concentration in dosage form, C_s represents the solubility in the matrix media, and D represents the drug diffusivity in the matrix.

- b) In heterogeneous and spherical, heterogeneous and planar, and homogeneous and spherical matrices, low concentrations of drugs are released and dissolved through the pores of the matrix, and the mathematical expression is as follows:

$$Q = \sqrt{\frac{D\varepsilon(2C - \varepsilon C_s)C_s}{\tau} * t}$$

Where Q is the amount of released drug at time t , C is the initial drug concentration in dosage form, C_s represents the solubility in the matrix media, D represents the drug diffusivity in the matrix, ε represents the porosity of matrix, and τ represents the capillary tortuosity.

- c) For the release and dissolution of drug in a matrix saturated with drug, it can be written as:

$$Q = \sqrt{\frac{2C_0\varepsilon D}{\tau\pi} * t}$$

Where Q is the amount of released drug at time t , C_0 represents the diffusing liquid concentration in the porous matrix media, D represents the drug diffusivity in the matrix, ε represents the porosity of matrix, and τ represents the capillary tortuosity.

The above three formulas can be simplified into one expression [208]:

$$Q = k * t^{0.5}$$

Dividing this equation on both sides by Q_0 , k remains a constant because Q_0 is the initial amount of drug which is a constant. One can get,

$$\frac{Q}{Q_0} = k * t^{0.5} \quad (3 - 7)$$

This is the simplest expression of Higuchi model, which indicates the released percentage of drug have a linear relationship with square root of time in Higuchi model.

- Korsmeyer-Peppas model

Korsmeyer and Peppas proposed a simple empirical equation to determine which type of dissolution the drug release from the system conforms to. The Korsmeyer-Peppas model is very appropriate for simulating the drug release process when the drug release mechanism is unclear or involves multiple mechanisms [214]. The equation is as follows:

$$\frac{Q}{Q_0} = k * t^n \quad (3 - 8)$$

Where Q is the amount of released drug at time t , Q_0 represents the initial amount of drug in the formulation or delivery system, k represents the release rate constant of Korsmeyer-Peppas model including geometric and structural characteristics of the delivery system and n represents the release exponent.

The n value is applied for the characterization of various drug release mechanisms. The table 3.2 provides the specific situations.

Table 3.2 Interpretation of drug transport and drug release mechanism [210]

Release exponent (n)	Drug transport mechanism	Drug release mechanism
$n < 0.5$	Quasi-Fickian diffusion	Diffusion
$n = 0.5$	Fickian diffusion (Case I transport)	Diffusion (Higuchi model)
$0.5 < n < 1.0$	Anomalous	Both diffusion and Relaxation
$n = 1.0$	Case II transport	Only relaxation or swelling (Zero order kinetics)
$n > 1.0$	Super case II transport	Breaking and crazing

Drug transport mechanism is mainly divided into the following two types, Fickian model (case I) and non-Fickian model (Case II, Anomalous case and Super case II). When $n=0.5$, the drug transport follows Fickian model (case 1), and the mechanism driving drug release is diffusion. The solvent diffusion rate is much higher than the rate of polymer chain relaxation. Besides, it is manifest that, when $n=0.5$, the Eqn. (3-8) becomes the Eqn. (3-7), which is the Higuchi model expression. When $0.5 < n < 1$, the model is anomalous transport, which means the drug release is controlled by both solvent diffusion and relaxation of the polymer chain. The diffusivity of the solvent is not much different from the swelling ratio of the polymer chain. In case II, where $n = 1.0$, the Eqn. (3-8) becomes Eqn. (3-4), which is the equation of zero order kinetics. The drug release is entirely due to the relaxation or swelling of polymer chains. In the Super Case II model, $n > 1$, which is an extreme form of drug transport, where the polymer chain breaks due to excessive tension and the solvent cracks [215] [216] [217]. Therefore, according to the table 3.2, the n values of fitting curves can offer a reference for the determination of the transport or release mechanism.

In addition, Eqn. (3-8) can also be written in logarithmic form [218]:

$$\log(Q/Q_0) = \log k + n \log t$$

As can be seen from the above equation, $\log(Q/Q_0)$ has a linear relationship with $\log t$, with a slope of n and an intercept of $\log k$. This provides another way to fit the curves of drug release.

- Weibull model

Weibull first proposed this model in 1951 [219], and then in 1972 Langenbucher et al. first adopted this function to describe drug release [220]. This equation is entirely an empirical model with no kinetic basis, so no inferences and assumptions about pharmacokinetics can be made. The parameters in this equation do not contain any drug-intrinsic dissolution factors and are not related to the *in vivo* or *in vitro* environment. This equation is mostly used to compare the drug release profiles of matrix systems, and it is as follows:

$$\frac{Q}{Q_0} = 1 - \exp \left[\frac{-(t - T_i)^a}{b} \right] \quad (3-9)$$

Where Q is the amount of released drug at time t , Q_0 represents the initial amount of drug in the formulation or delivery system, T_i represents the lag time measured of the drug dissolution or release process, which is normally zero. b is a scale parameter which describes the time dependence, a is a shape parameter that characterizes the type of curve [219].

- 1) $a = 1$, the shape of curve fits exactly the shape of the exponential profile.
- 2) $a > 1$, the shape of curve becomes sigmoidal with a turning point.

3) $\alpha < 1$, the shape demonstrated a parabolic curve with a high initial slope [220].

When $T_i = 0$, the Eqn. (3-9) becomes:

$$\frac{Q}{Q_0} = 1 - \exp\left(\frac{-t^\alpha}{b}\right)$$

Where $1/b$ is a constant, so the above equation can also written as:

$$\frac{Q}{Q_0} = 1 - e^{(-b*t^\alpha)} \quad (3-10)$$

Eqn. (3-10) is the one in the table 3.1, which will be applied for the fitting of release curves in the results section.

3.2.8 Determination of fitting parameters

The most commonly-used fitting parameters such as R^2 (COD), RSS and BIC were selected to determine which model has the best goodness-of-fit and is the most suitable to fit calcein or doxorubicin release curves of plain liposome and chitosan coated liposome. The detailed description of each parameter are as follows:

- R^2 (COD)

The R-squared represents the coefficient of determination (COD), another name is the Nash–Sutcliffe model efficiency coefficient. The meaning of R-squared is that it measures how well the predicted value fits the true value, which is a statistic that measures the goodness of fit. R-squared uses the least squares method for parameter estimation, which is the ratio of the regression sum of squares to the sum of squares of the total deviation,

indicating the proportion of the sum of squares of the total deviation that can be explained by the sum of squares of the regression. The larger the ratio, the more accurate the model, the more significant the regression effect. The R-squared is between $-\infty$ and 1, and the closer it is to 1, the better the regression fitting effect. It is generally considered that the model with more than 0.8 has a high goodness of fit [210]. Given a series of true values y_i and corresponding predicted values $\hat{y}_i$, the R-squared is defined as:

$$R^2 = 1 - \frac{\sum_i (\hat{y}_i - y_i)^2}{\sum_i (y_i - \bar{y})^2}$$

Where $\hat{y}_i$ represents the regression value of the dependent variable, which is the predicted value, $\bar{y}$ represents the mean of the dependent variable, y_i represents the true dependent variable.

Different R-squared values indicate different situations [221]:

- $R^2 = 1$: Ideally, all predicted values are equal to the true value.
- $R^2 = 0$: All $\hat{y}_i$ values are equal to $\bar{y}$.
- $R^2 < 0$: The model has poor predictive ability, even worse than the prediction that all $\hat{y}_i$ values are equal to $\bar{y}$. This indicates that the wrong model may be applied, or assumptions of the model are not reasonable.

There is no lower bound on the minimum value of the R-squared since predictions can be arbitrarily bad. As a result, the range of the R-squared is $(-\infty, 1]$. Despite its name R-squared is not the square of a number and can therefore be negative.

- Chi²/DOF

Reduced chi-squared is a very universally applied parameter for convergence diagnostic, model comparison, error estimation and model assessment, which is defined as [222]:

$$X_v^2 = \frac{X^2}{v}$$

Where X_v^2 means Chi-squared per degree of freedom, v represents the degree of freedom, which can be expressed as:

$$v = n - m$$

Where n is the number of observations, m is the number of parameters in fit.

Besides, X^2 represents Chi-squared, a weighted sum of squared deviations, which can be written as:

$$X^2 = \sum_i \frac{(O_i - C_i)^2}{\sigma_i^2}$$

Where O_i is the observed value, C_i is the predicted value, σ_i^2 is variance related to the measurement error for observed value.

The value of Chi^2/DOF can be used to evaluate the fitting goodness of mode [223],

- $\text{Chi}^2/\text{DOF} \approx 1$: The selected model fits perfectly with the data.
- $\text{Chi}^2/\text{DOF} > 1$: The selected model could be improved.
- $\text{Chi}^2/\text{DOF} < 1$: The selected model is too complicated, or errors of data are too large.

● RSS

The residual sum of squares (RSS), also known as the sum of squared estimate of errors (SSE) or the sum of squared residuals (SSR). The difference between the y value of each actual data point and the y value of its corresponding position on the regression curve is called the residual, and the sum of the squares of each residual is called the residual sum of squares. It is used to measure the difference between the actual data and the estimated value predicted by the model to help determine whether the model fits the selected data.

The smaller the residual sum of squares, the better the model fits the data; the larger the RSS, the less the model fits the data. An RSS value of zero indicates that the model fits this dataset perfectly.

The expression for RSS can be written as:

$$RSS = \sum_{i=1}^n (y_i - \hat{y})^2$$

Where y_i is the observed variable value, $\hat{y}$ is the predicted value estimated by the regression curve.

If the regression model is linear, the above expression can be expressed as:

$$RSS = \sum_{i=1}^n (y_i - (\alpha + \beta x_i))^2$$

Where α is the intercept coefficient of the linear regression model, β is the slope coefficient of the linear regression model.

- BIC

Gideon E. Schwarz first invented the Bayesian Information Criterion (BIC), also known as the Schwarz Information Criterion (SIC), in a 1978 paper [224]. BIC is developed based on the likelihood function and is used to select the optimal model among several models. The model with the lowest BIC value is listed as the first choice. BIC can be defined as [225]:

$$BIC = k \ln(n) - 2 \ln(\hat{L})$$

Where $\hat{L}$ represents the maximized value of the likelihood function of the model M , which can be expressed as:

$$\hat{L} = p(x|\hat{\theta}, M)$$

Where $\hat{\theta}$ are the parameters which maximize the likelihood function, x is the observed data, n is the number of observed data x , k is the number of predicted parameters using model M .

BIC is closely related to AIC, which refers to Akaike's Information Criteria, developed by Hirotugu Akaike in 1973. AIC can be used to evaluate the fit of any model, while BIC is mainly applied to select the best model from models with different number of parameters. Compared with AIC, BIC penalizes a larger proportion of free parameters. In addition, BIC is more suitable for consensus estimation, while for cross-validated asymptotic equivalence, AIC is more appropriate [226].

3.3 Results and discussion

3.3.1 The influence of pH on the Calcein fluorescence intensity standard curve

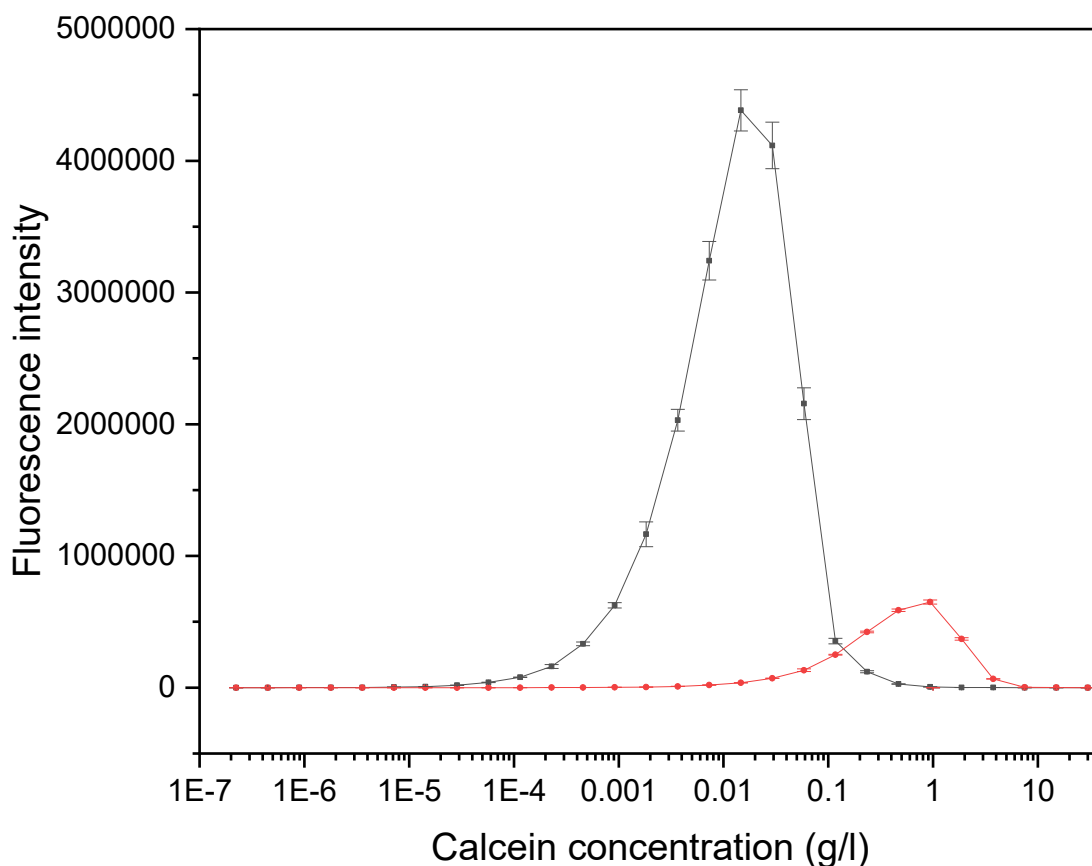


Figure 3.2 Calcein fluorescence standard curves in acetic acid solution (pH=3.0) (●) and HEPES buffer solution (pH=7.4) (■) measured by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) (Slit width=1). Data represents mean $\pm$ SD. $n=3$ independent batches.

As illustrated in Figure 3.2, both fluorescence standard curves had peaks as calcein concentration varied. In HEPES buffer solution, below 2.86×10^{-5} g/l, there was nearly no strong fluorescence displayed from calcein. When the solution concentration increased, a surge of fluorescence intensity can be observed, with a peak of around 4.5 million at 1.46×10^{-2} g/l. Instead of continuous ascending, the fluorescence intensity plummeted when the concentration rose after 1.46×10^{-2} g/l, reaching merely 0.5 million at 1.71×10^{-1} g/l. This collapse of fluorescence intensity is a consequence of the self-quenching effect of calcein.

After that, the fluorescence intensity declines marginally to almost 0 at initial calcein concentration of 30 g/l.

In contrast, the peak of fluorescence intensity of calcein in acetic acid solution (pH=3.0) was much lower (merely 0.7 million), which is only 1/6 of the intensity in HEPES buffer solution. In addition, the concentration of the intensity peak rose to 9.38 E-01 g/l rather than 1.46 E-02 g/l in the HEPES buffer solution. Also, no sharp rise or fall of fluorescence intensity was demonstrated in acetic acid solution. Below 1.46 E -02 g/l, the fluorescence intensity of calcein in acetic acid solution was negligibly small. The significant differences between two fluorescence intensity curves means the fluorescence intensity of calcein is sensitive to the pH of its surrounding solution. Some research also has been undertaken to investigate the influence of pH on the fluorescence intensity of calcein. Four calcein fluorescence standard curves with four pH values (pH 5.0, pH 6.0, pH 7.0 and pH 8.2) were constructed and the results demonstrated the decrease of pH induced the decline of fluorescence intensity of calcein at all concentrations which is consistent with these results [227]. It is important to note too that the existence of specific ions such as Fe^{3+} , Co^{2+} , Cu^{2+} and Mn^{2+} at pH 7 can cause dramatic quenching on fluorescence intensity of calcein [228] [229]. Therefore, during the whole experiments regarding to the measurements of calcein fluorescence, the pH and ion levels were carefully monitored.

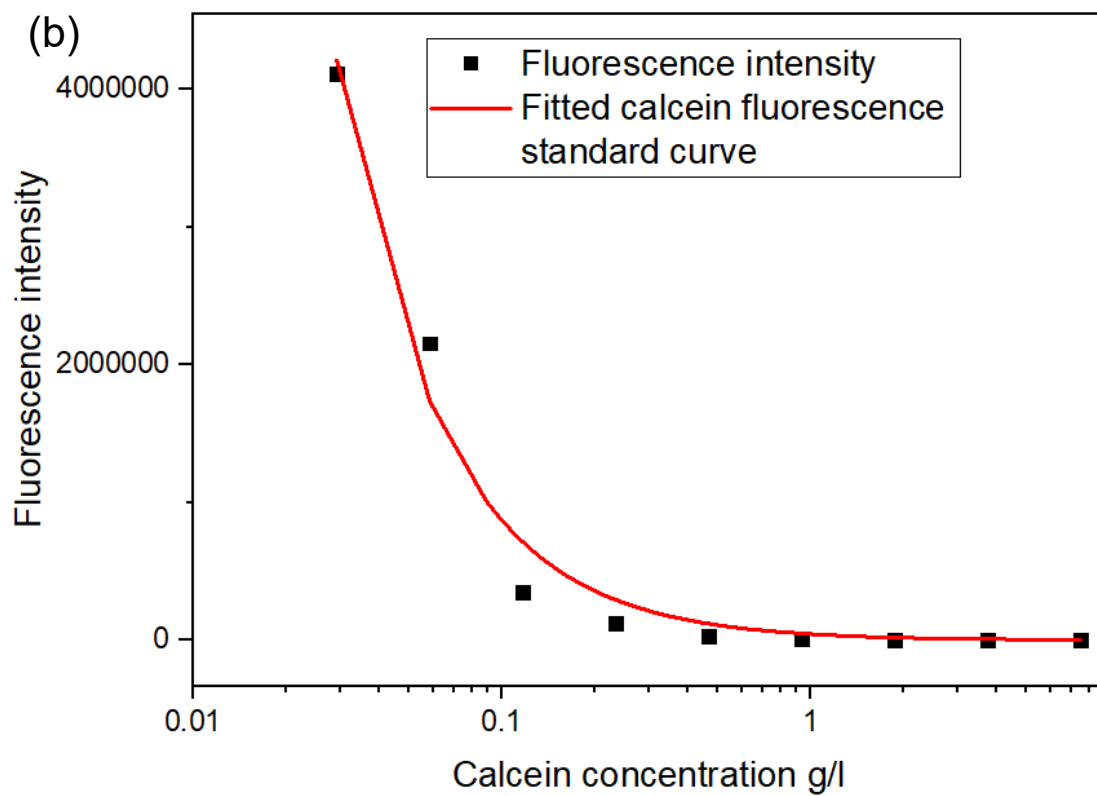
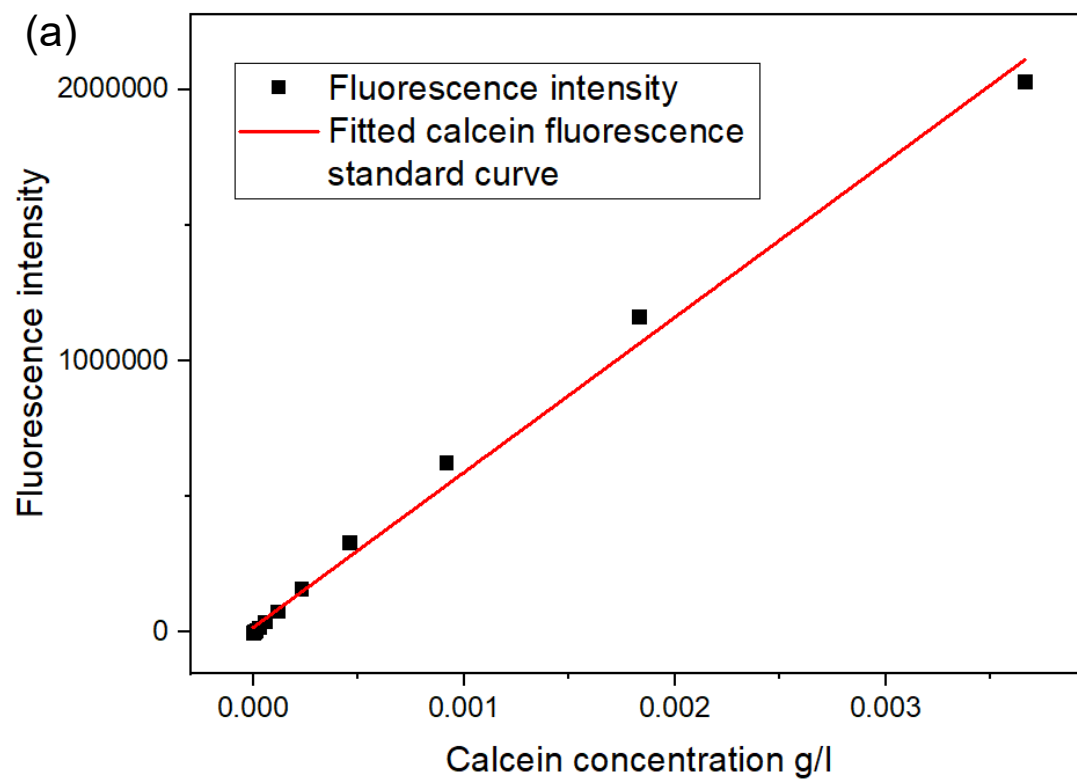


Figure 3.3. Fitted calcein fluorescence standard curves in HEPES buffer solution (pH=7.4) measured by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA). (a) Calcein concentration ranging from 0 g/L to 3.66 E-3 g/L. (b) Calcein concentration ranging from 2.93 E-2 g/L to 7.5 g/L.

Because it was difficult to fit the whole calcein fluorescence standard curve with only one model, two models were applied to fit two separate parts of this standard curve. As can be observed from Figure 3.3 (a), data points of calcein concentration ranging from 0 g/L to 3.66 E-3 g/L formed a linear relationship, thus linear equation was applied in the first part of the standard curve. Besides, linear scale in horizontal axis was used in the Figure 3.3 (a) to demonstrate the linear relationship clearly. On the other hand, the exponential function was utilised to fit the second part of the standard curve with calcein concentration ranging from 2.93 E-2 g/L to 7.5 g/L, of which the horizontal axis was a 'log 10' scale as same as that in the Figure 3.2 for a better observation. Most importantly, the selected two calcein concentration ranges can cover all concentrations that will be referred in this chapter.

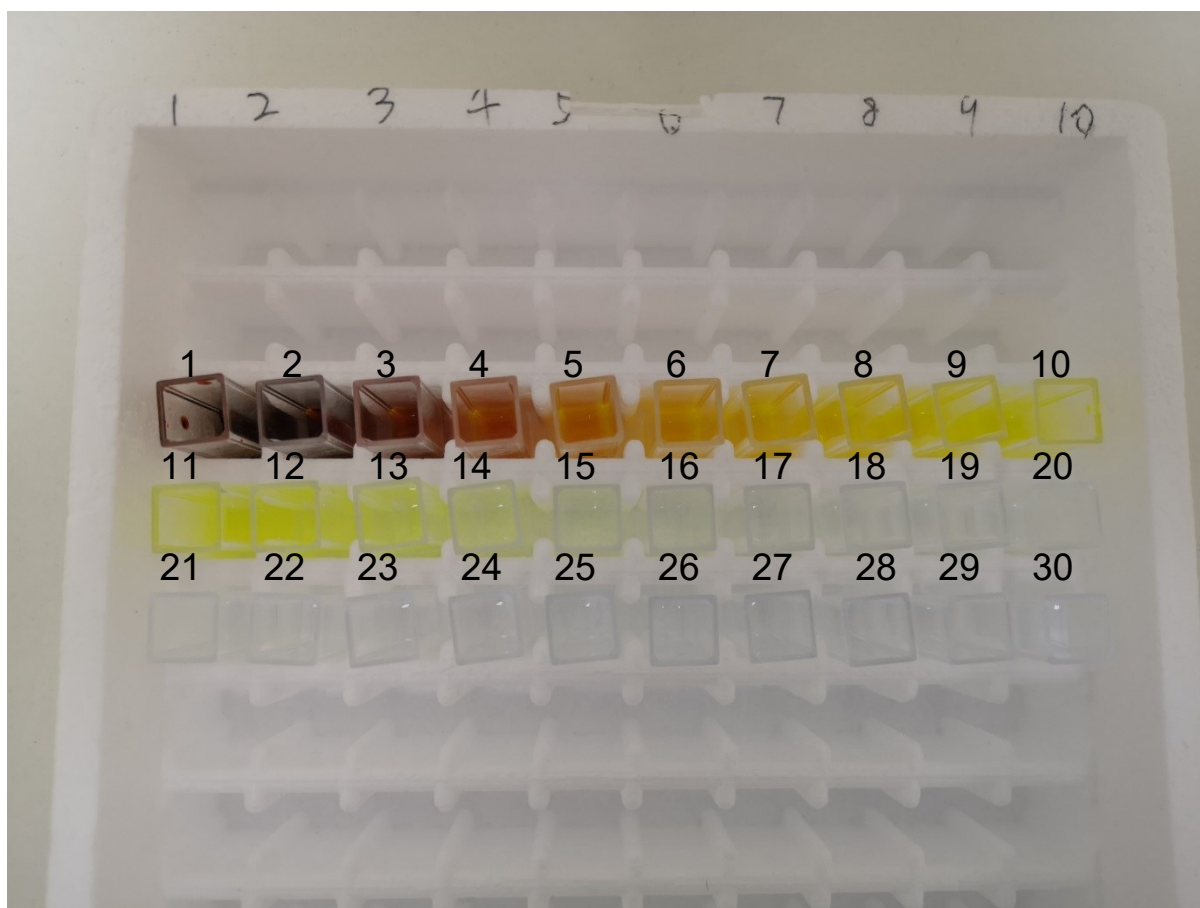


Figure 3.4 The photograph of calcein in HEPES buffer solution (pH 7.4) with gradient concentration for plotting calcein fluorescence standard curves (the calcein concentration of the following sample was the half of the previous one and the calcein concentration of first sample was 30 g/l).

As can be observed from Figure 3.4, the initial calcein solution sample in HEPES buffer solution with a concentration of 30 g/l has a dark red colour. With the decrease of the calcein concentration, the colour of solution turned into orange in the No. 5 sample at 1.88 g/l and subsequently turned into bright green in the No. 12 sample at 1.46 E-02 g/l where the strongest fluorescence intensity was found, which matched the data in Figure 3.2. After reaching the peak at 1.46 E-02 g/l, the fluorescence intensity declined gradually along with the fading of its colour.

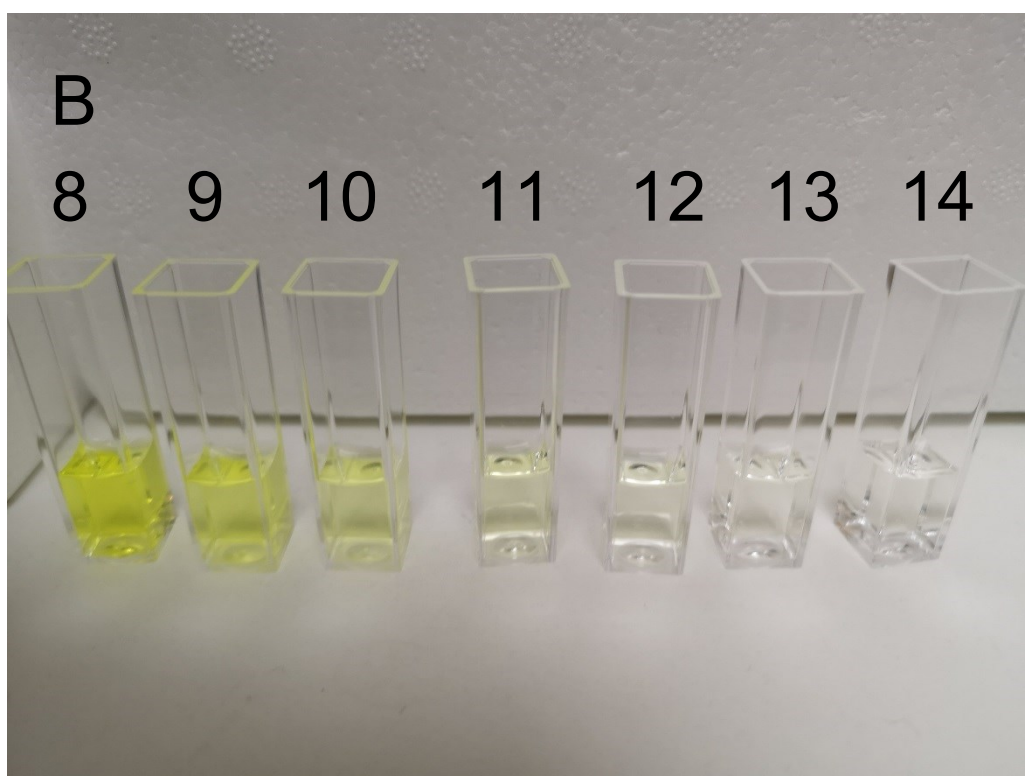
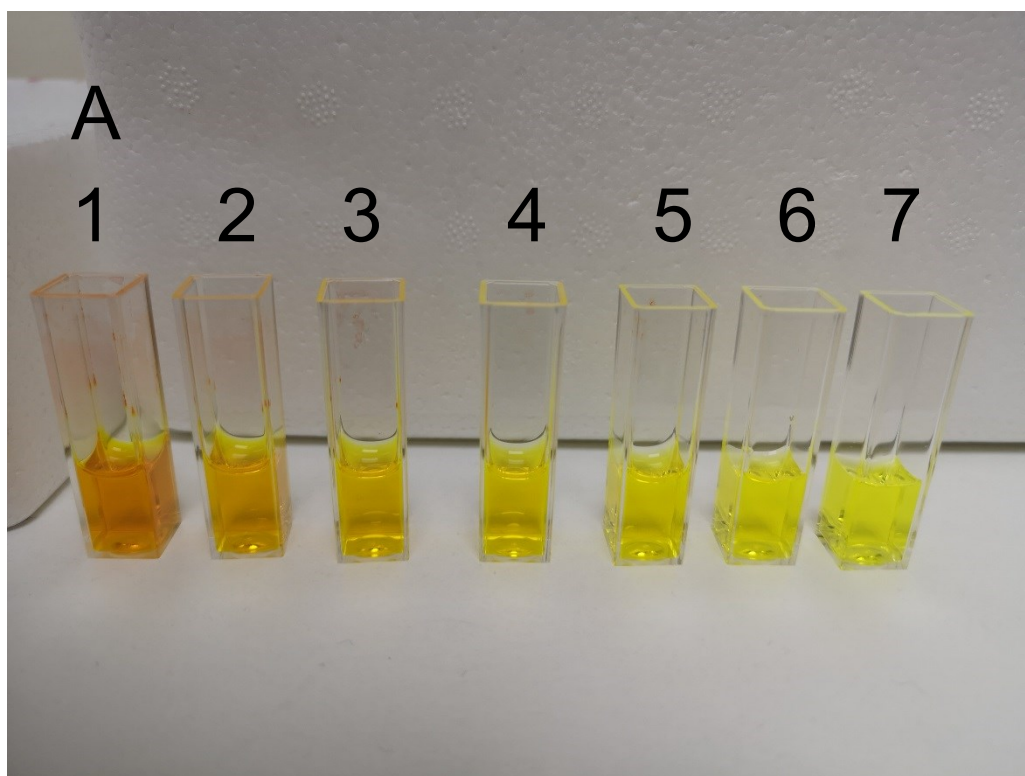


Figure 3.5 Photograph of calcein in acetic acid solution (pH=3.0) with gradient concentration for plotting calcein fluorescence standard curves (the calcein concentration of the following

sample was the half of the previous one and the calcein concentration of first sample was 30 g/l). Samples from No. 1-7 (A) and No. 8-14 (B).

Figure 3.5 depicts the colour variation of calcein in acetic acid solution (pH 3.0) with the dilution of calcein concentration. It is evident that the colour of initial calcein solution at 30 mg/ml in acetic acid solution was lighter than that in HEPES buffer solution in Figure 3.4, which means the addition of acidic solution changed calcein fluorescence property as well as its colour instantly. With the dilution of calcein concentration, its colour turned into bright green as well. Nevertheless, the strongest fluorescence intensity was observed in the 6th sample at 9.38 E-01 g/l rather than the 12th sample at 1.46 E-02 g/l in HEPES buffer, which signifies the decline of pH value of calcein solution could elevate the calcein concentration for its peak fluorescence intensity. In addition, the colour in acetic solution become colourless after the 13th sample at 7.32 E-03 g/l, whereas light green colour can still be observed before the 20th sample at 5.72 E-05 g/l in HEPES buffer in Figure 3.4.

3.3.2 Encapsulation efficiency of calcein encapsulated liposome

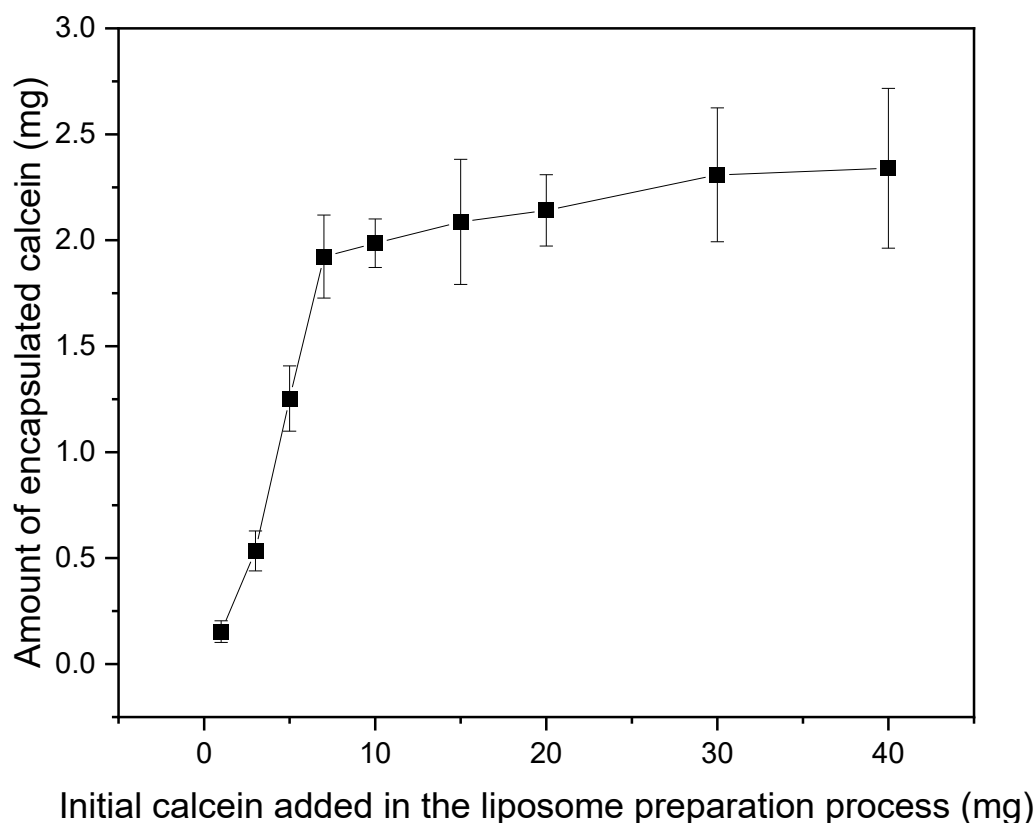


Figure 3.6 Effect of initial added calcein in the liposome preparation process on the amounts of encapsulated calcein inside liposomes. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 3.6 features the variation of the amount of encapsulated calcein depends on the initial added calcein in the liposome preparation process. As can be observed from this line chart, the maximum calcein loaded capacity is around 2.3 mg. The amount of encapsulated calcein surged from 0.153 mg to 1.923 mg as the initial calcein increased from 1 mg to 7 mg, before reaching a plateau at roughly 2 mg, which indicates the rise of initial added calcein higher than 7 mg did not produce an obvious higher amount of calcein encapsulated inside liposome. Calcein was dissolved in 1 ml deionised water in the preparation process, so its correspondent concentration was 7 mg/ml.

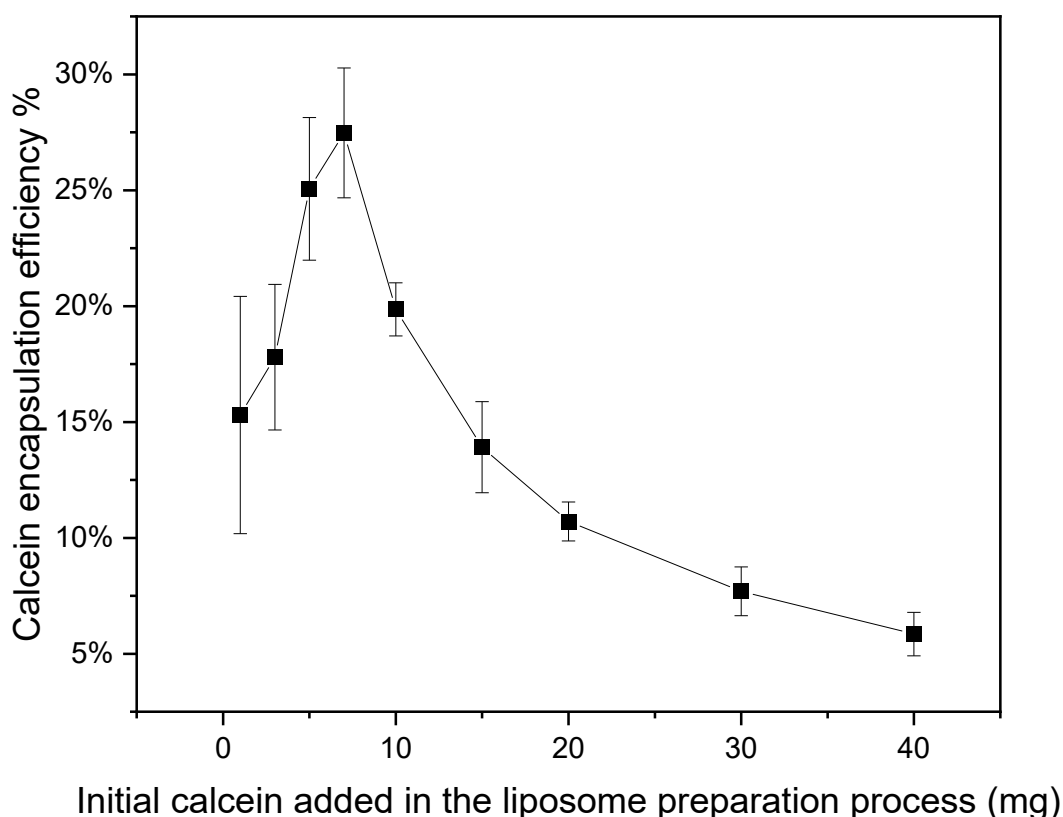


Figure 3.7 Effect of initial added calcein in the liposome preparation process on the encapsulation efficiency of calcein. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 3.7 outlines how calcein encapsulation efficiency is influenced by the initial calcein added in the liposome preparation procedure. It is clear that the highest encapsulation efficiency (27.47%) was achieved when 7 mg of initial calcein was added in the liposome preparation process. When the initial calcein was less than 7 mg, the encapsulation efficiency rose gradually from 15.30% to 25.06% as the amount of initial calcein increased. The encapsulation efficiency collapsed dramatically as the initial calcein beyond 7 mg, reaching the lowest percentage of 5.85% at 40 mg because the maximum amount can be encapsulated inside liposome was around 2.3 mg as described in Figure 3.6. This indicates that the calcein added in the preparation more than 7 mg would cause unnecessary waste.

Therefore, 7 mg of initial calcein was determined in the preparation of calcein encapsulated liposome procedure and characterisations of chitosan coated liposome in the following sections. Besides, the high standard deviations were discovered between the 1 mg to 5 mg of initial calcein. This is because the initial calcein amount was located at the denominator when calculating the percentage of calcein encapsulation efficiency. The smaller denominator results in larger deviation of percentage with similar numbers of numerator.

3.3.3 Release property of calcein encapsulated liposome

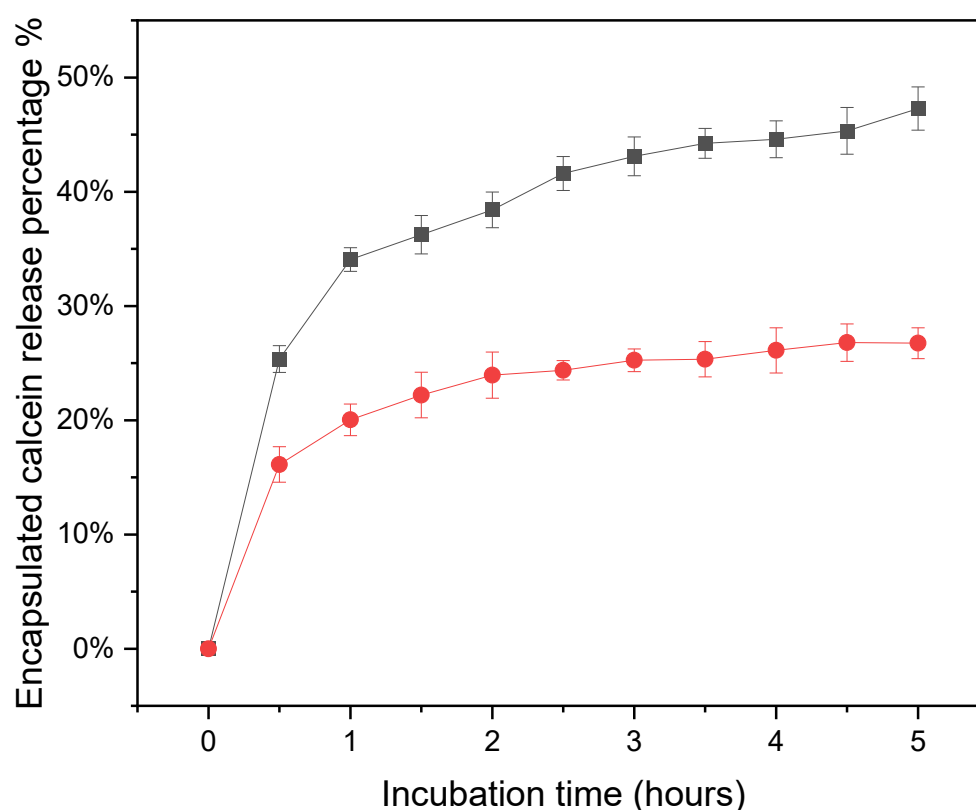


Figure 3.8 The percentages of encapsulated calcein released from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C in

HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 3.8 depicts the encapsulated calcein release percentage at 42 °C from plain liposomes without chitosan coating and liposomes coating with 0.1% (w/w) medium molecular weight chitosan at pH 7.4. As can be observed from this graph, both encapsulated calcein release percentages of two liposome samples experienced sharp rises in the first half hour, increasing from 0% to 25% for plain liposome and 16% for chitosan coated liposome separately. After the leap in the first half hour, release percentage from plain liposome ascended moderately from 25% to 47% after 5 hours incubation at 42 °C. Whereas, only slight increase of calcein release was observed from 0.5 hours to 3 hours in chitosan coated liposome, before the percentage reached a plateau at around 25% from 3 hours to 5 hours. In addition, the gap between two percentages were also widened slightly, climbing from less than 10% at 0.5 hours point to roughly 21% at 5 hours point.

As reported, the transition temperature of DPPC has been shown to be 41.3 °C [230]. Besides, HaT liposome has a similar transition temperature (T_m) of (41-41.5 °C) and the increase amount of Brij S20 incorporated in the HaT liposome did not alter the T_m dramatically [98] [231]. Above transition temperature of HaT liposome, large amount of encapsulated calcein released from plain liposome (47 %), which means that the liposome membrane transformed from solid phase into liquid phase.

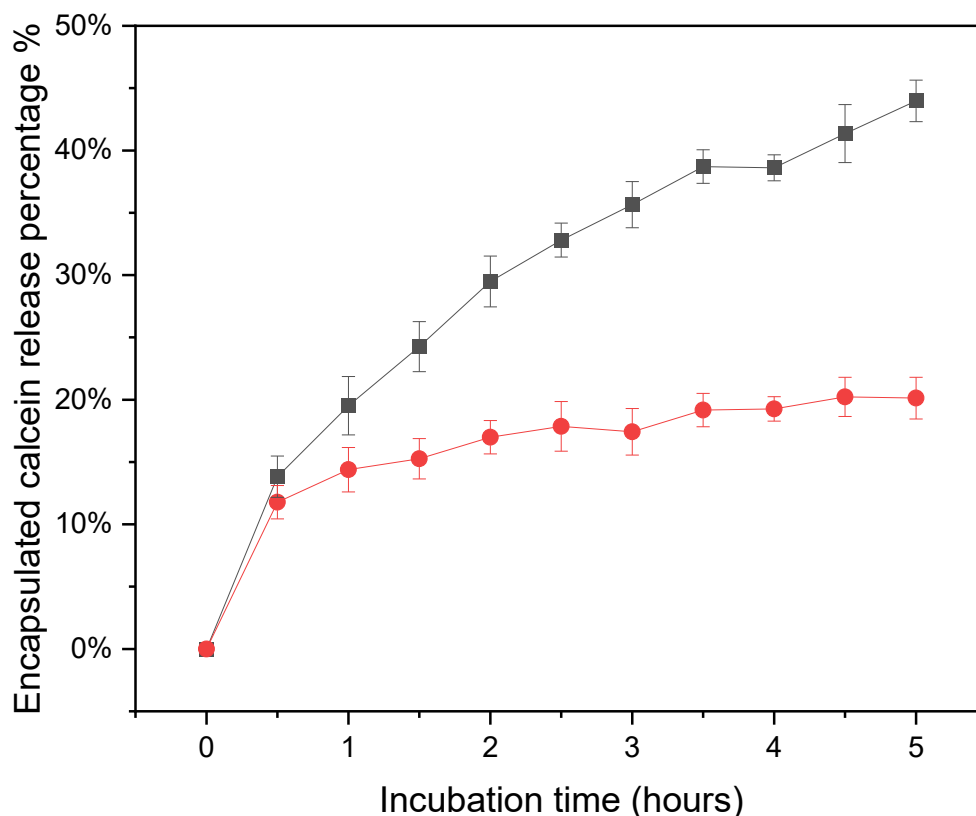


Figure 3.9 The percentages of encapsulated calcein released from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 3.9 outlines the variation of calcein release percentage depending on various incubation time of plain liposomes and liposomes coated with 0.1% (w/w) medium molecular weight chitosan at pH 7.4 at 40 °C. It is clear that, in the first half hour, both calcein release percentage saw dramatic growths, rising from 0% to 13.8% for plain liposome and 11.8% for chitosan coated liposome, with merely 2% difference. After the first half hour, the percentage of plain liposome kept the same growth rate, climbing constantly and substantially to 44.0% after 5 hours incubation. Nevertheless, the growth on the percentage of chitosan coated

liposomes after the first half hour was much lower than that of plain liposome, a moderate increase of less than 9% can be observed from the period of 0.5 to 5 hours. Besides, the difference value between two curves rose more drastically than that in Figure 3.7., from around 2% at 0.5 hour to more than 23% at 5 hours.

As can be observed from this graph, plain HaT liposome still released large amount of encapsulated calcein (44.0%) at 40 °C which is slightly lower than its transition temperature. Our results are consistent with the original paper of HaT liposome, whose release curve at 40 °C were similar with that at 42 °C [99].

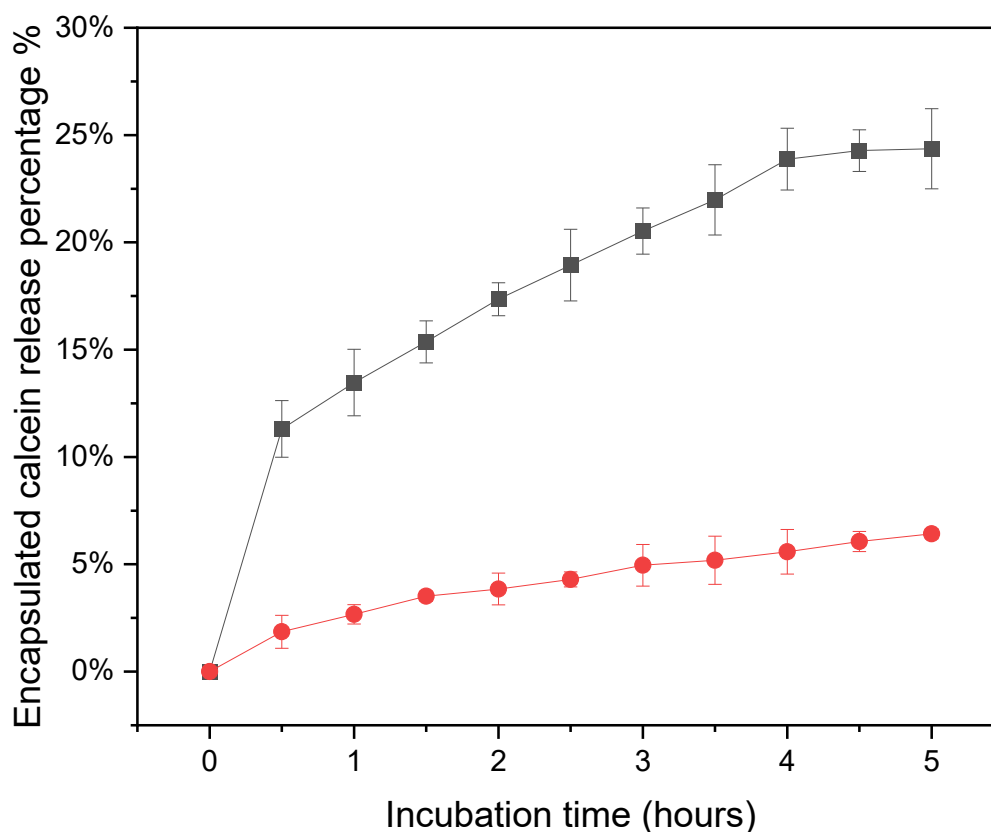


Figure 3.10 The percentages of encapsulated calcein released from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 3.10 features the comparison of two calcein release percentages of liposomes coated with and without chitosan at pH 7.4 at 37 °C over a time span of 5 hours. Unlike two other graphs (Figure 3.8 and Figure 3.9), a sharp rise can only be observed from the calcein release percentage of plain HaT liposomes in the first half hour rather than both two curves of plain and chitosan coated liposomes. Instead, merely a slight increase was found in the percentage of chitosan coated liposome in the first 0.5 hour. Then, the percentage continued to rise with the same low growth rate until reaching to 6.4% after 5 hours.

At physiological temperatures of 37 °C, the plain HaT liposome released merely half of that at 40 °C and 42 °C. Nevertheless, the coating of 0.1% (w/w) medium molecular weight chitosan enhance its stability further, reducing the leakage dramatically from 24.4% in plain liposome to 6.4%.

The rapid releases in the first half hour can be observed in all three figures, this is because the calcein concentration gradient between the interior liposome and external buffer solution is the highest in the beginning. As more calcein released gradually into the HEPES buffer solution, the concentration gradient decreased, and the release rate dropped correspondingly until the equilibrium is achieved.

3.3.4 Shelf life of calcein encapsulated liposome

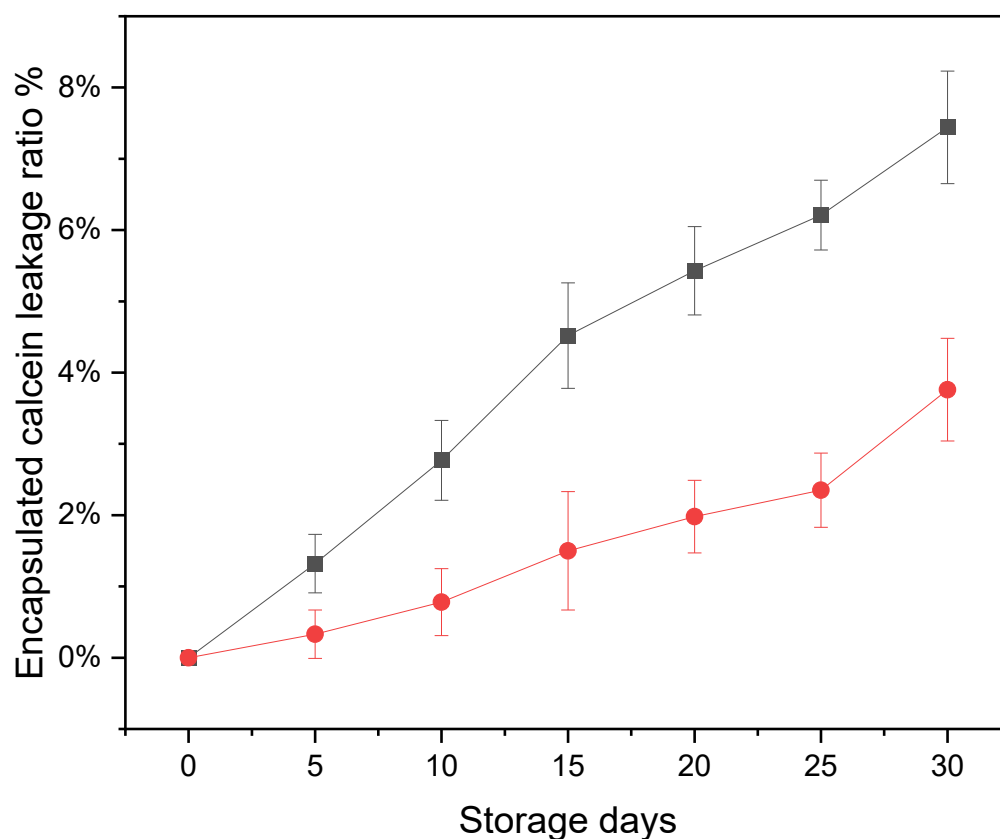


Figure 3.11 The percentages of encapsulated calcein leaked from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 4 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean $\pm$ SD. n=3 independent batches.

The Fig 3.11 contrasts the shelf life of plain liposome and chitosan coated liposome suspension at pH 7.4 at 4 °C. It is manifest that, at 4 °C, both liposome suspensions leaked relatively slowly (around 7.5% for plain liposome and 3.7% for chitosan coated liposome) after 30 days storage. It is also clear that the leakage is approximately linear over this time scale. The coating of 0.1% (w/w) medium molecular weight chitosan on the liposome surface successfully enhance the stability of liposome by reducing the leakage to roughly 4% of calcein from the liposome. Moreover, throughout whole period of 30 days, the chitosan

coated liposome leaked less than plain HaT liposome. The gap between leakage ratio of two samples increased from around 1% to 4% as the storage time extended.

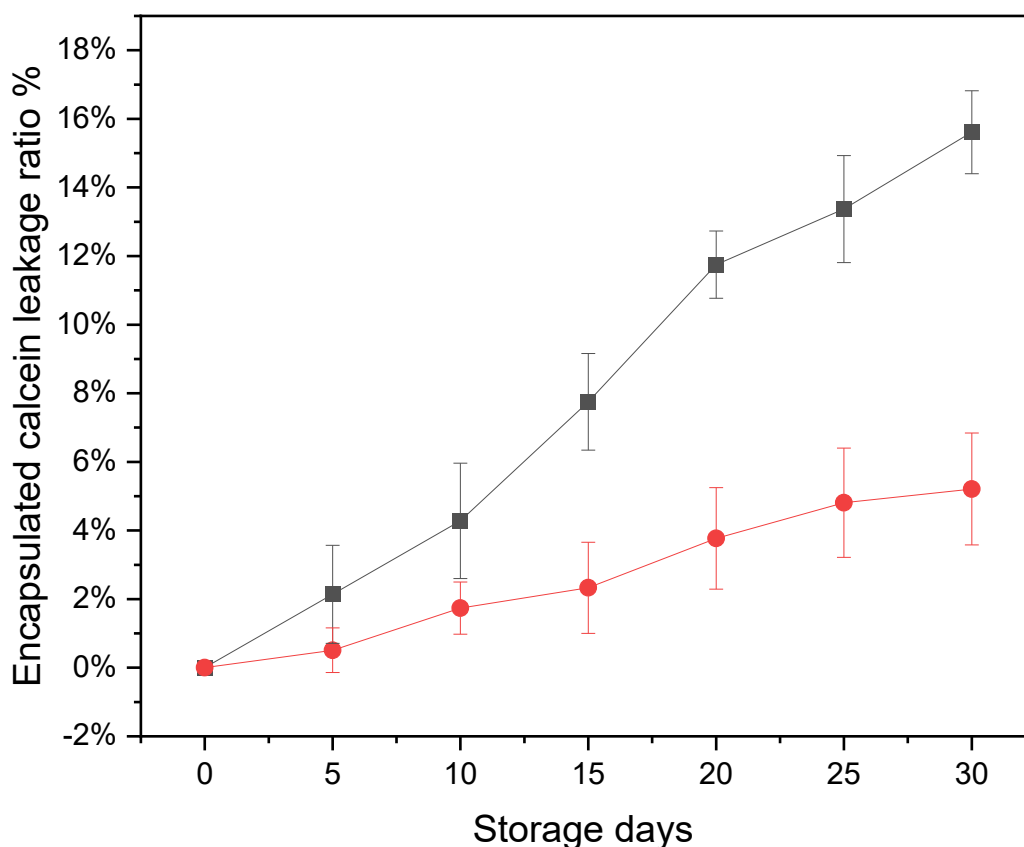


Figure 3.12 The percentages of encapsulated calcein leaked from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 25 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 3.12 outlines the comparison of leakage properties of liposome with and without chitosan coating at pH 7.4 at 25 °C. As can be observed from this graph, both liposomes stored at room temperature experienced more leakage than those stored at 4 °C (around

15% leakage for plain liposome and 5% for chitosan coated liposome). Especially for the standard HaT liposome, the release percentage from this plain liposome at room temperature (15.8%) was more than two times as much as that stored at 4 °C (7.3%). Although the leakage of the chitosan coated liposome was greater than at 4 °C, after 30 days at 25 °C. The leakage is only around 5%, compared to just under 4% at 4 °C. Again, the rate of leakage is approximately linear over the whole range of time, with the leakage from chitosan coated liposome always being lower than that from plain HaT liposome during the whole period.

3.3.5 Fitting of release curves of calcein encapsulated liposomes using different pharmaceutical models

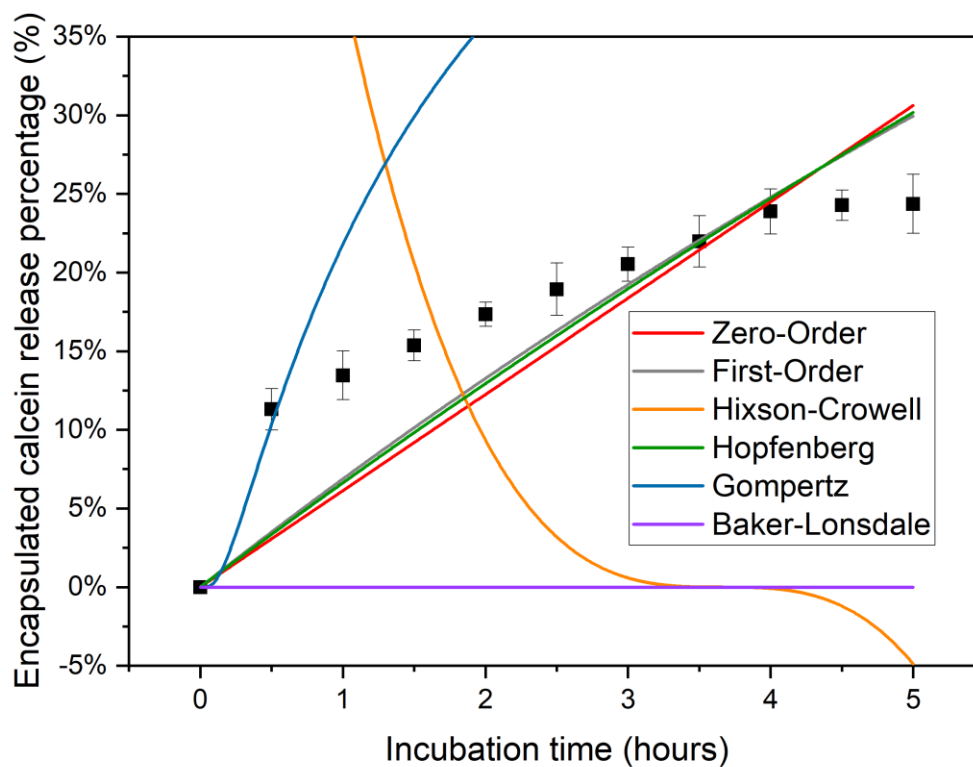
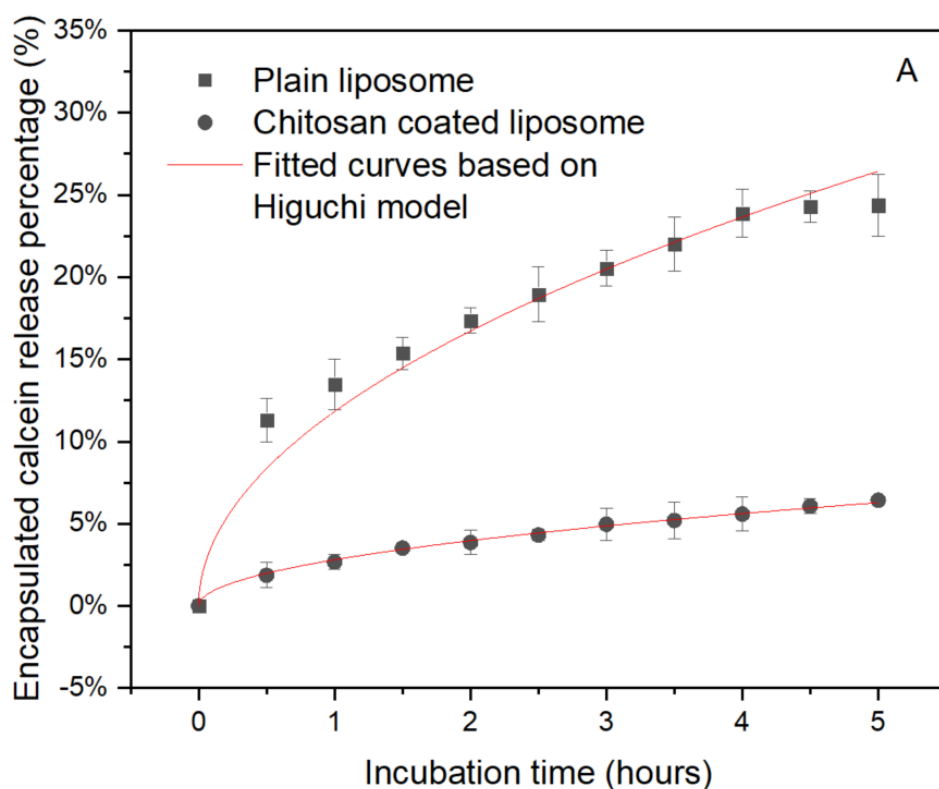


Figure 3.13 Zero-Order, First-Order, Hixson-Crowell, Hopfenberg, Gompertz and Baker-Lonsdale models are utilised to fit calcein release from plain liposomes (■) at 37 °C. Data represents mean $\pm$ SD. n=3 independent batches.

Take the calcein release curve from plain liposomes at 37 °C as example, Zero-Order, First-Order, Hixson-Crowell, Hopfenberg, Gompertz and Baker-Lonsdale models are used for the initial trials of fitting. Apparently, as can be observed from the Figure 3.13, these models are not suitable or cannot converge on calcein release curves in this case. Therefore, Higuchi, Korsmeyer-Peppas and Weibull models are selected for the final detailed comparison and discussion in the following section.



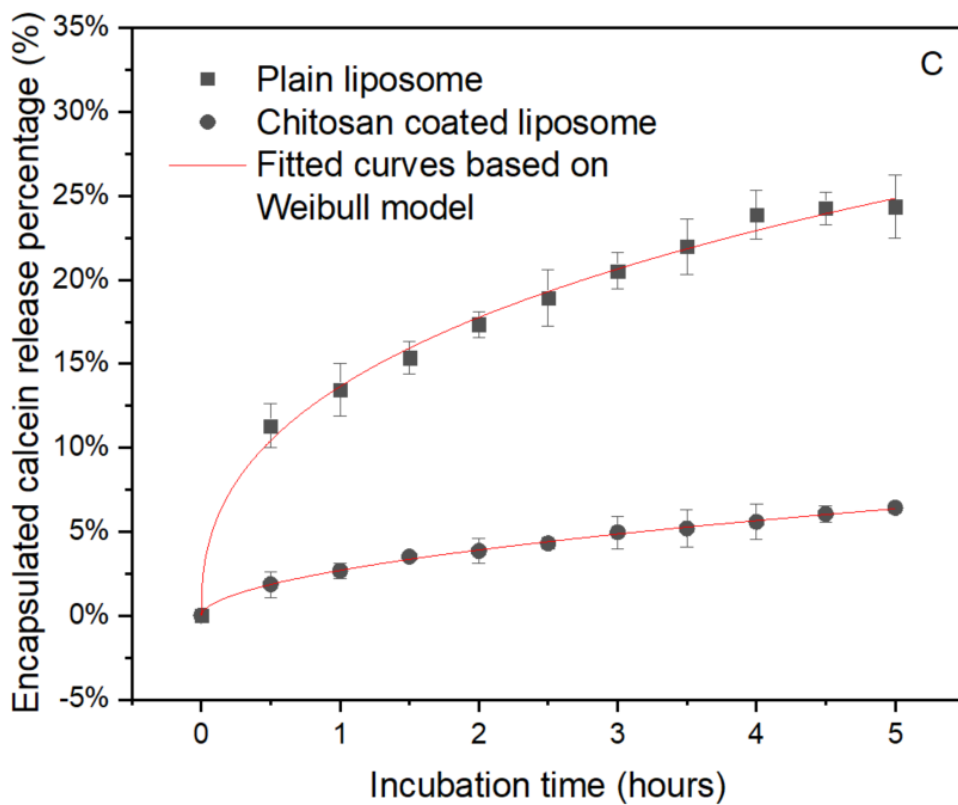
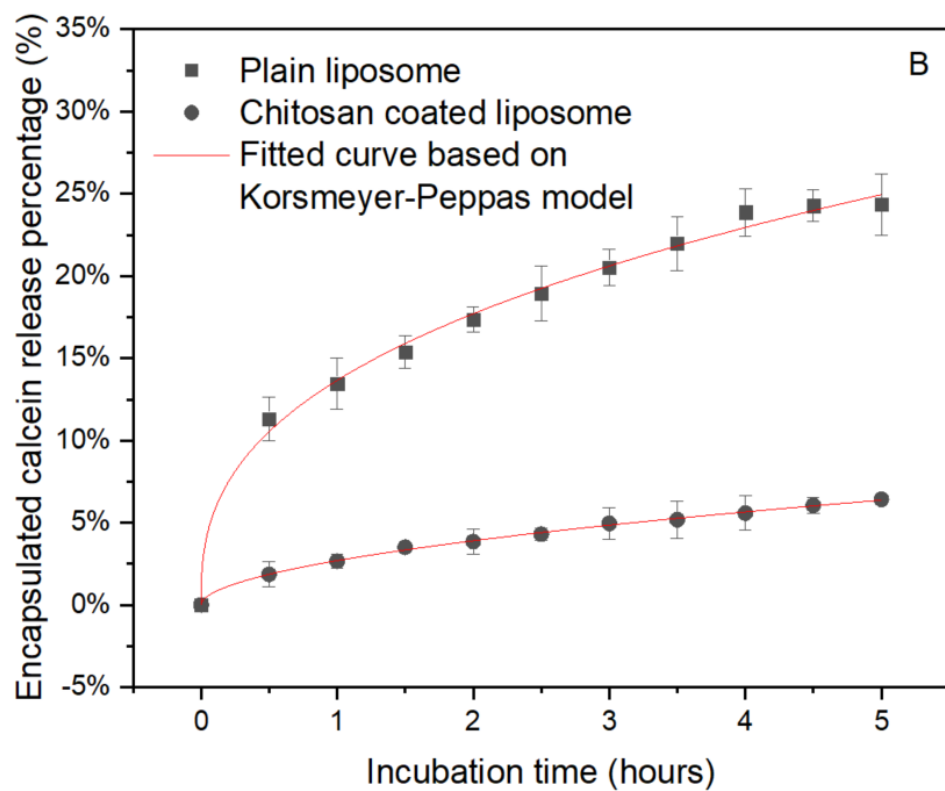
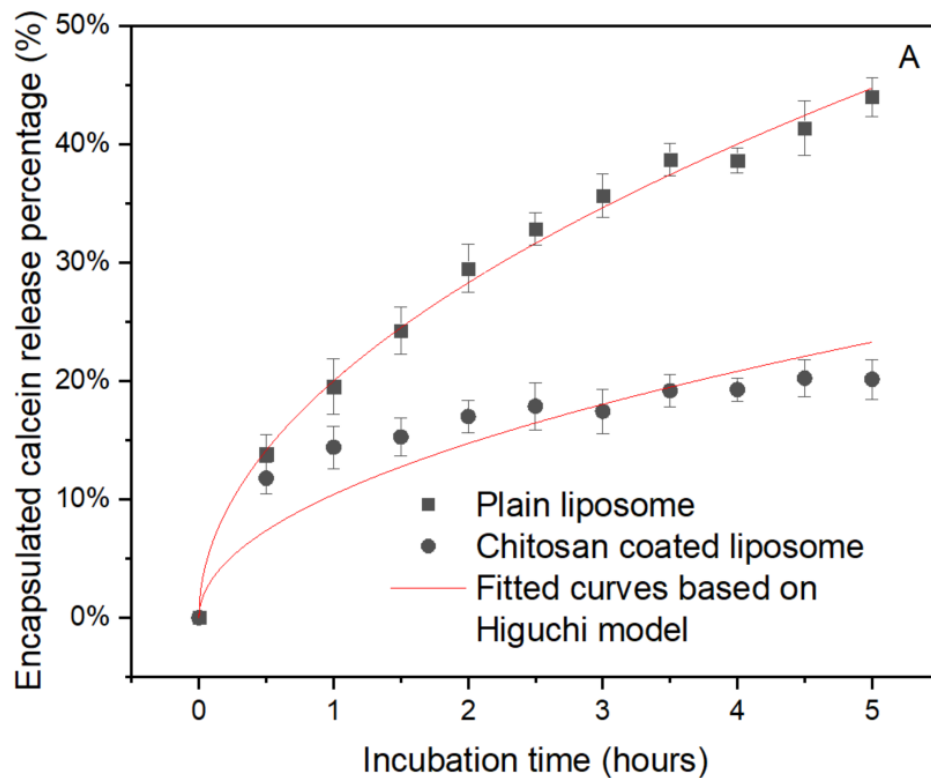


Figure 3.14 Higuchi (A), Korsmeyer-Peppas (B) and Weibull (C) models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium

molecular weight chitosan (●) at 37 °C. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 3.14 outlines the fitted curves using three models respectively. Apparently, Weibull model and Korsmeyer-Peppas model fit well to both calcein release curves of plain liposome and chitosan coated liposome at 37 °C. For the Higuchi model, it fits relatively well to release profile of chitosan coated liposome while many data points of plain liposome deviate from the fitted curve.



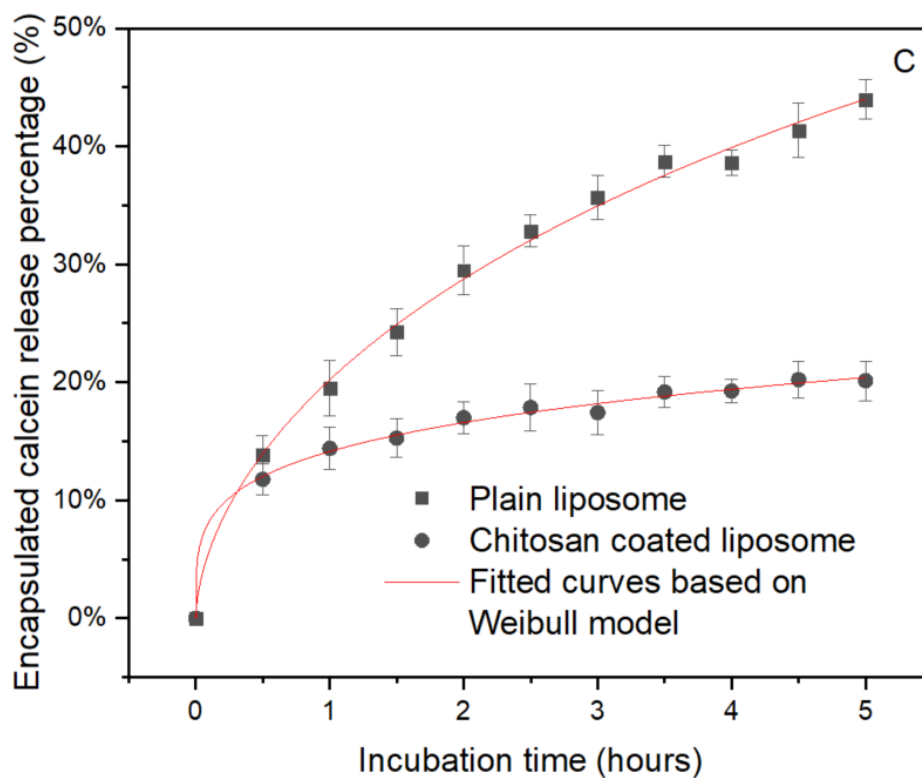
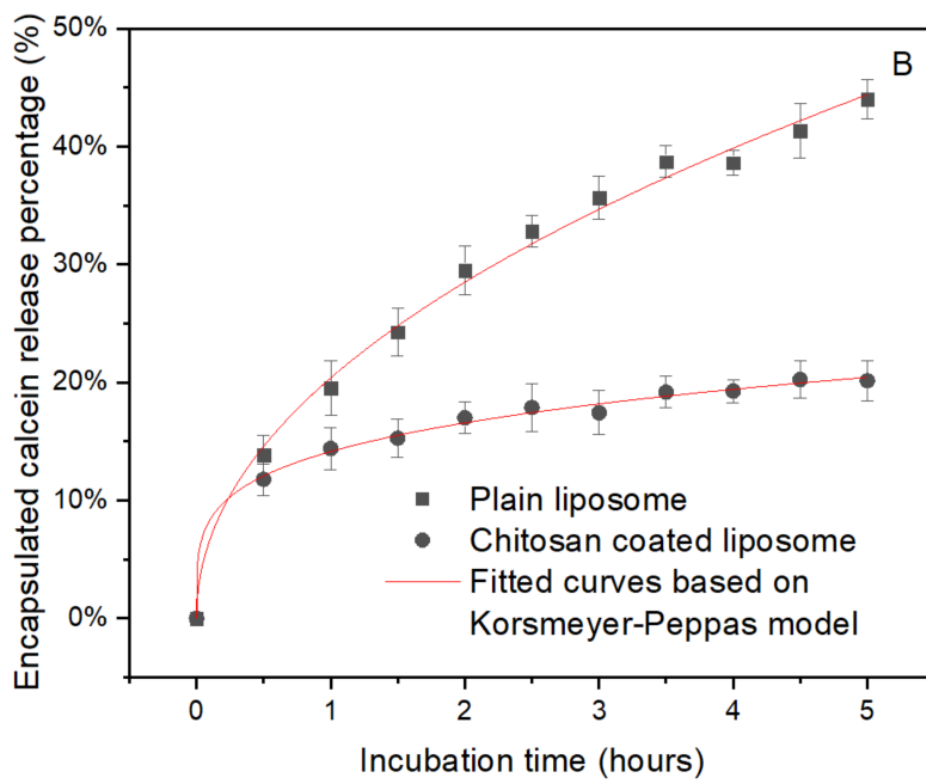
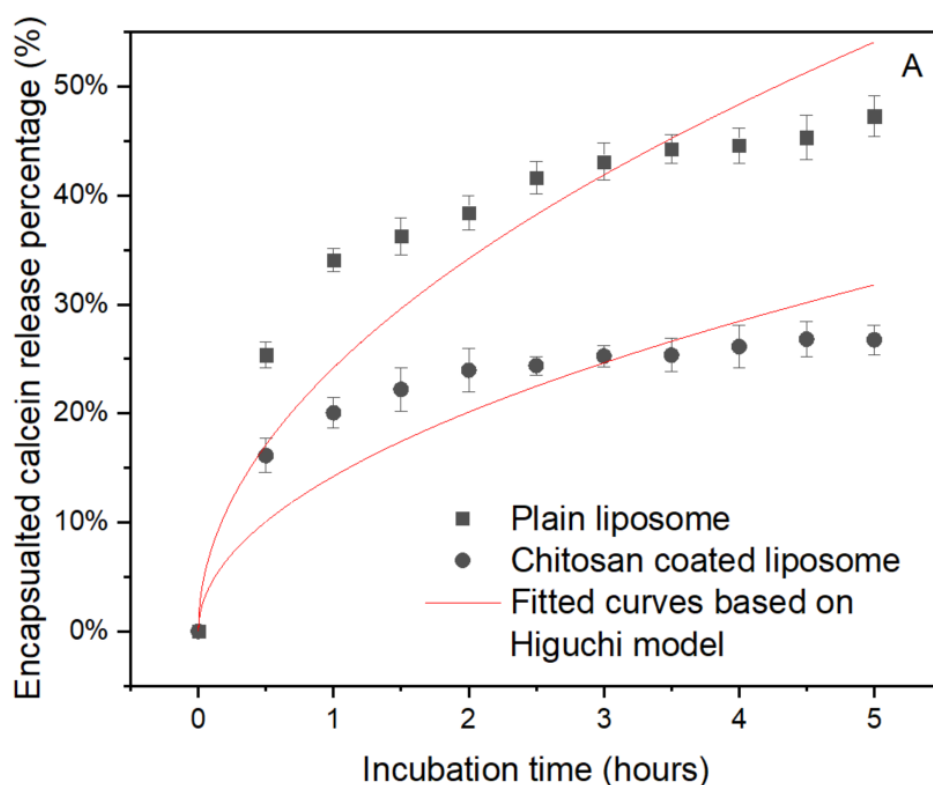


Figure 3.15 Higuchi (A), Korsmeyer-Peppas (B) and Weibull (C) models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 3.15 compares fitted curves of calcein release from both plain liposome and chitosan coated liposome at 40 °C by three pharmaceutical models. It is clear that Korsmeyer-Peppas model and Weibull model have superior goodness-of-fit than Higuchi model, which is consistent with the fitting results in Figure 3.14. To be specific, Higuchi model fits better with plain liposome release curve than chitosan coated liposome at 40 °C, where most of the data points are not on the fitting curve of Higuchi model.



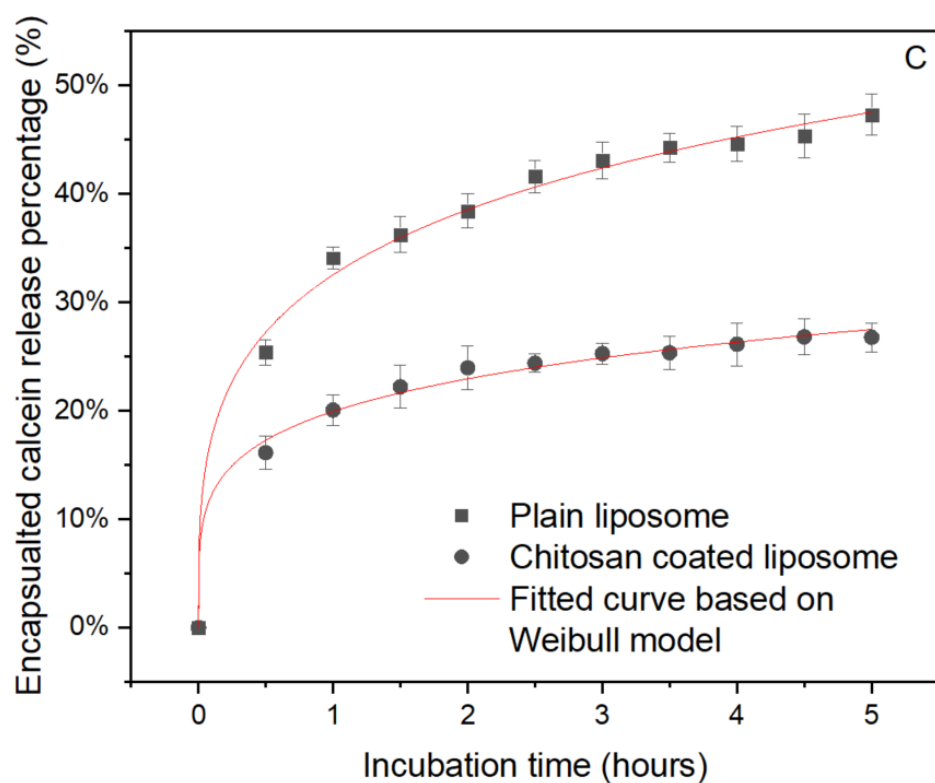
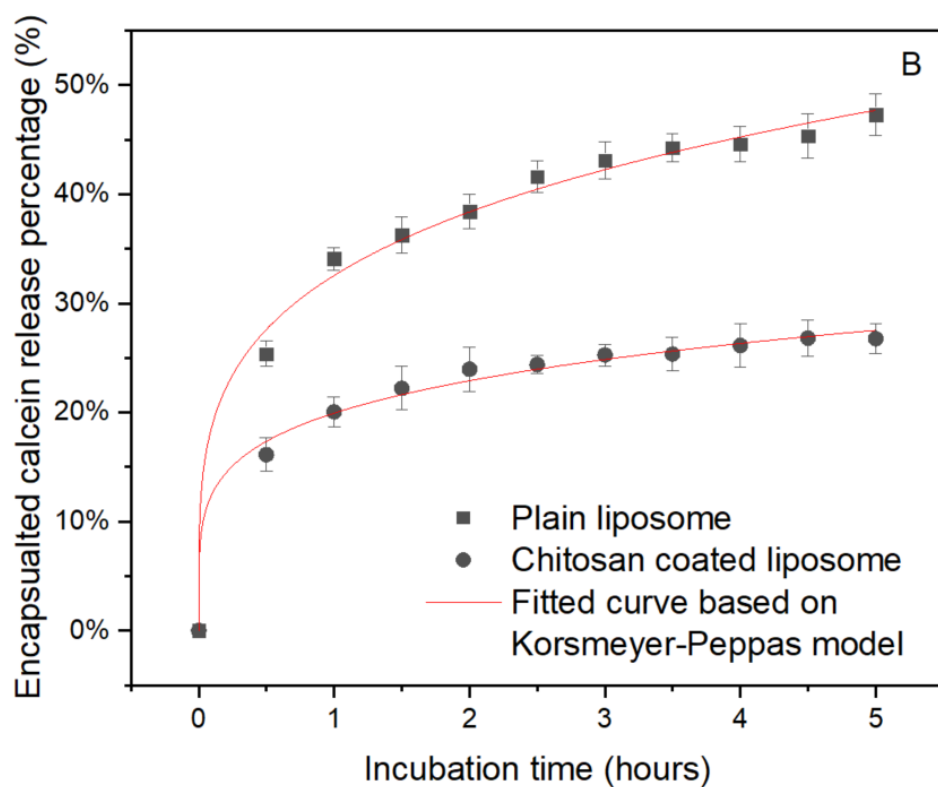
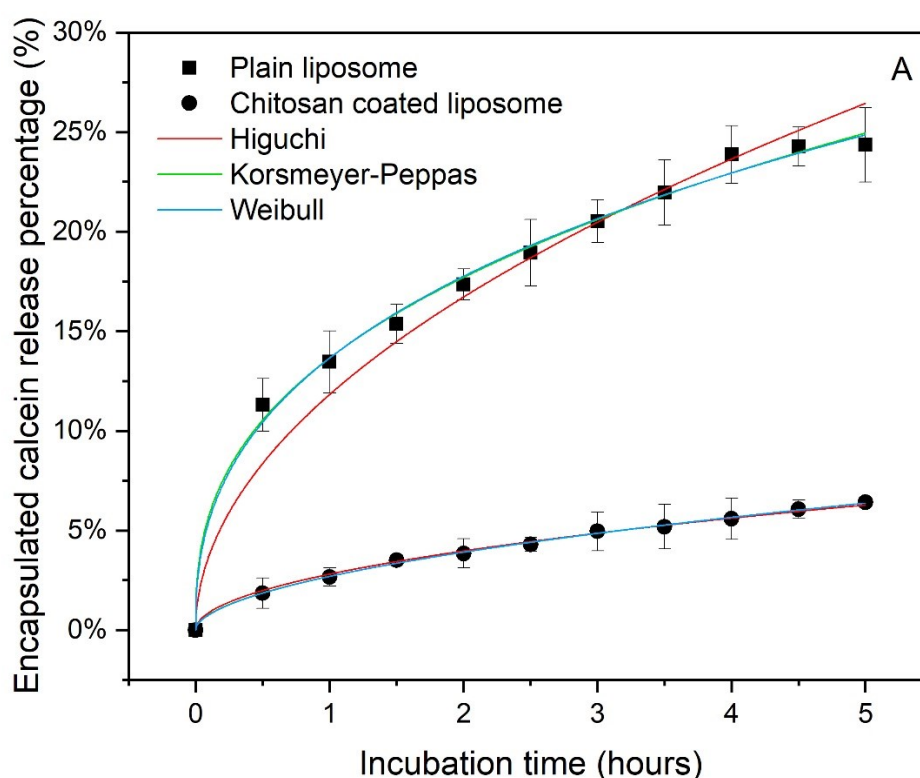


Figure 3.16 Higuchi (A), Korsmeyer-Peppas (B) and Weibull (C) models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 3.16 depicts the comparison of release curves fitted by three models. Similar to the results of Figure 3.14 and Figure 3.15, Korsmeyer-Peppas model and Weibull model have an obviously better goodness-of-fit than Higuchi model. Furthermore, in this case, Higuchi model is not fitted well to both release curves of plain liposome and chitosan coated liposome at 42 °C. Nevertheless, from the results of above fitting graphs, it is not clear which model is superior between Korsmeyer-Peppas model and Weibull model.



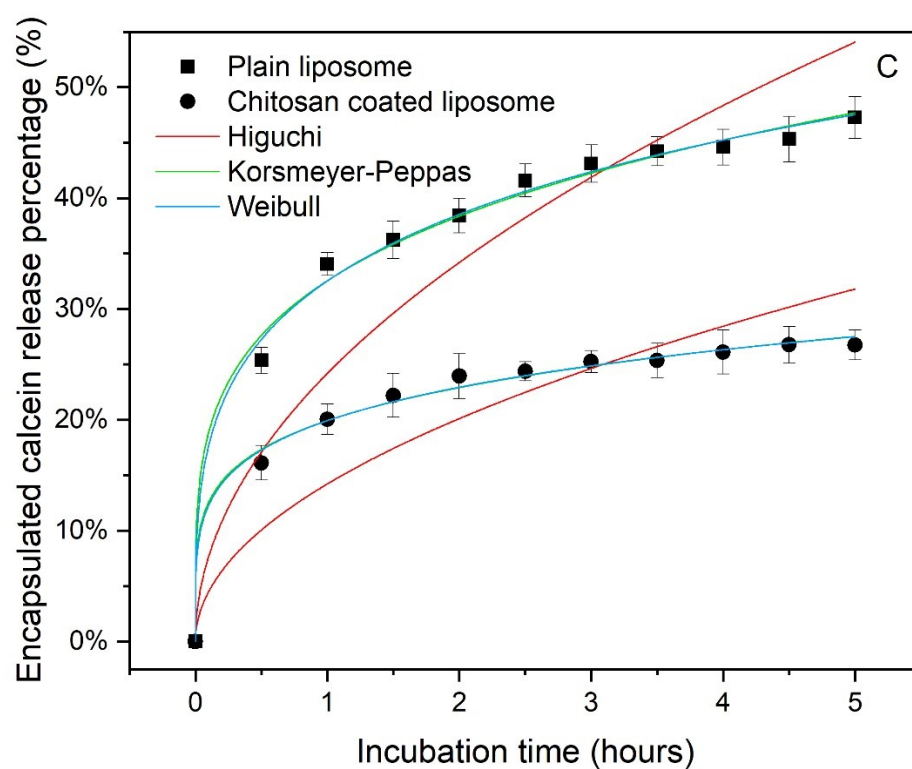
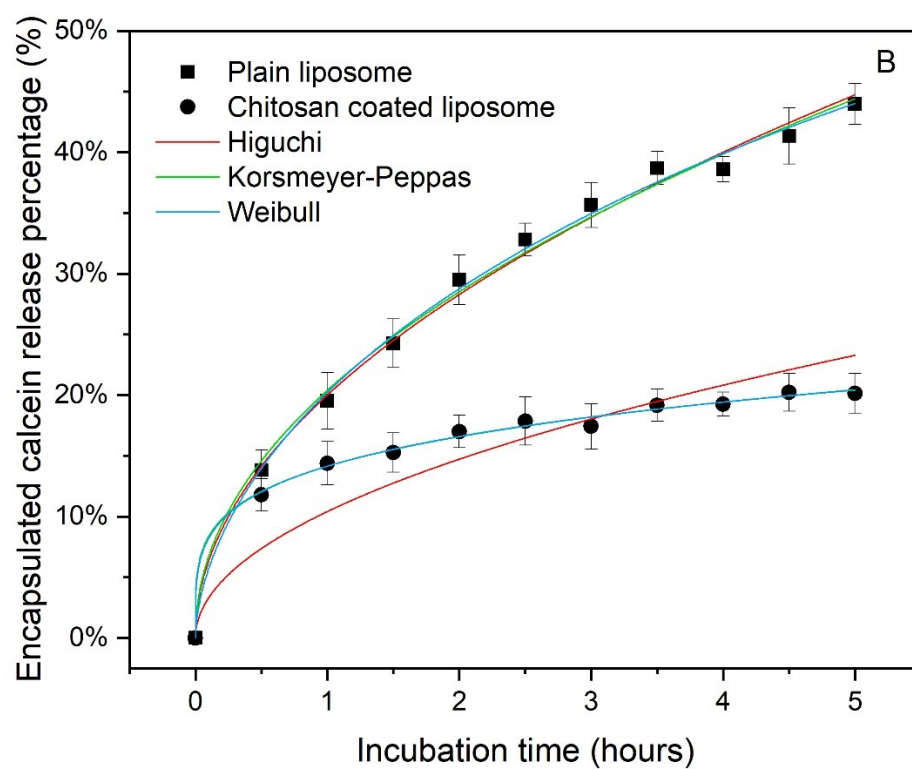


Figure 3.17 Higuchi, Korsmeyer-Peppas and Weibull models are utilised to fit calcein release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C (A), 40 °C (B) and 42 °C (C). Data represents mean $\pm$ SD. n=3 independent batches.

As can be observed from the Figure 3.17, both Korsmeyer-Peppas and Weibull models can fit the calcein release curves from both plain liposomes and chitosan coated liposomes at all three temperatures. It is difficult to distinguish which model is better because the two fitted curves almost overlap. Therefore, more specific and quantified parameters such as R^2 , BIC value and RSS are requisite for further analysis, which will be discussed in the following section. On the other hand, it is apparent that Higuchi model does not fit well with the release curve of plain liposome at 37 °C, that of chitosan coated liposome at 40 °C, and release curves of both plain and chitosan coated liposomes at 42 °C.

3.3.6 Comparison of fitting parameters

Table 3.3 The R^2 (COD) of each fitted curves using Higuchi, Korsmeyer-Peppas and Weibull models. Abbreviations: CPL, plain liposomes encapsulated with calcein; CCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsulated with calcein.

Model	37 °C CPL	40 °C CPL	42 °C CPL	37 °C CCL	40 °C CCL	42 °C CCL
Higuchi	96.7%	99.5%	81.8%	99.7%	80.8%	74.5%
Korsmeyer-Peppas	99.5%	99.5%	99.4%	99.8%	99.6%	99.3%
Weibull	99.5%	99.7%	99.5%	99.8%	99.6%	99.4%

Table 3.3 depicts the R^2 (COD) values of release curves of CPL and CCL at 37 °C, 40 °C and 42 °C fitted by Higuchi, Korsmeyer-Peppas and Weibull models. It is manifest that the R^2 of most curves fitted by Higuchi model are below 0.99, apart from '40 °C CPL' and '37 °C CCL'. As a contrast, the R^2 of all six curves fitted by Korsmeyer-Peppas and Weibull models are more than 99%, which indicates these two models fit data well. To be specific, Weibull model is slightly superior than Korsmeyer-Peppas model since it has higher values of R^2 than Korsmeyer-Peppas model in most cases. Moreover, all R^2 of curves fitted by Weibull model are above 99.4%, among which '37 °C CCL' has the highest value of 99.8% while the lowest number is in '42 °C CCL' (99.4%). Therefore, based on the analysis of R^2 , Weibull model is the most appropriate model to fit calcein release curves of liposomes with and without chitosan coating at three temperatures.

Table 3.4 The BIC values of each fitted curves using Higuchi, Korsmeyer-Peppas and Weibull models. Abbreviations: CPL, plain liposomes encapsulated with calcein; CCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with calcein.

<i>Model</i>	<i>37 °C CPL</i>	<i>40 °C CPL</i>	<i>42 °C CPL</i>	<i>37 °C CCL</i>	<i>40 °C CCL</i>	<i>42 °C CCL</i>
<i>Higuchi</i>	-91.3	-98.0	-58.9	-145.9	-77.0	-67.3
<i>Korsmeyer-Peppas</i>	-110.6	-96.4	-93.4	-150.1	-116.5	-105.2
<i>Weibull</i>	-109.5	-100.8	-96.0	-149.9	-116.8	-106.2

Table 3.4 features the BIC values of all fitted curves. As can be observed from this table, most BIC values of curves fitted by Higuchi model are greater than other two models. Apart

from '37 °C CPL' and '37 °C CCL', Weibull model has the lowest BIC values among other four curves. Specifically, smallest BIC value (-149.9) of Weibull model is discovered in '37 °C CCL' and the highest is for '42 °C CPL' at -96.0. Because smaller BIC value represents better goodness of fit, Weibull model is considered to be the most suitable model for these release profiles and Korsmeyer-Peppas takes up the second position while Higuchi is not well compatible to these curves apparently.

Table 3.5 The RSS of each fitted curves using Higuchi, Korsmeyer-Peppas and Weibull models. Abbreviations: CPL, plain liposomes encapsulated with calcein; CCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsulated with calcein.

<i>Model</i>	<i>37 °C CPL</i>	<i>40 °C CPL</i>	<i>42 °C CPL</i>	<i>37 °C CCL</i>	<i>40 °C CCL</i>	<i>42 °C CCL</i>
<i>Higuchi</i>	1.76E-03	9.59E-04	3.37E-02	1.23E-05	6.50E-03	1.56E-02
<i>Korsmeyer-Peppas</i>	2.45E-04	8.90E-04	1.17E-03	6.81E-06	1.44E-04	4.03E-04
<i>Weibull</i>	2.71E-04	6.01E-04	9.29E-04	6.93E-06	1.40E-04	3.67E-04

Table 3.5 outlines the RSS of all calcein release data fitted by three pharmaceutical models. Obviously, all RSS of Higuchi models are much higher than those of other two models and Weibull model has the lowest RSS in most data sets. The differences of RSS among each curve are far significant than BIC and R^2 . For instance, the RSS value of '42 °C CPL' fitted by Higuchi model (3.37E-02) is around 2700 times more than that of '37 °C CCL' (1.23E-05). Besides, it is manifest that the smallest RSSs fitted by all three models are in the group of '37 °C CCL', in the meantime, the '42 °C CPL' group rank the top.

Overall, based on analysis of all three essential parameters (R^2 , BIC and RSS), we can conclude that Weibull model is the best model in this scenario, the next one is Korsmeyer-Peppas model, and Higuchi model has the worst goodness of fit, which is not appropriate to fit these data sets. The worst fitting results of Higuchi model ($Q/Q_0 = k * t^{0.5}$) is because its exponent is fixed at 0.5 and the only constant is 'k'. By contrast, the Korsmeyer-Peppas model, ($Q/Q_0 = k * t^n$), provides two constants including the exponent 'n', which can fit the release curves better. Even though Korsmeyer-Peppas model and Weibull model ($Q/Q_0 = 1 - e^{(-b*t^a)}$) have completely different mathematical expressions, both models have two constants and can offer "Γ" shape-like fitted profiles and it is difficult to distinguish the better one by the rough observation. Nevertheless, the fitting parameters proves the Weibull model has the best goodness of fit, this may be because it is an entirely empirical model without any pharmacokinetic basis, as illustrated in 3.2.7. In that case, this model would be compatible in more fitting scenarios than Korsmeyer-Peppas model.

3.4 Conclusion

1. The calcein fluorescence intensity standard curve obtained from Horiba FluoroMax-4 spectrofluorometer proved the fluorescence intensity of calcein is sensitive to pH and acidic environment impaired fluorescence intensity of calcein significantly. These data were used to plot the fluorescence intensity standard curve as a reference for the following measurements of calcein encapsulation efficiency, release property and shelf life.
2. 7 mg of initial calcein added in the preparation process was shown to have the highest encapsulation efficiency and this concentration was used in all subsequent release studies.

3. The coating of 0.1% (w/w) medium molecular weight chitosan on the liposome can reduce release percentage from liposome drastically at all three temperature (37 °C, 40 °C and 42 °C). Especially at 37 °C, the coating of chitosan enhanced the stability of liposome significantly. Even though the coating of chitosan decreased the release percentage from liposome above 40 °C, the release percentage is still substantial.
4. At 4 °C, both plain liposome and chitosan coated liposome leaked slowly, with the chitosan coated lipid still able to enhance the liposome. Moreover, at room temperature, plain liposome demonstrated a relatively high leakage of 15%, after 30 days, while the coating of chitosan prevented this leakage significantly to around 5% over 30 days.
5. Finally, Higuchi, Korsmeyer-Peppas and Weibull model were selected for fitting of calcein release curves from plain HaT liposome and chitosan coated liposome. Detailed fitting curves were presented and three quantified fitting parameters (R^2 , BIC and RSS) were calculated by the Origin software. According to the analysis of these parameters, Weibull model has the greatest values of R^2 and lowest figure for BIC and RSS, which proves the Weibull model is the most appropriate model to simulate the fitting of all calcein release curves at 37, 40 and 42°C.

Chapter 4 Characterization of chitosan-coated liposome with doxorubicin encapsulation

4.1 Introduction

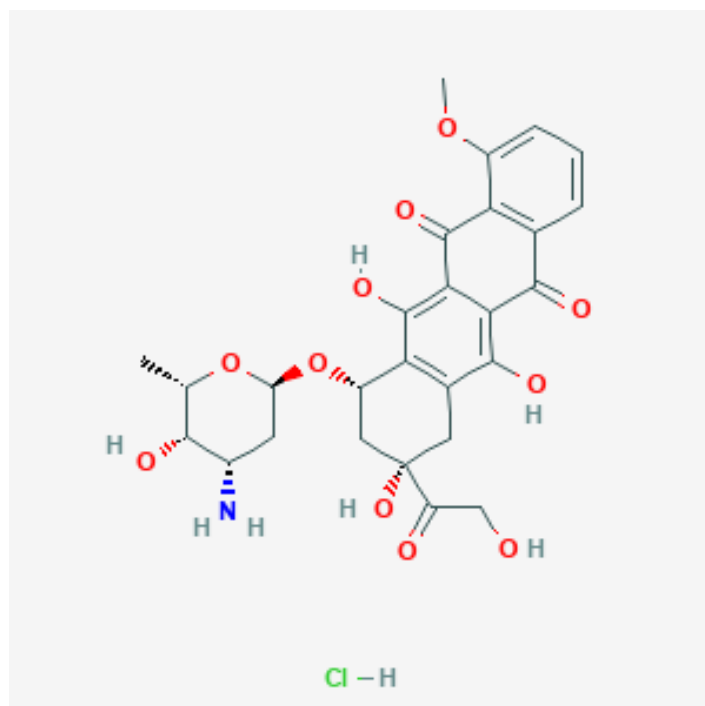


Figure 4.1 Schematic of doxorubicin hydrochloride [232]

Doxorubicin is an anthracycline antibiotic for cancer chemotherapy. It has been widely used for the treatment of breast, lung, gastric, ovarian, thyroid, soft tissue sarcomas, and paediatric cancers [233] [234] [235]. It takes effect by intercalating itself into DNA to prevent DNA replication [236]. Doxorubicin cannot be absorbed by intestines and stomach and can cause strong irritation to tissues. Thus, intravenous administration was the only approach for doxorubicin [237]. Doxorubicin molecule has both outstanding cancer-therapeutic and fluorescence imaging capabilities, making it an excellent therapeutic agent which can be

studied readily for controlled release. Many doxorubicin related drugs have been approved to be sold on markets, such as Doxil® [238], Myocet® [239] and Lypodox®.

Doxil®, also known as Caelyx®, is a pegylated liposome formulation of doxorubicin, and was the first long-circulating liposome encapsulating doxorubicin in the world. The preparation approach used an ammonium sulphate gradient to load doxorubicin into liposomes, and the presence of polyethylene glycol on the liposome surface was there to reduce uptake by the reticuloendothelial system. Preclinical experiments have indicated that the elimination half-life of Doxil® is about 20-30 hours, and the area under the concentration-time curve (AUC) of Doxil® is more than 60 times higher than that of free doxorubicin. Its clinical research focused on AIDS-related Kaposi's sarcoma (ARKS) and solid tumours such as prostate, ovarian and breast cancer. Its pharmacokinetic behaviour in humans is similar to that in animals, which has two phases. The first phase is an initial phase with a half-life of 1-3 hours, and the second phase has a half-life of 30-90 hours. The outstanding ability of Doxil® to penetrate through the tumour vasculature and its significantly prolonged circulation time *in vivo* allows for increased drug concentration delivered to the tumour site. Side effects of Doxil® include mild myelosuppression, mucosal and cutaneous toxicity, cardiotoxicity, and alopecia. Nevertheless, compared with free doxorubicin, the side effects of cardiotoxicity and alopecia caused by Doxil® are remarkably mitigated, thus allowing higher doses for administration than free doxorubicin. Therefore, the invention of Doxil® can significantly improve the therapeutic index of doxorubicin [240].

Another marketed doxorubicin encapsulated liposome is Myocet®, which is composed of egg phosphatidylcholine and cholesterol (55:45 molar ratio), and the pH gradient method was used to load doxorubicin into liposomes to obtain a higher drug to lipid ratio. Results from experiments in mouse and human cancer models indicated that Myocet® persisted longer and accumulated more in tumours than conventional free doxorubicin because

Myocet® can penetrate the leaky vasculature of tumours. As an anthracycline drug, doxorubicin has strong cardiotoxicity, which has become the biggest factor limiting its use in large doses. Nevertheless, preclinical studies have revealed that Myocet® targets far from the heart, thus significantly reducing cardiotoxicity without affecting the antitumor efficacy of encapsulated doxorubicin [239].

Nevertheless, to the best of our knowledge, studies regarding the comparison of doxorubicin encapsulated HaT liposome with and without chitosan coating have not been previously reported. Therefore, in this chapter, 0.1% (w/w) medium molecular weight chitosan was selected to coat on the liposome surface as well. The standard fluorescence curve of doxorubicin and encapsulation efficiency, release property and potential shelf life of doxorubicin encapsulated liposomes with and without a chitosan coating will be investigated. Besides, several commonly used pharmaceutical models will be chosen to fit these release curves for the determination of the best fitting model in this case.

4.2 Materials and Methods

4.2.1 Materials

1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) was purchased from Avanti Polar lipid, Alabaster, AL, USA.

Brij S20, low molecular weight Chitosan (50-190 kDa), medium molecular weight Chitosan (190–310 kDa), high molecular weight Chitosan (310-375 kDa), Triton X-100, Sigma® cell culture plate size 96 wells (flat bottom, sterile), doxorubicin hydrochloride and 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich.

Sephadex G-50 (medium) was purchased from GE healthcare.

Spectrum™ Spectra/Por™ Float-A-lyzer™ G2 dialysis devices was purchased from Fisher Scientific

All water used was de-ionised and had a conductivity less than 80 mS.

4.2.2 The determination of doxorubicin fluorescence intensity standard curve

Two doxorubicin fluorescence intensity standard curves were plotted using two instruments: Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) and Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega). Both curves were obtained by measuring the fluorescence intensity of each sample with specific doxorubicin concentration. In both cases, the first sample was prepared by dissolving 5 mg doxorubicin in 3 ml HEPES buffer solution with a concentration of 1.67 g/l in a 4.5 ml plastic standard cuvette. Each following sample was made by diluting the previous one with same volume of solution. In that case, the doxorubicin concentration of the following sample was the half of the previous one. A total of 24 samples were measured with a minimum concentration of 3.97×10^{-7} mg/ml and maximum concentration of 1.67 g/l. The pure solution (HEPES buffer solution or acetic acid solution) without doxorubicin was measured as well for contrast.

For the doxorubicin fluorescence intensity standard curve based on the data from Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA), 3 ml of each sample with various concentrations were loaded in a 4.5 ml plastic standard cuvette which was then inserted into the spectrofluorometer one by one for the fluorescence intensity measurement. Doxorubicin was dissolved in HEPES buffer solution (pH 7.4) and acetic acid solution (pH 3.0) separately in order to obtain two doxorubicin fluorescence intensity standard curves at these pH's.

Excitation and emission wavelength of 470 and 560 nm were setting in this spectrofluorometer.

For the doxorubicin fluorescence intensity standard curve based on the data from Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega), 200 µl was extracted from each 3 ml doxorubicin sample with gradient concentration and loaded into Sigma® cell culture plate size 96 wells (flat bottom, sterile). Then, the 96 wells cell culture plate containing all samples was inserted into the Microplate reader for the fluorescence intensity measurements.

The optimum excitation and emission wavelength of doxorubicin is 470 and 560 nm respectively. Nevertheless, Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega), can only provide several fixed options of excitation and emission wavelengths rather than any accurate values of wavelength. Hence, the excitation wavelength of 475 nm and emission wavelength of 500-550 nm offered by this machine were selected.

4.2.3 Liposome characterisation: Encapsulation efficiency

The encapsulation efficiency was calculated by the percentage of doxorubicin encapsulated in liposomes relative to the total amount of doxorubicin added in the liposome preparation process. Pure doxorubicin encapsulated liposome suspension was collected after passing through a Sephadex G 50 column (0.8 cm*25 cm) equilibrated with HEPES buffer (pH 7.4). To determine the total amount of doxorubicin encapsulated in the liposomes, firstly, 1 v/v % Triton X-100 surfactant was added in to doxorubicin encapsulated liposome suspension to disrupt the liposomal membrane and release encapsulated doxorubicin thoroughly. Then, 200 µl was extracted from this solution and added into Sigma® cell culture plate size 96

wells (flat bottom, sterile) to measure its fluorescence intensity in the Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega). The concentration of encapsulated doxorubicin released in this solution can be obtained by referring to the doxorubicin fluorescence standard curves in HEPES buffer solution (pH=7.4) in Figure 4.3. Finally, the amount of encapsulated doxorubicin inside liposomes (M_{de}) should be the concentration of doxorubicin obtained from Microplate reader times the volume of liposome suspension collected from gel filtration.

The doxorubicin encapsulation efficiency was calculated using the following equation (4-1):

$$EE (\%) = \frac{M_{de}}{M_{dt}} \times 100\% \quad (4-1)$$

Where $EE (\%)$ is the encapsulation efficiency, M_{de} is the mass of doxorubicin encapsulated in liposomes and M_{dt} is the mass of initial doxorubicin added in the liposome preparation procedure.

4.2.5 Liposome characterisation: Release Property

The release property of liposomes with and without a monolayer coating of chitosan was investigated using a dialysis tube diffusion technique. 2 ml of liposome suspension was placed in Spectrum™ Spectra/Por™ Float-A-lyzer™ G2 dialysis device with a molecular weight cut-off (MWCO) of 300 kDa and immersed in 15 ml HEPES buffer solution in 50 ml centrifugal tube. The whole unit was then incubated in a water bath at the desired temperature (37 °C, 40 °C and 42 °C). Aliquots of 200 µl dissolution medium were extracted

every half an hour and the same volume of fresh medium was replaced every half an hour. The 200 µl extracted medium was added into the Sigma® cell culture plate size 96 wells (flat bottom, sterile) and this 96 well culture plate was inserted into Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega) for the measurement of fluorescence intensity. By referring to the doxorubicin fluorescence standard curves in HEPES buffer solution (pH=7.4) in Figure 4.3, the released doxorubicin concentration (C_{di}) after dialysis can be obtained. In addition, 1 v/v % Triton X-100 surfactant was added into original liposome solution sample in order to disrupt the liposomal membrane and release encapsulated doxorubicin completely. Then, the doxorubicin concentration (C_{dt}) in this solution was analysed by the same approach.

The release percentage of doxorubicin from liposomes was calculated using the following equation (4-2):

$$RR (\%) = \frac{C_{di} \times V_1 + \sum_{i=1}^{n-1} C_{di} \times V_2}{C_{dt} \times V_3} \times 100\% \quad (4 - 2)$$

Where RR is the release ratio, C_{di} is the concentration of total doxorubicin released at moment i , V_1 is the volume of buffer solution in centrifugal tube (15 ml), n is the number of samples removed from the release medium, V_2 is the volume of removed release medium (200 µl), C_{dt} is the concentration of initial liposome solution after the addition of 1 v/v % Triton X-100 and V_3 is the volume of initial liposome suspension loaded inside dialysis tube (2 ml).

4.2.6 Liposome characterisation: Shelf life

2 ml of plain liposome and 0.1% (w/w) medium molecular weight chitosan coated liposome suspensions were loaded separately in the same self-assembly dialysis unit as demonstrated in Figure 4.1. Both samples were stored at 4 °C and room temperature (25 °C) in a dark environment *i.e.* without light. Aliquots of 200 µl dissolution medium were extracted and same volume of fresh medium was replaced every 5 days until one month. The 200 µl of extracted medium was loaded into 96 wells culture plate and measured by Microplate reader as stated in the section 4.2.5. Finally, the leakage ratio (release percentage) was calculated with the equation (4-2) as well and each experiment was repeated in triplicate (n=3).

4.2.7 Determination of fitting models and fitting parameters

Among all frequently used kinetic models in Table 3.1, Higuchi model, Korsmeyer-Peppas model, Weibull model and First-order model were chosen for the detailed comparison in the following section. Besides, R^2 , BIC and RSS were selected as fitting parameters. All fitting procedure and parameters calculation were carried out using Origin spreadsheeting (9.65 Version).

4.3 Results and discussion

4.3.1 The influence of pH on the doxorubicin fluorescence intensity standard curve

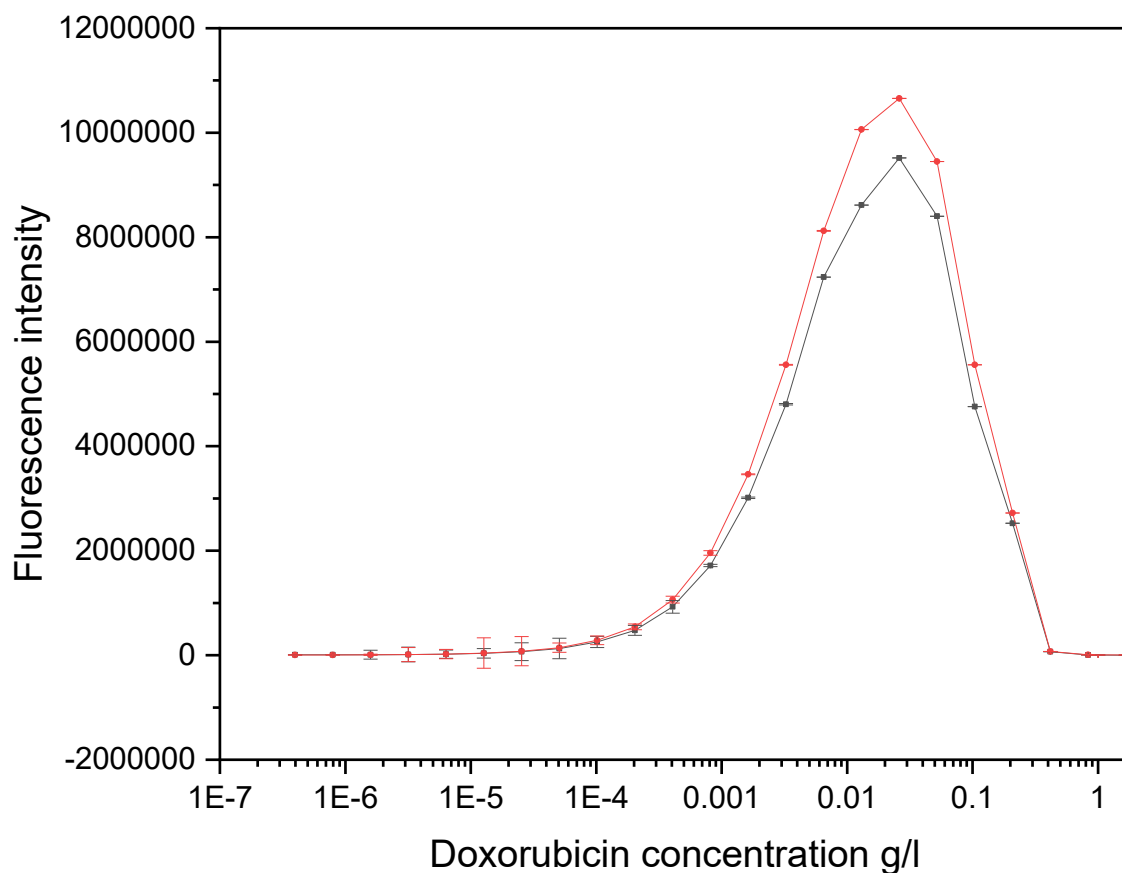
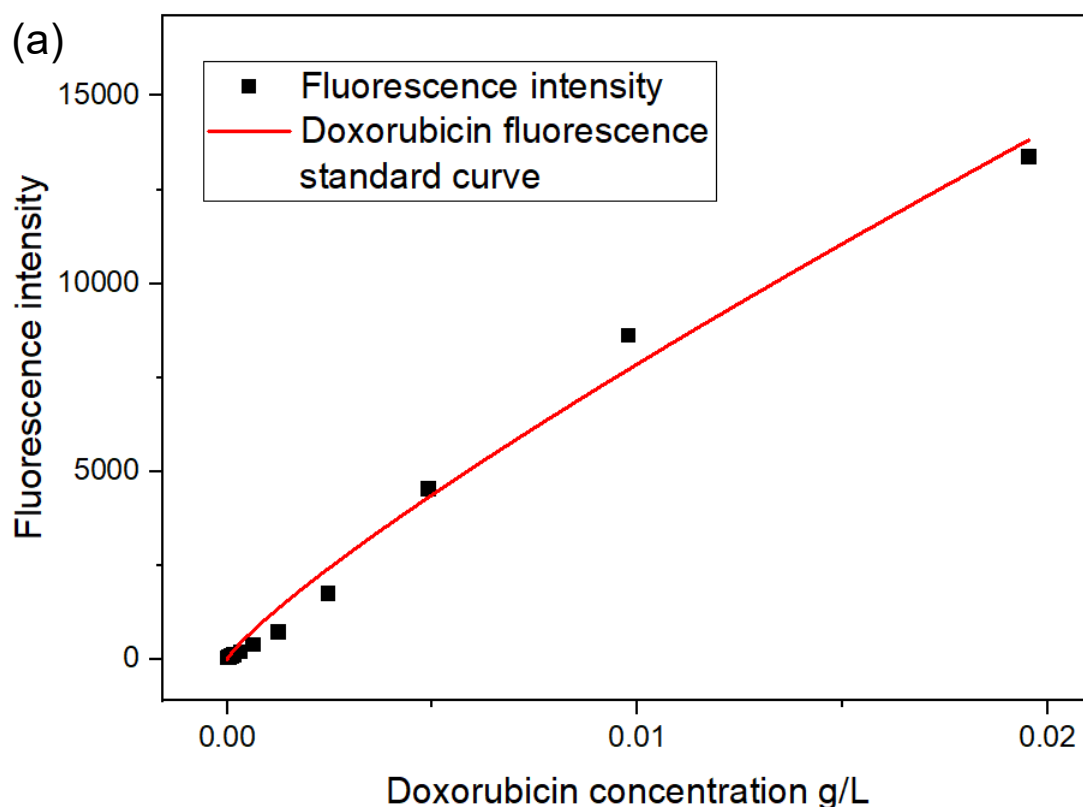


Figure 4.2 Doxorubicin fluorescence standard curves in acetic acid solution (pH=3.0) (●) and HEPES buffer solution (pH=7.4) (■) measured by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) (Slit width=4). Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 4.2 compares two doxorubicin fluorescence standard curves at pH 3.0 and pH 7.4. As can be observed from this graph, fluorescence intensities of both curves rapidly increased to around 9.5 million at pH 7.4 and 10 million at pH 3.0 at 0.026 g/l doxorubicin before dropping substantially to nearly 0 as the doxorubicin concentration increased. This drop of the fluorescence intensity is due to the self-quenching effect of doxorubicin. Throughout the whole concentration, the fluorescence intensities of doxorubicin at pH 3.0 in acetic acid

solution was slightly higher than that at pH 7.4 in the HEPES buffer solution, although the pH sensitivity was much less than for calcein, as illustrated in Figure 3.2. Even so the pH of doxorubicin solution was monitored throughout this study.

Both figures of fluorescence standard curves were measured in the same instrument, Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) with same procedure. Yet different values of slit width were selected in two experiments. A larger slit width produces a higher fluorescence intensity using the same sample. In the doxorubicin fluorescence standard curve experiment, a peak intensity of 10 million was observed when the slit width was setting at 4 and a peak intensity of 4.5 million was discovered in calcein experiment when the setting was at 1. Similar magnitudes of peak fluorescence intensities were obtained in doxorubicin experiment using a much higher value of slit width indicates that the fluorescence intensity performance of doxorubicin was much lower than calcein's.



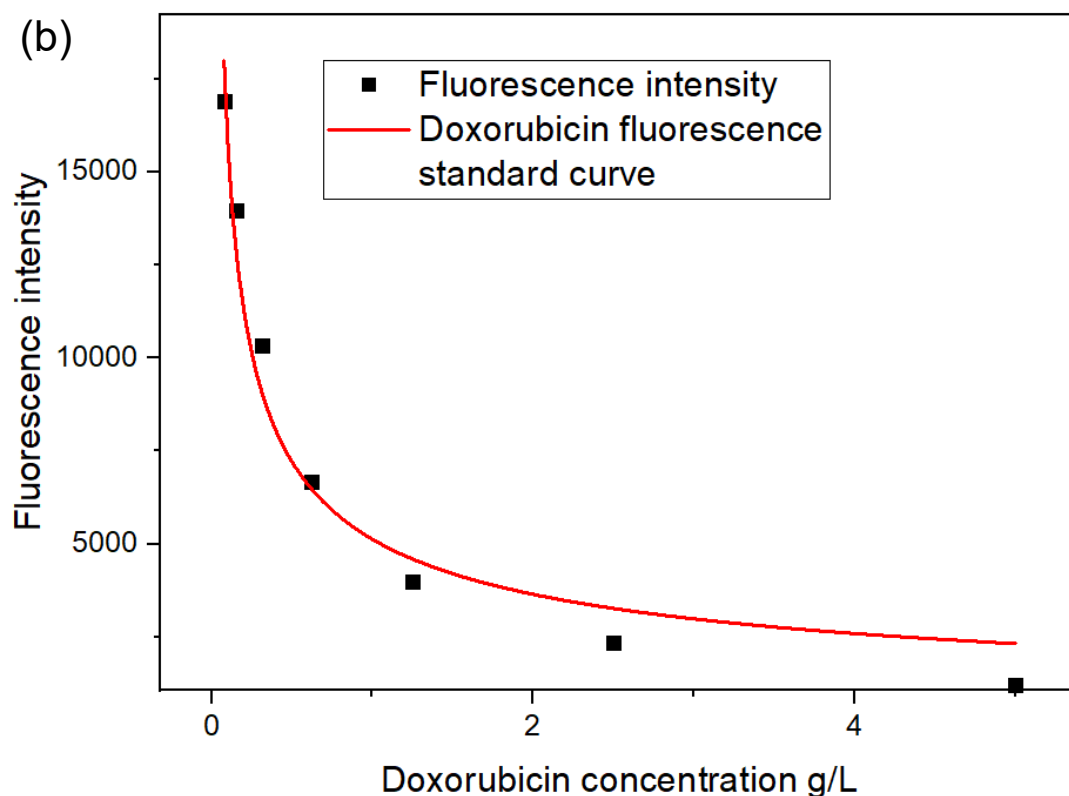


Figure 4.3. Fitted doxorubicin fluorescence standard curves in Hepes buffer solution (pH=7.4) measured by Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega). (a) Doxorubicin concentration ranging from 2.98×10^{-7} g/L to 0.019 g/L. (b) Doxorubicin concentration ranging from 0.039 g/L to 5 g/L.

As can be observed from above graph, both Figure 4.3 (a) and Figure 4.3 (b) applied exponential equation to fit data points in their respective ranges. In addition, their horizontal axis were both transferred to the linear scale rather than the 'log 10' scale in Figure 4.2 for more convenient observation. The chosen doxorubicin concentration ranges can cover all referred concentrations in the following sections.

The rest of the experiments in this chapter including encapsulation efficiency, release property and shelf life of doxorubicin were obtained using Figure 4.3 rather than Figure 4.2 as a reference. The doxorubicin fluorescence standard curve in Figure 4.3 was measured by Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega). This is due to its excellent advantage, which is it can measure large numbers of samples simultaneously with the use of 96 well plates (well working volumes 25-340 μ L for each well) and enhance the efficiency of measurements. Nevertheless, there is one disadvantage of this equipment which is its low maximum measuring range. In the experiment of plotting calcein fluorescence standard curve, when the calcein concentration is above 0.015 g/L, its corresponding fluorescence intensity was too large to be recorded, also on this instrument the slit width could not be adjusted. Hence, the validation of self-quenching effect of calcein and doxorubicin and the influence of pH on their standard curve were examined by Horiba FluoroMax-4 spectrofluorometer (HORIBA, USA) in Figure 3.2 and Figure 4.2. Whereas, the shortcoming of Horiba FluoroMax-4 spectrofluorometer is that it can only measure one sample at a time in a cuvette with a minimum sample volume of 3 ml. Thus, the sample with a small volume has to be diluted to 3 ml for the measurement. Nevertheless, if the solution containing doxorubicin whose fluorescence intensity is much lower than calcein is diluted to 3 ml, the relatively low fluorescence intensity would cause undesired inaccuracy. Therefore, the doxorubicin fluorescence standard curve in Figure 4.3 measured by Microplate reader (GloMax-Multi +Microplate Multimode Reader, Promega) was selected as a reference for the rest of the doxorubicin containing liposome characterisation in this chapter.

4.3.2 Encapsulation efficiency of doxorubicin encapsulated liposome

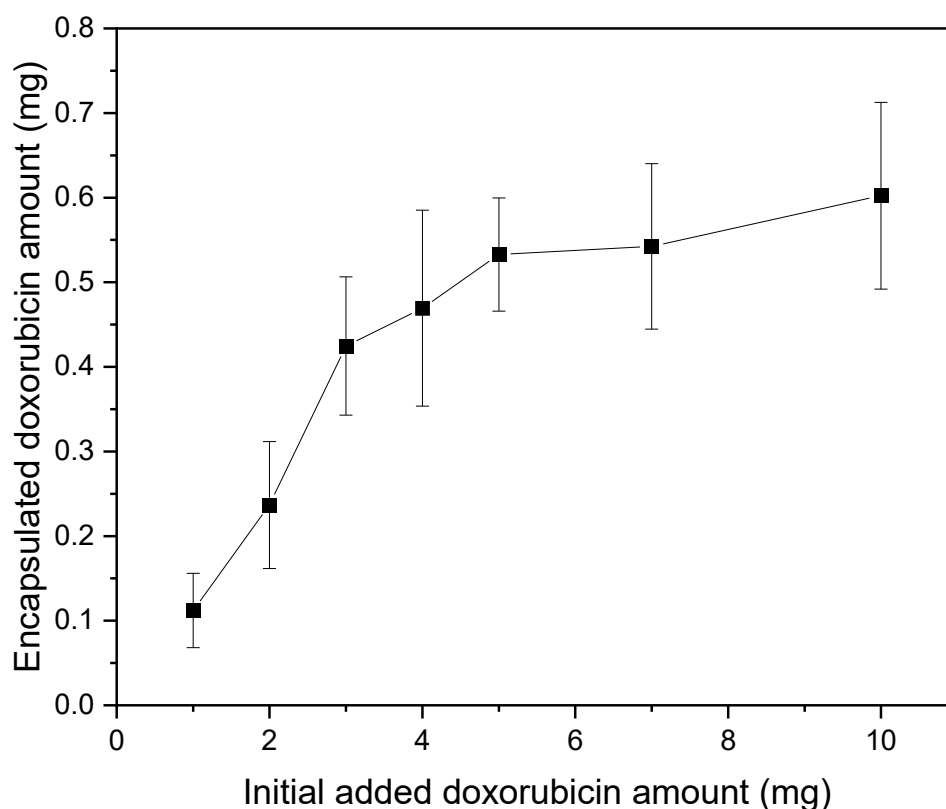


Figure 4.4 Effect of initial added doxorubicin in the liposome preparation process on the amounts of encapsulated calcein inside liposomes. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 4.4 outlines the variation of the amount of encapsulated doxorubicin depends on the initial added doxorubicin in the liposome preparation process. The amount of encapsulated doxorubicin surged dramatically from 0.11 mg to around 0.42 mg as the initial added doxorubicin amount rose from 1 mg to 3 mg, before the encapsulated doxorubicin rose gradually to its maximum amount (0.60 mg) at 10 mg initial added doxorubicin amount. Figure 4.4 indicates that increasing the amount of doxorubicin above 3 mg did not result in a sharp rise on the encapsulated doxorubicin amount. Doxorubicin was dissolved in 1 ml deionised water in the preparation process, so its correspondent concentration was 3 mg/ml.

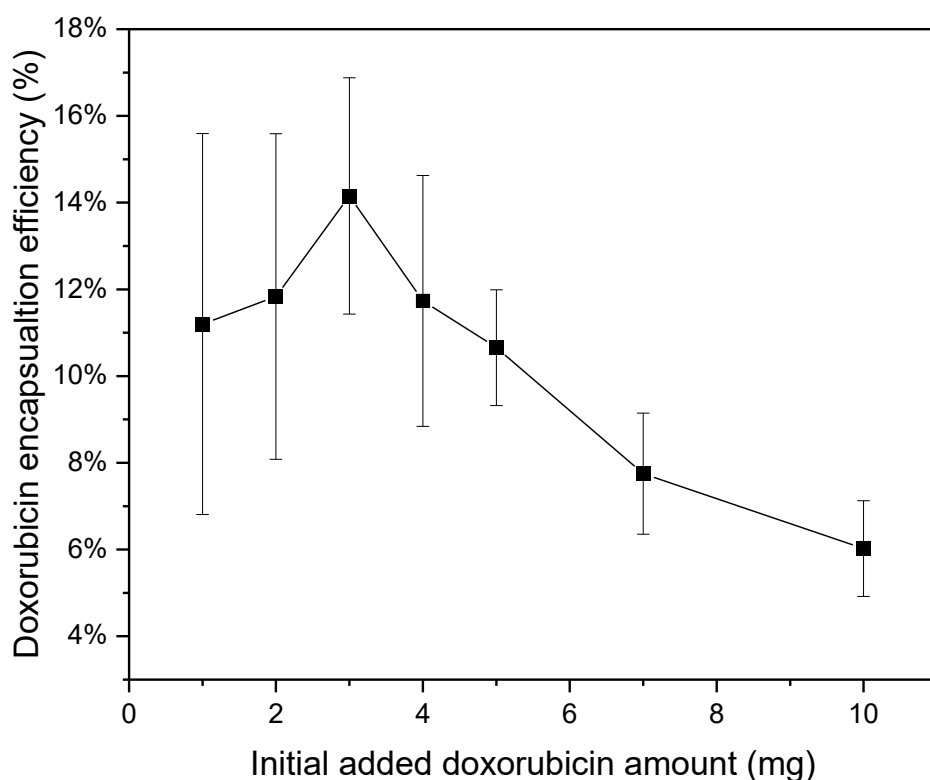


Figure 4.5 Effect of initial added doxorubicin in the liposome preparation process on the encapsulation efficiency of doxorubicin. Data represents mean $\pm$ SD. n=3 independent batches.

Furthermore, Figure 4.5 features how the doxorubicin encapsulation efficiency is affected by the amount of initial added doxorubicin. It is clear that when 3 mg of initial doxorubicin was added in the preparation process, its encapsulation efficiency reached the peak of roughly 14%. Specifically, the doxorubicin encapsulation efficiency plummeted from 14% to 6% when the initial amount ascended from 3 mg to 10 mg after a moderate growth from 11% to 14%. Therefore, 3 mg of initial added doxorubicin was selected for the following experiments

because the excessive added amount above 3 mg would cause vast waste and did not produce a higher encapsulation efficiency. The relatively high standard deviation in the data whose initial added amount was below 4 mg was because the initial added amount which is located at the denominator in equation 4.1 was comparatively low.

4.3.3 Release property of doxorubicin encapsulated liposome

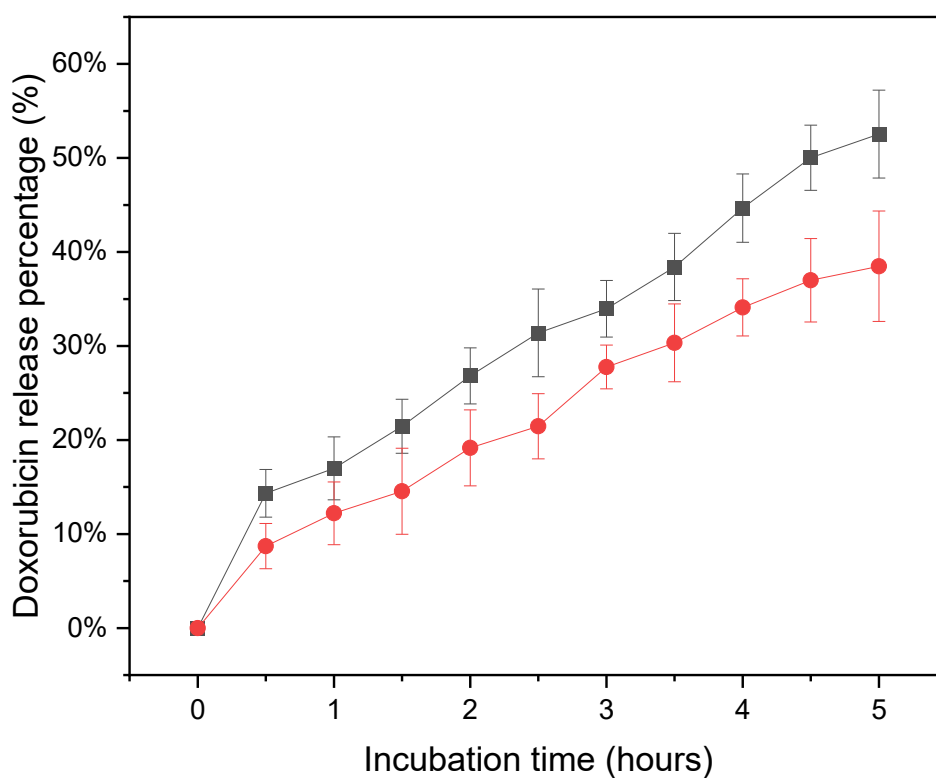


Figure 4.6 The percentages of encapsulated doxorubicin released from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean ± SD. n=3 independent batches.

Figure 4.6 compares the release percentage of doxorubicin encapsulated liposomes with and without coating of chitosan at 42 °C in HEPES buffer (pH 7.4). As can be observed from this graph, the release percentage of plain liposome was consistently higher than that of chitosan coated liposome. Both curves saw relatively sharp rises in the first half hour, with a 15% and 9% growth respectively. The release percentage of plain liposome ascended gradually from 15% to roughly 54% after 5 hours incubation, while the chitosan coated liposome released 39% of encapsulated doxorubicin after 5 hours. Besides, the difference value between two curves rose slightly from 6% at 0.5 hours point to 14% at 5 hours point with some fluctuations.

Our results are in accordance with other researches investigating the effect of coating chitosan on the liposome stability [241] [242] [243]. The enhancement by polymer coating on the liposome surface is determined by the bonding strength between polymer and liposome [244].

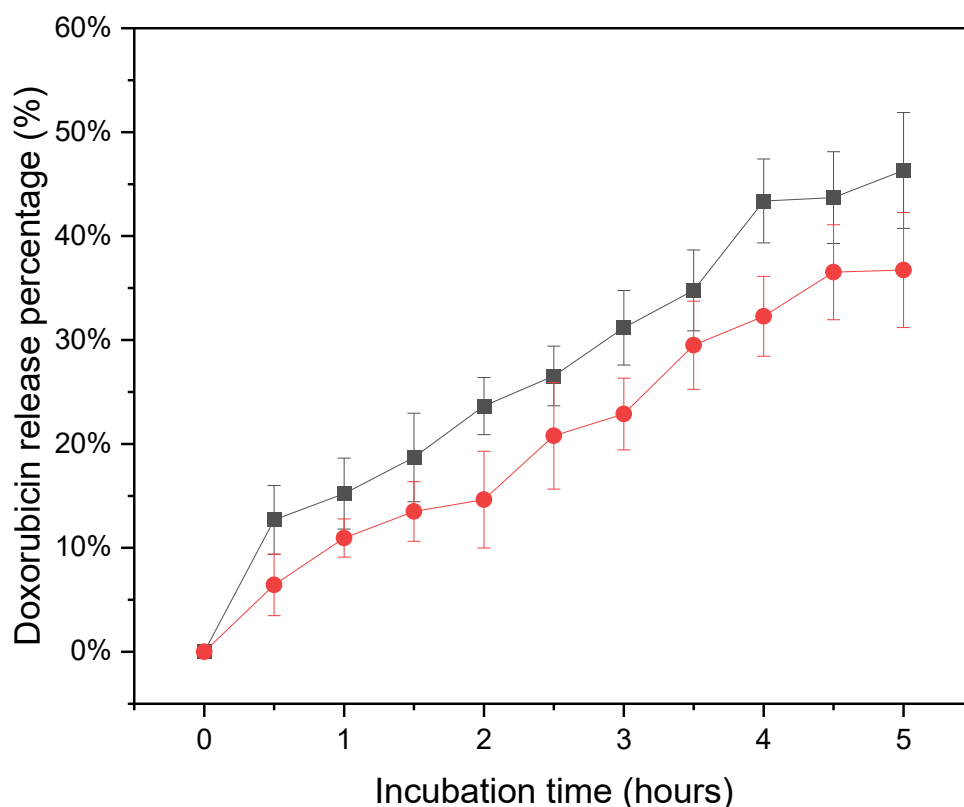


Figure 4.7 The percentages of encapsulated doxorubicin released from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 4.7 outlines the variations of release percentages of encapsulated doxorubicin from plain liposome and chitosan coated liposome over 5 hours. After a leap to 13% in the first half hour, the percentage of doxorubicin released from plain liposome rose constantly to around 45% after 5 hours incubation. Whereas chitosan coated liposome released its encapsulated doxorubicin gradually without obvious surge until 35% of entrapped doxorubicin was released after 5 hours. The gap between two curves remained relatively stable over the whole period ranging from 5% to 10%.

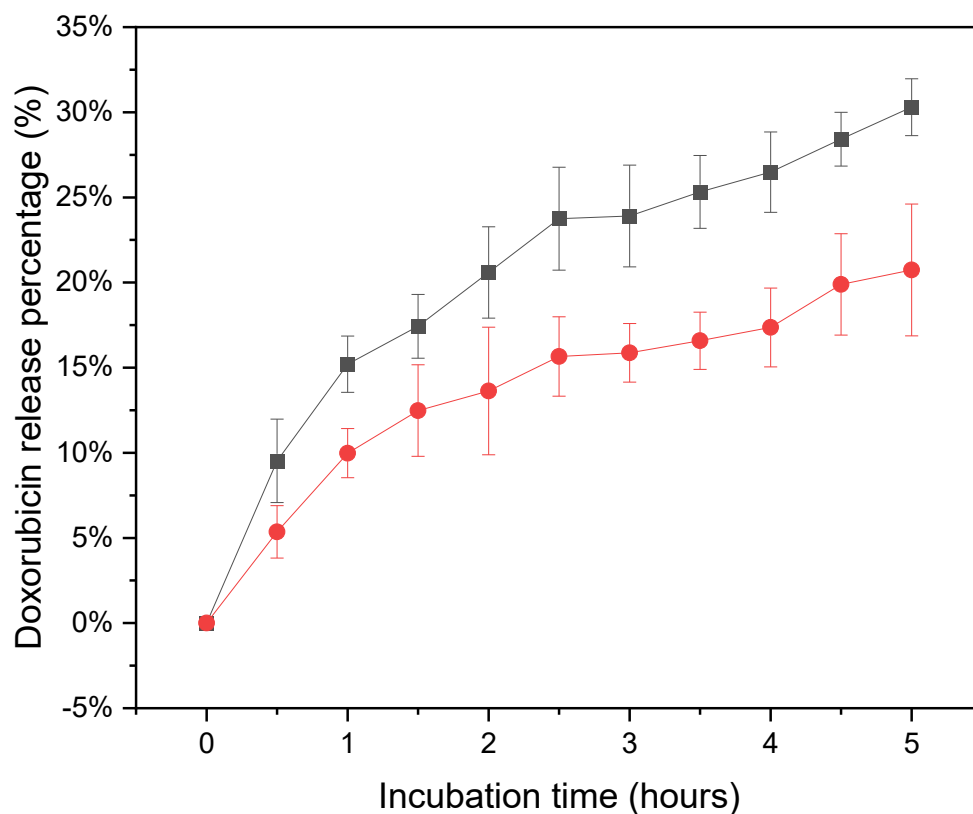


Figure 4.8 The percentages of encapsulated doxorubicin released from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C in HEPES buffer (pH 7.4) over a period of 5 hours every half an hour. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 4.8 depicts how the percentages of entrapped doxorubicin released from liposome with and without coating chitosan varied over 5 hours. The plain liposome experienced a leap in the first 2.5 hours, rising to nearly 25%, before increasing modestly to 30% after 5 hours. Nevertheless, the surge was only observed in the first hour for chitosan coated liposome, climbing to 10% after one hour. After that, the release proportion of entrapped

doxorubicin from chitosan coated liposome increased gradually to 20% after 5 hours. In addition, the difference value was widened slightly. At the end of period, plain liposome released roughly 10% more encapsulated doxorubicin than chitosan coated liposome.

In the original paper of HaT liposome, 10%-20% of encapsulated doxorubicin was released in the first half hour [99]. The coating of 0.1% (w/w) medium molecular weight chitosan on the HaT liposome in this research successfully enhanced its stability at 37 °C (merely 5% was released in the first half hour and 20% in 5 hours).

4.3.4 Shelf life of doxorubicin encapsulated liposome

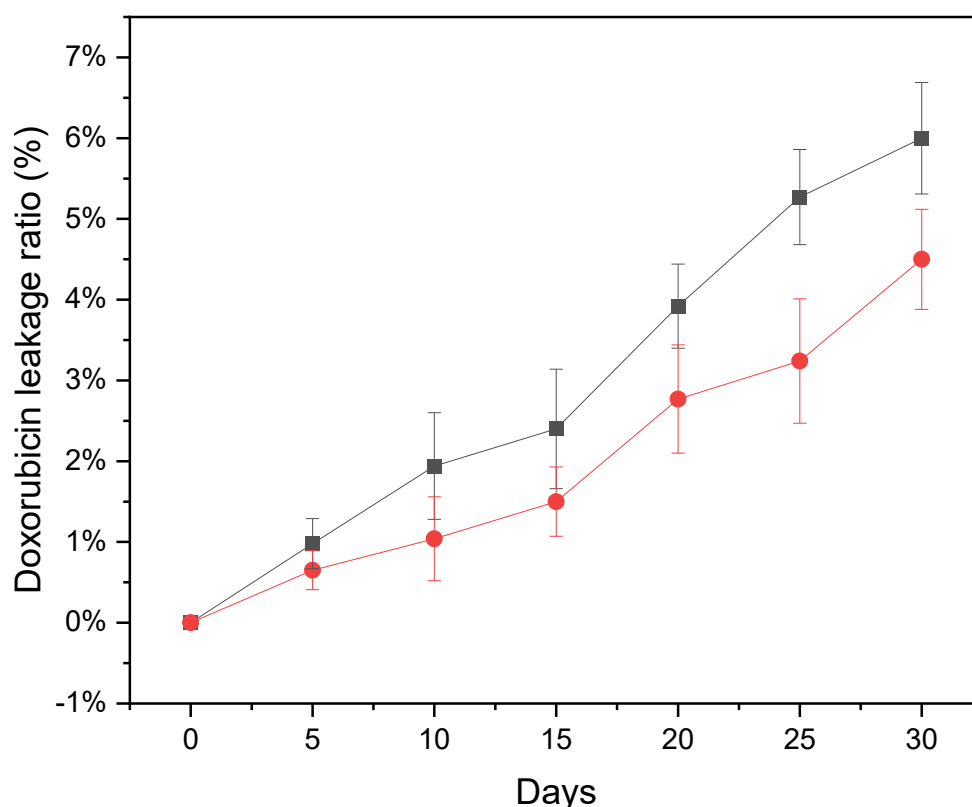


Figure 4.9 The percentages of encapsulated doxorubicin leaked from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 4 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean $\pm$ SD. n=3 independent batches.

Figure 4.9 outlines the shelf life of both plain liposome and chitosan coated liposome stored in fridge at 4 °C. As can be observed from this graph, both liposomes leaked little at 4 °C after one-month storage (merely 6% for plain liposomes and 4.5% for chitosan coated liposomes). The leakage ratio for both liposomes rose gradually with small fluctuations. Throughout whole period of 30 days, chitosan coated liposome released less than plain liposome even though the difference value was not significant ranging from 0.3% to 2.0%. This proves that the coating of chitosan can improve the HaT liposome stability at 4 °C.

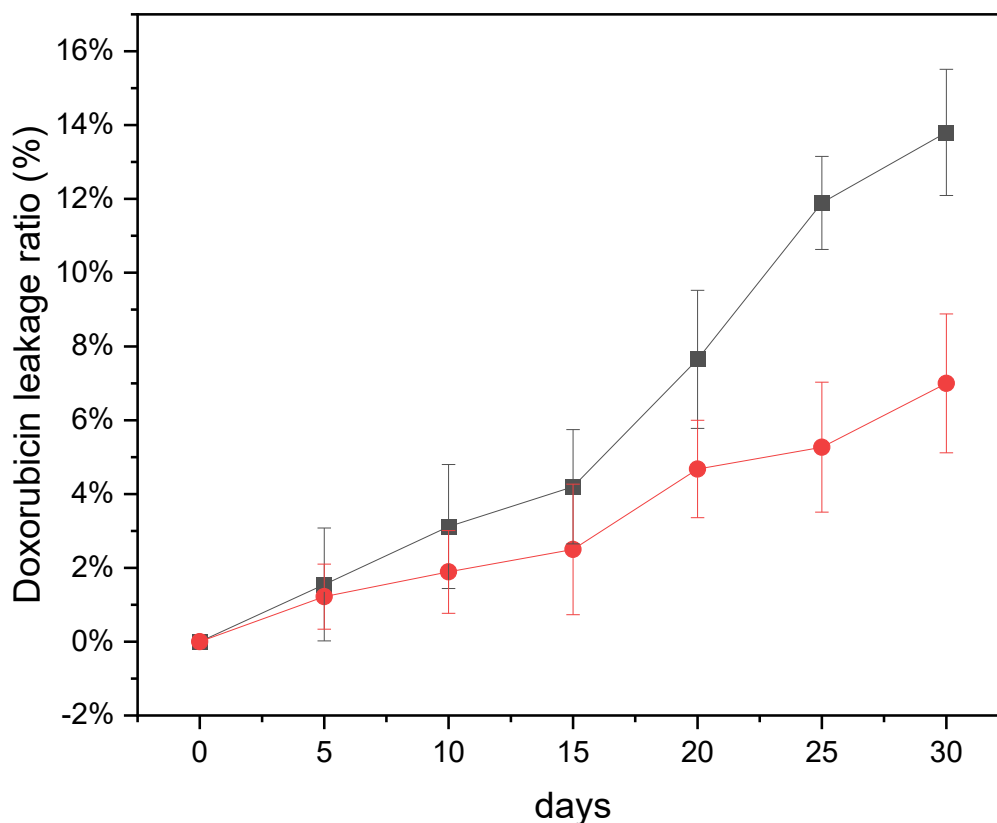


Figure 4.10 The percentages of encapsulated doxorubicin leaked from plain liposome (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 25 °C in HEPES buffer (pH 7.4) over a period of 30 days every 5 days. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 4.10 depicts that the leakage ratios of plain liposome and chitosan coated liposome stored at room temperature in HEPES buffer (pH 7.4). As can be seen from this graph, after a moderate growth to 4.2% in first 15 days, the leakage ratio of plain liposome soared to 13.8% after one month stored at 25 °C. Nevertheless, there was no significant sharp increase in the release curve of chitosan coated liposome. Chitosan coated liposomes leaked its encapsulated doxorubicin gradually to around 7% after one month. In other words, the coating of chitosan prevented nearly 7% compared to plain liposomes without chitosan

coating. This stability enhancement of chitosan coating is more effective at room temperature than at 4 °C because merely 1.5% of entrapped doxorubicin was reduced by the chitosan coating at 4 °C as discussed in Figure 4.9.

4.3.5 Fitting of release curves of doxorubicin encapsulated liposomes using different pharmaceutical models

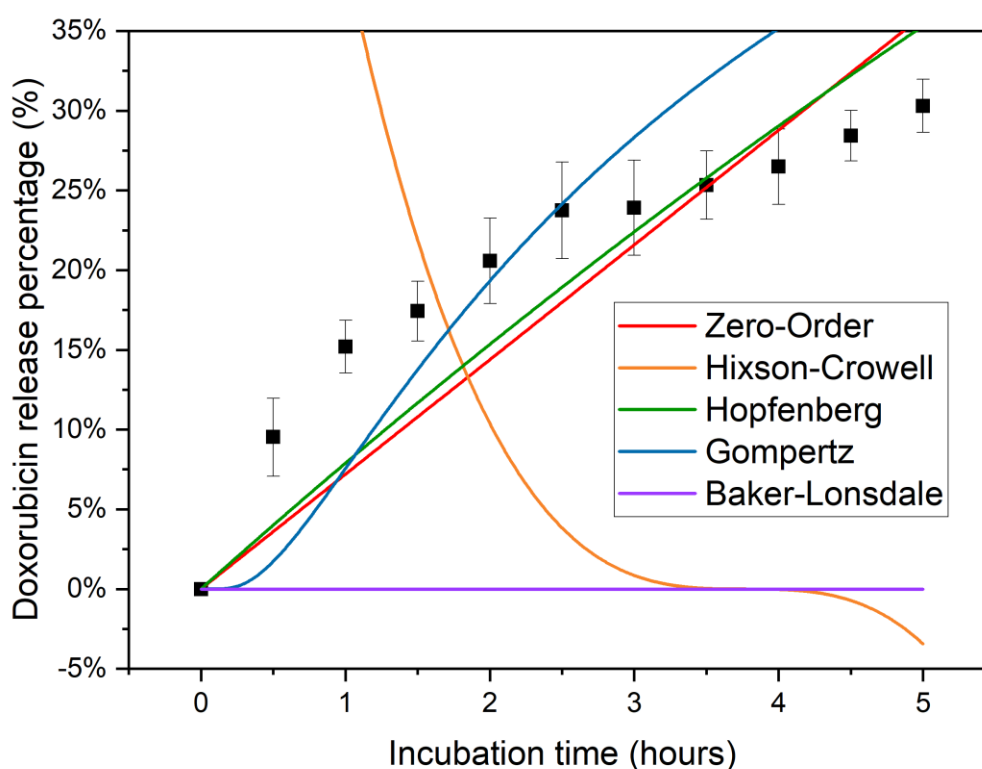
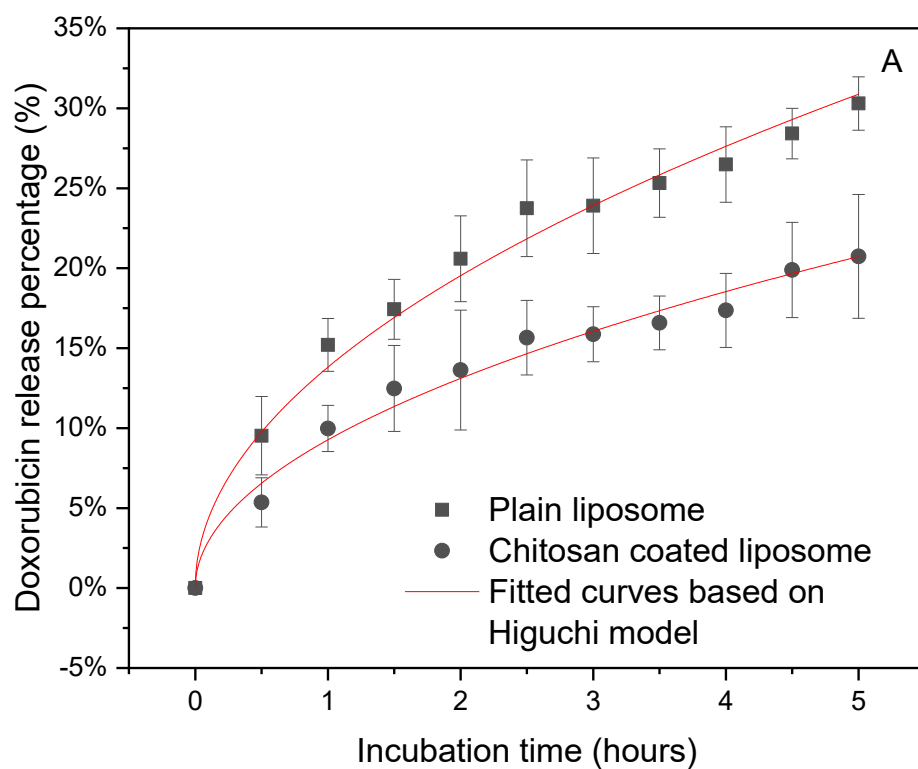
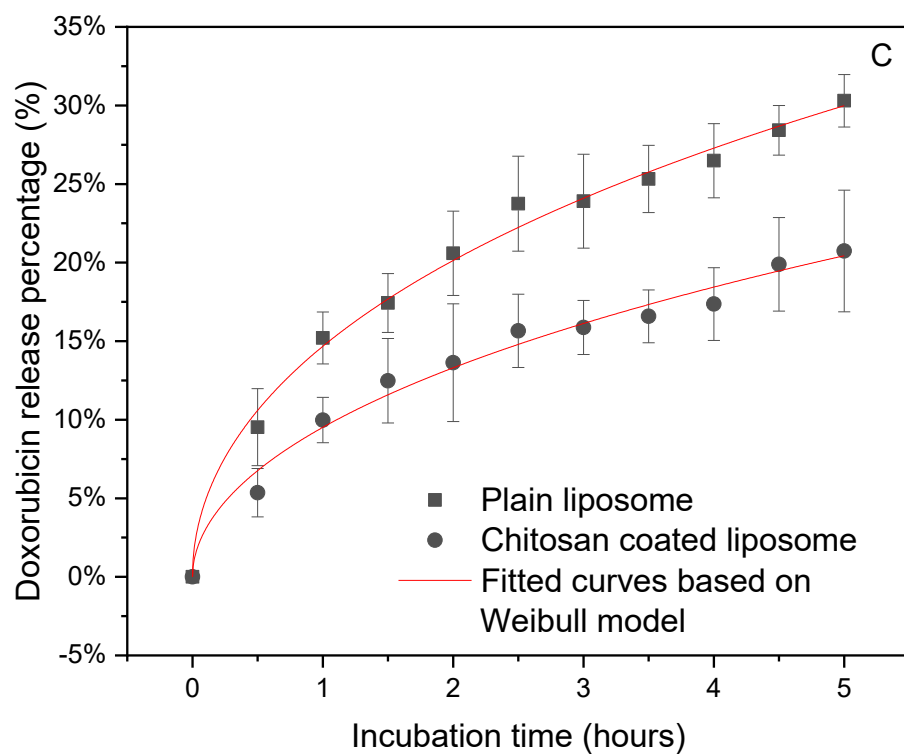
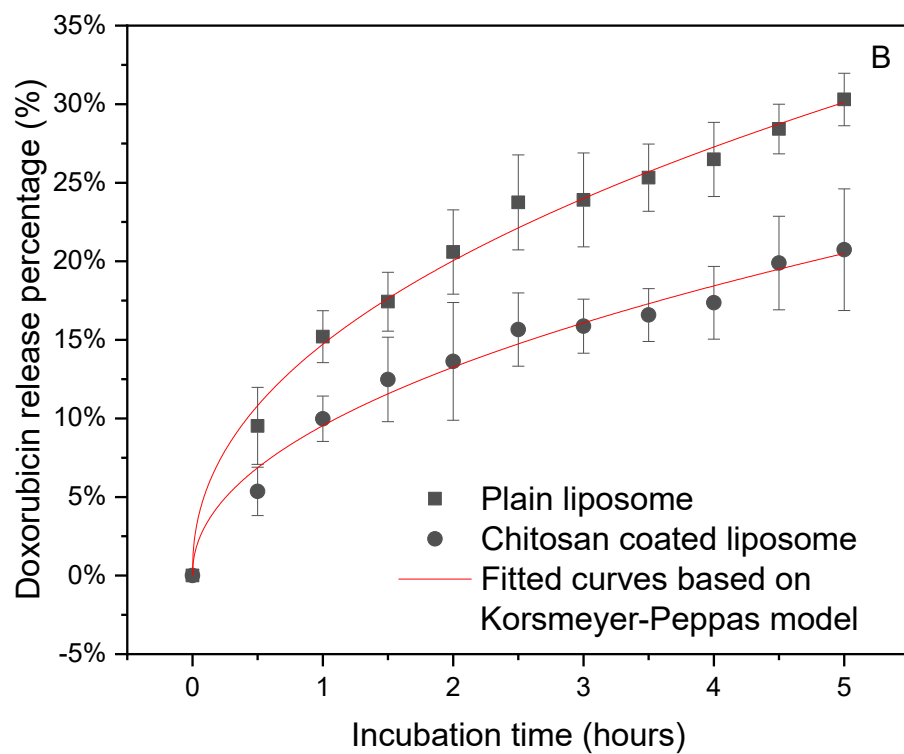


Figure 4.11 Zero-Order, Hixson-Crowell, Hopfenberg, Gompertz and Baker-Lonsdale models are utilised to fit doxorubicin release from plain liposomes (■) at 37 °C. Data represents mean $\pm$ SD. $n=3$ independent batches.

As can be seen from the above figure, it is manifest that Zero-Order, Hixson-Crowell, Hopfenberg, Gompertz and Baker-Lonsdale models cannot converge on the doxorubicin release curve from plain liposome at 37 °C, after initial fittings. Hence, First-Order, Higuchi, Korsmeyer-Peppas and Weibull models are chosen for the detailed comparison and discussion to determine the best model in this case.





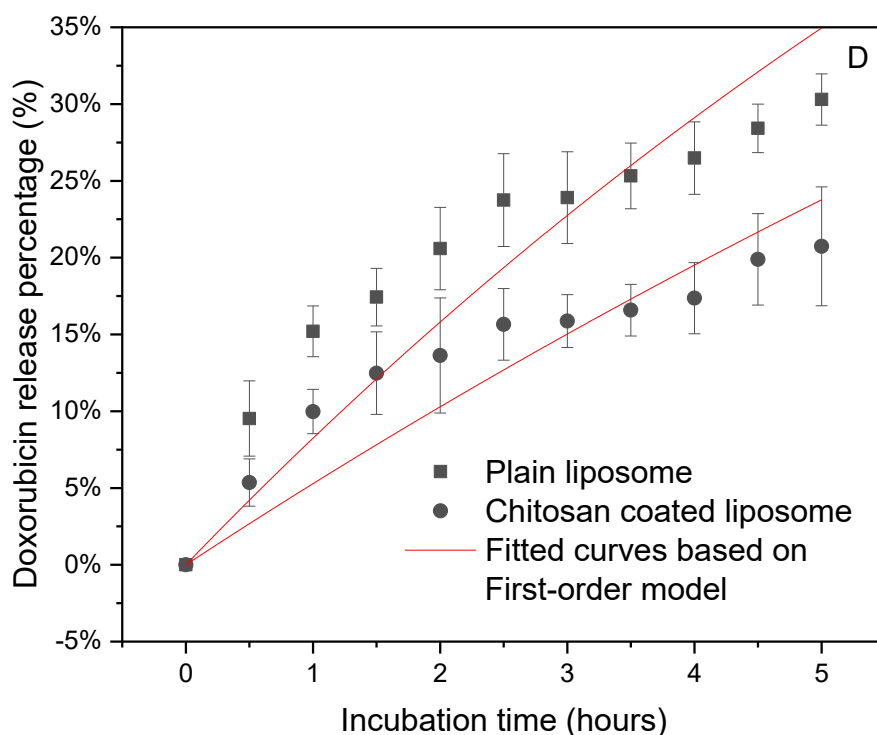
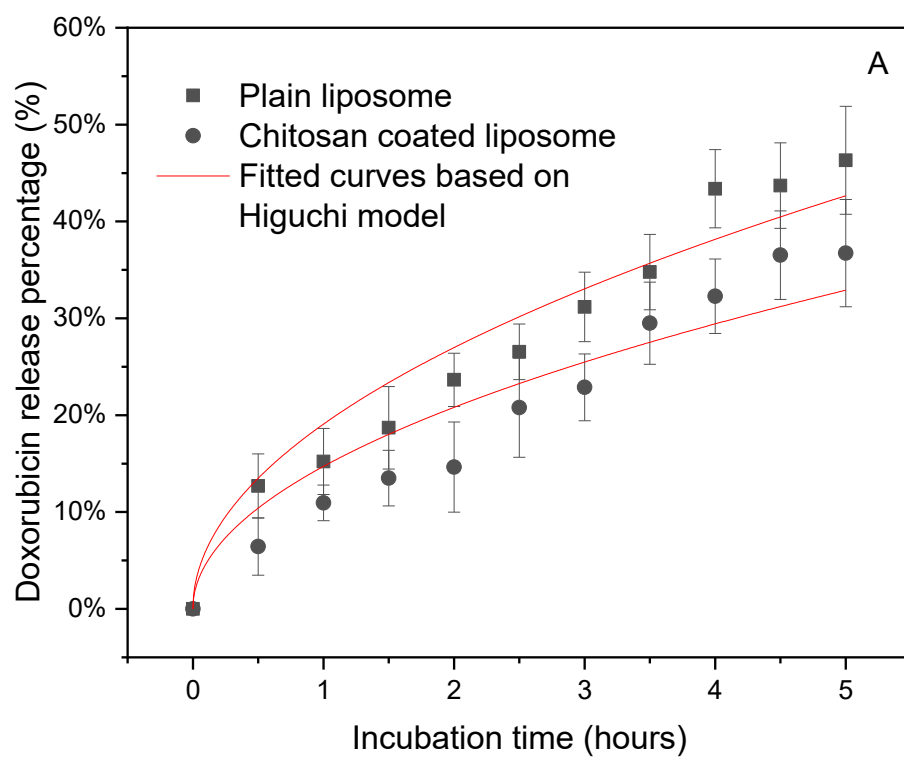
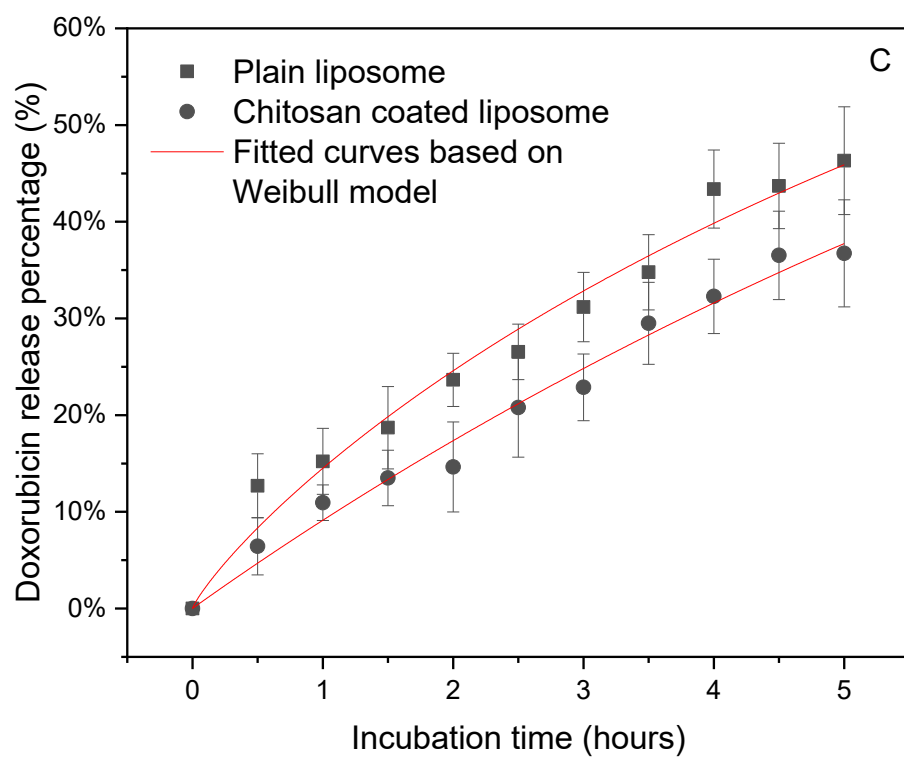
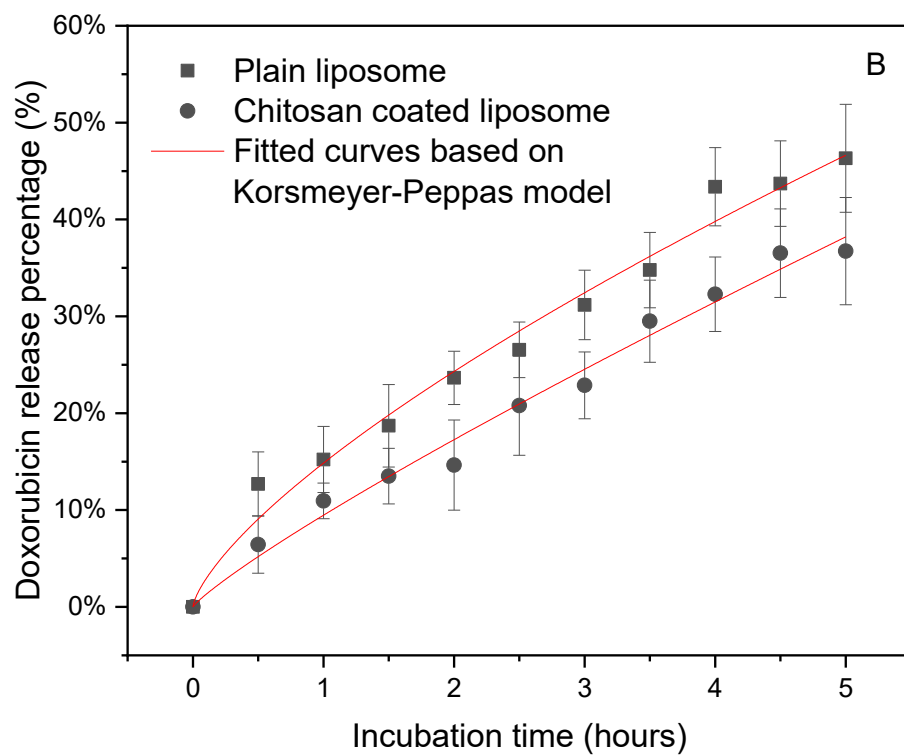


Figure 4.12 Higuchi (A), Korsmeyer-Peppas (B), Weibull (C) and First-order (D) models are utilised to fit doxorubicin release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 37 °C. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 4.12 depicts the doxorubicin release curves fitted by four commonly used pharmaceutical models at 37 °C. It is apparent that First-order model does not fit these curves, because most of data points are located far from the fitted curve. As a contrast, other three models seem to fit these curves well. Nevertheless, it is difficult to distinguish which model is the best for simulation from the observation of these fitting graphs. As a result, three fitting parameters (R^2 , BIC and RSS) are required to quantify the difference between

the results of each fitting mode in order to select the most appropriate model in this case, which will be discussed further in the following section.





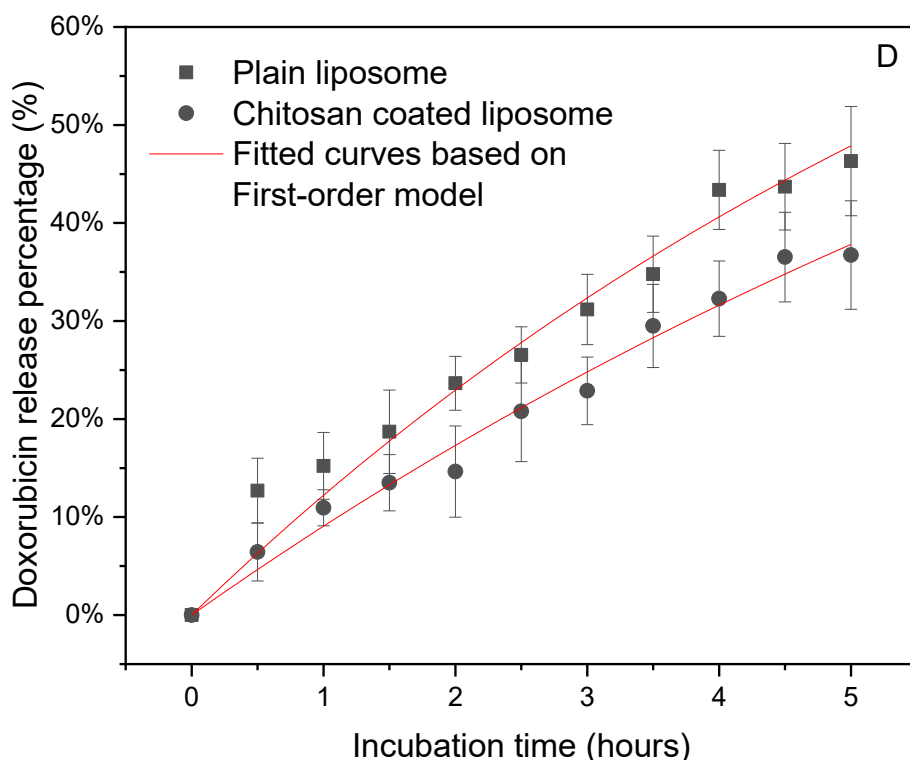
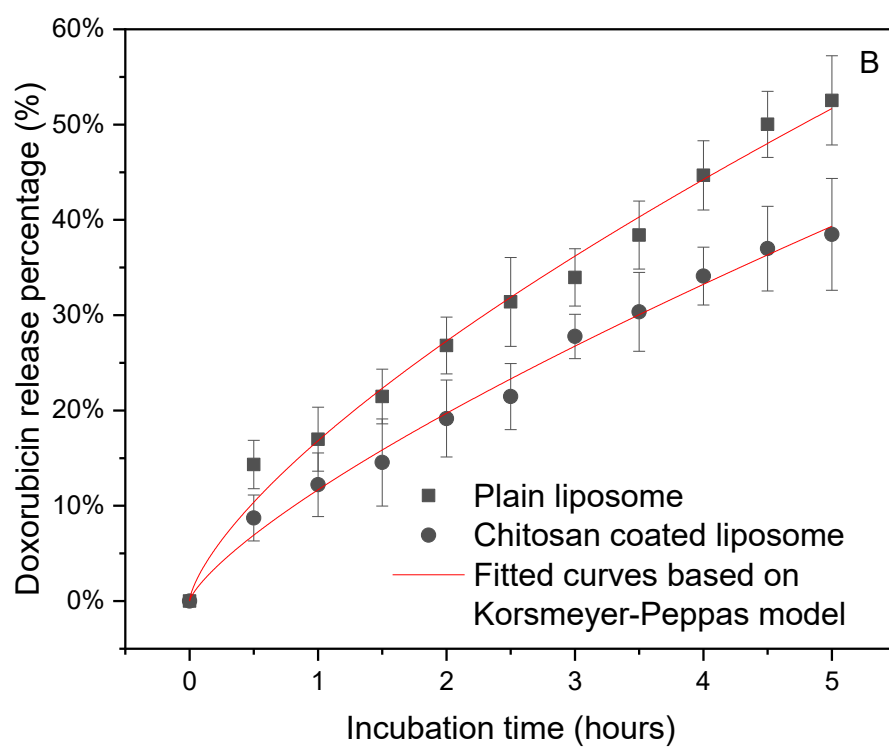
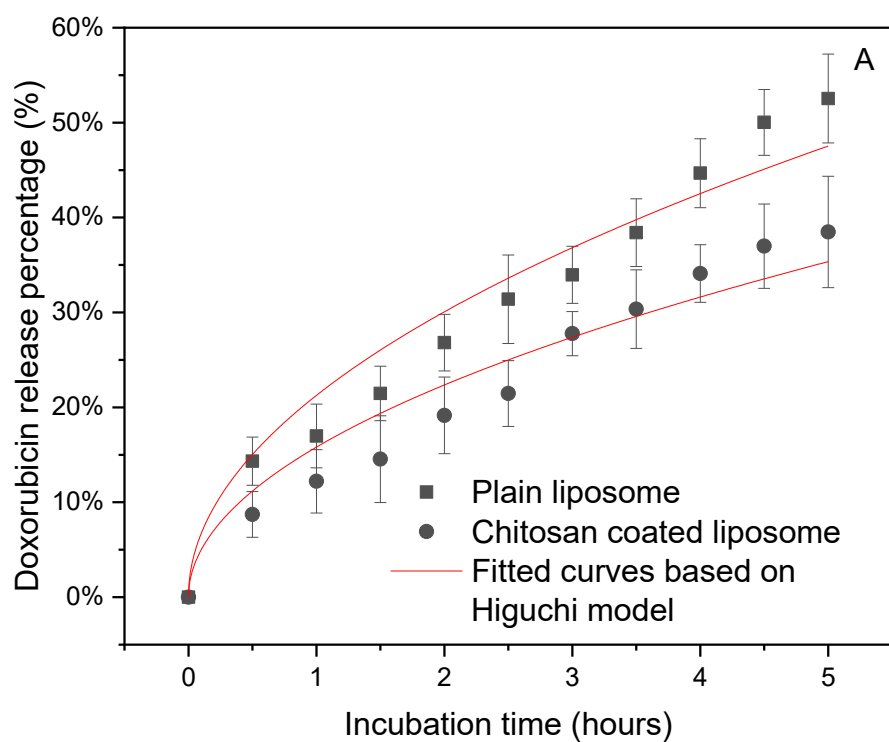


Figure 4.13 Higuchi (A), Korsmeyer-Peppas (B), Weibull (C) and First-order (D) models are utilised to fit doxorubicin release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 40 °C. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 4.13 compares the fitting curves simulated by four models at 40 °C. As can be observed from above graph, obviously, Higuchi model is not suitable in this case, because the shape of the fitting curve is different with that of other three models and most data point deviate from the fitting curve. Other three models, Korsmeyer-Peppas, Weibull and First-order model, have similar shape of fitting curve and it is not easy to determine the best model from simple observation. Thus, it also needs more evaluation on the basis of three parameters.



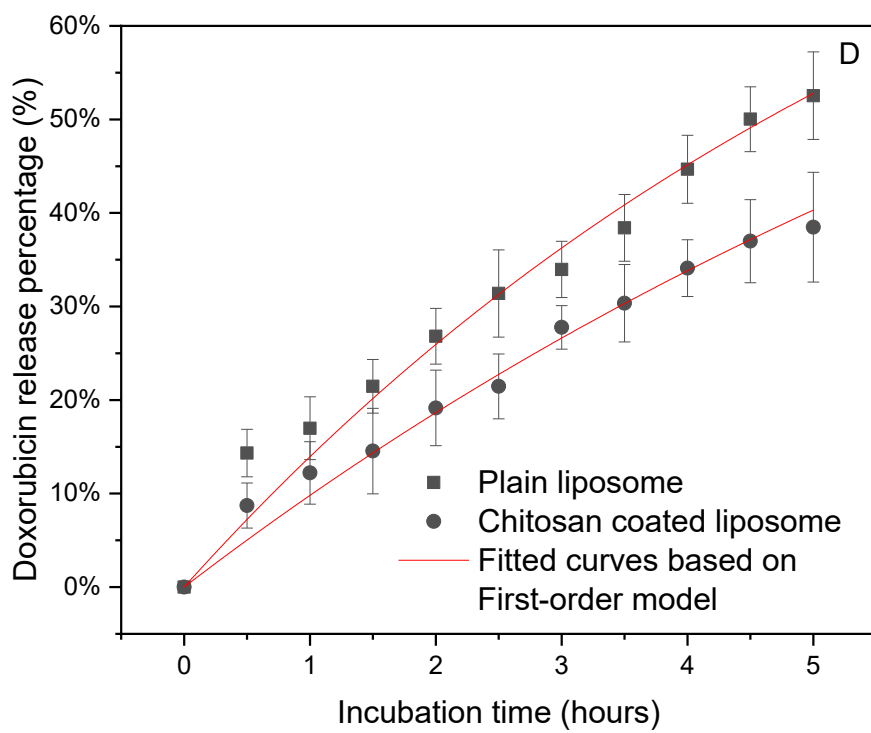
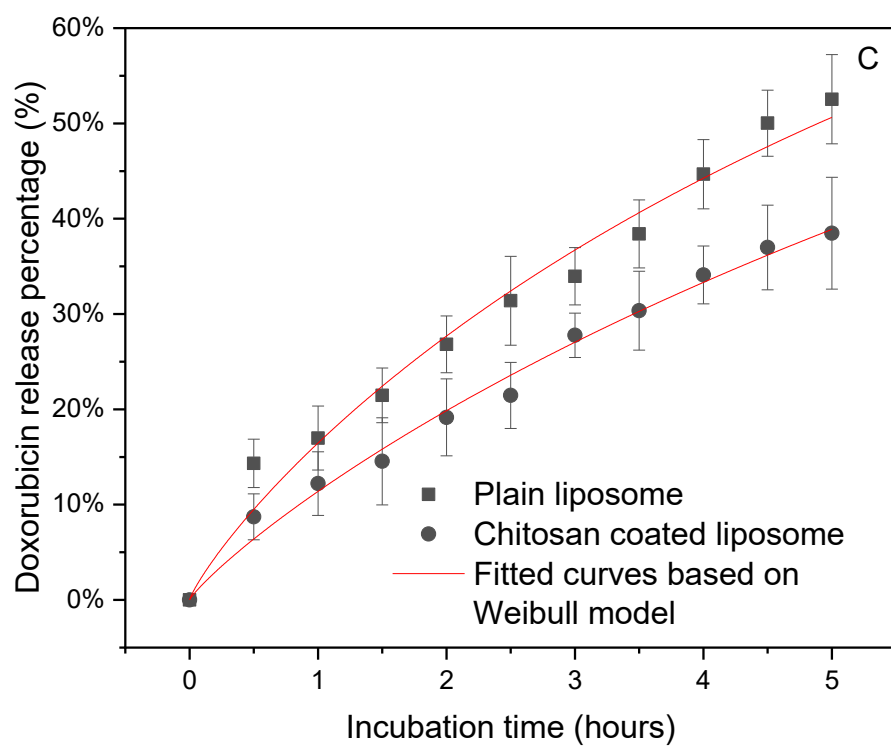
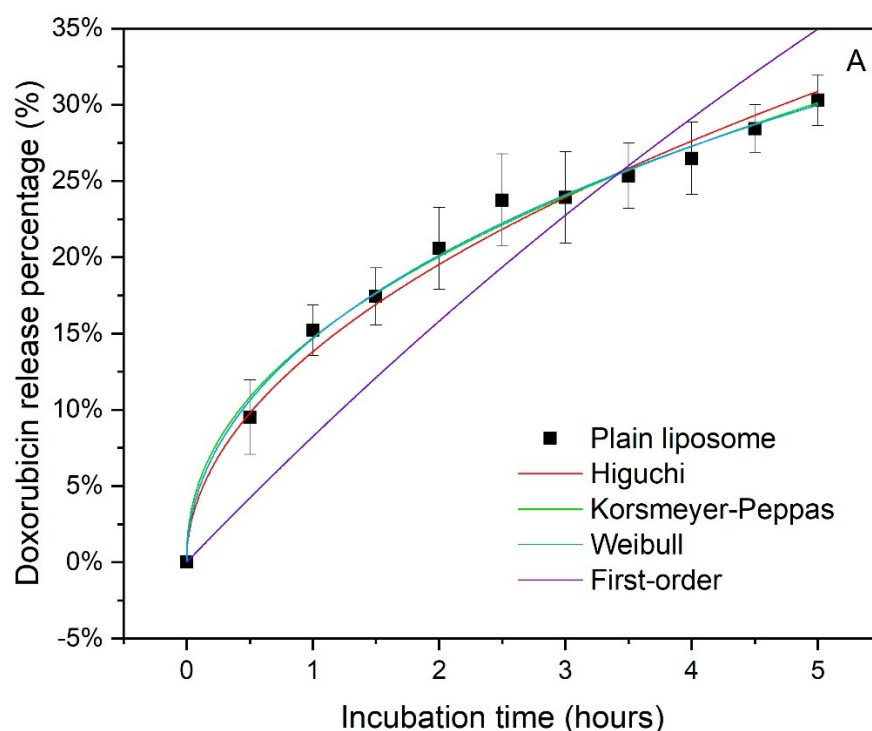


Figure 4.14 Higuchi (A), Korsmeyer-Peppas (B), Weibull (C) and First-order (D) models are utilised to fit doxorubicin release from plain liposomes (■) and liposomes coated with 0.1% (w/w) medium molecular weight chitosan (●) at 42 °C. Data represents mean $\pm$ SD. $n=3$ independent batches.

Figure 4.14 outlines the fitting circumstances by four models at 42 °C. It is manifest that Higuchi model doesn't fit the release curves well while other three models give better goodness-of-fits, which is same with the fitting result of release curves at 40 °C. In order to get more accurate and quantified comparison of fitting results, the analysis of fitting parameters are needed as well.



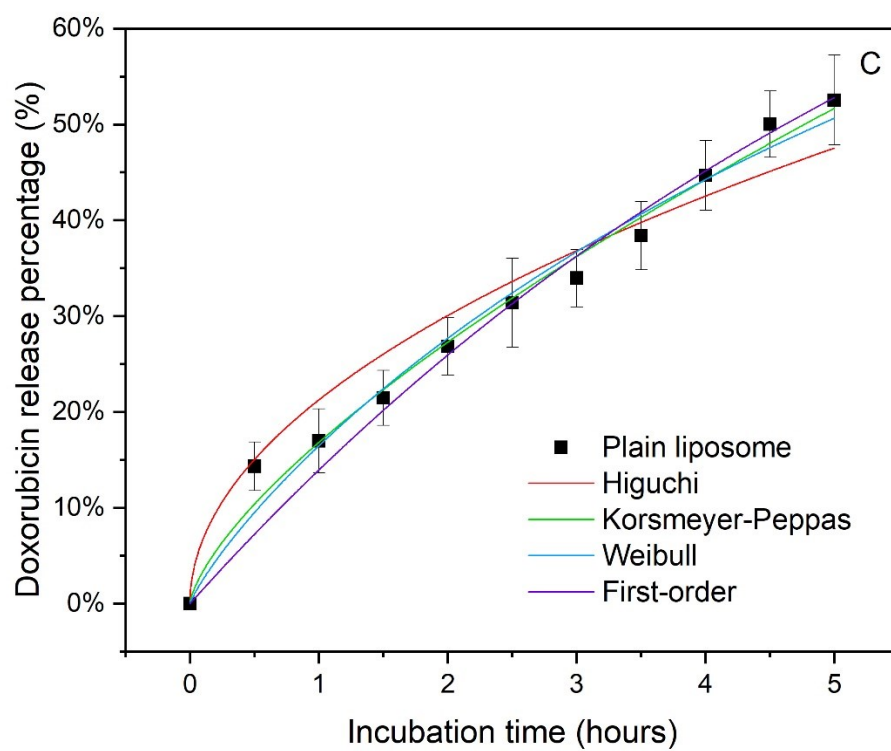
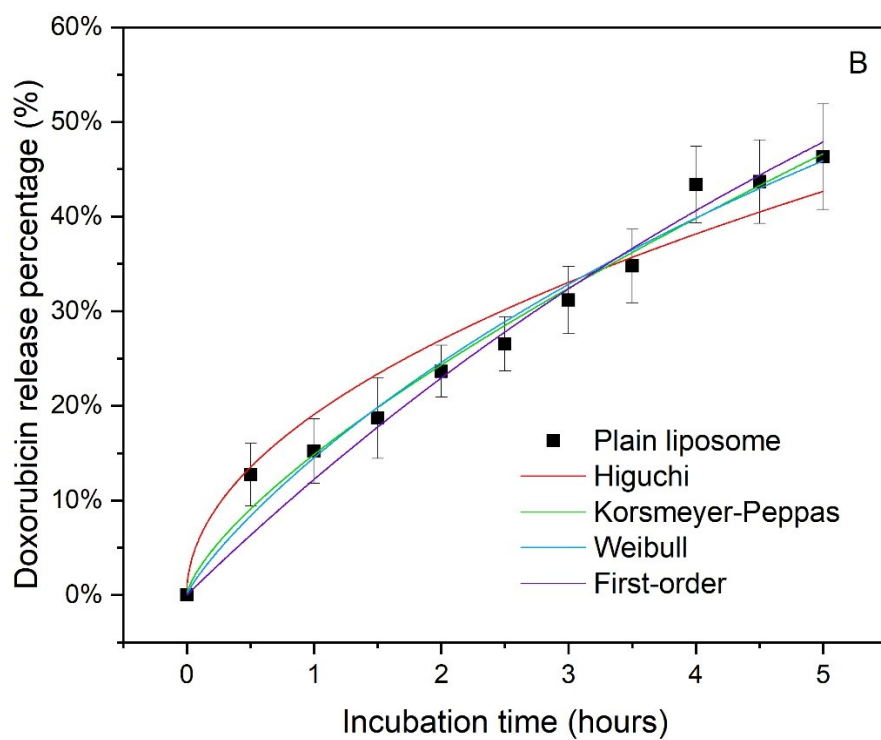
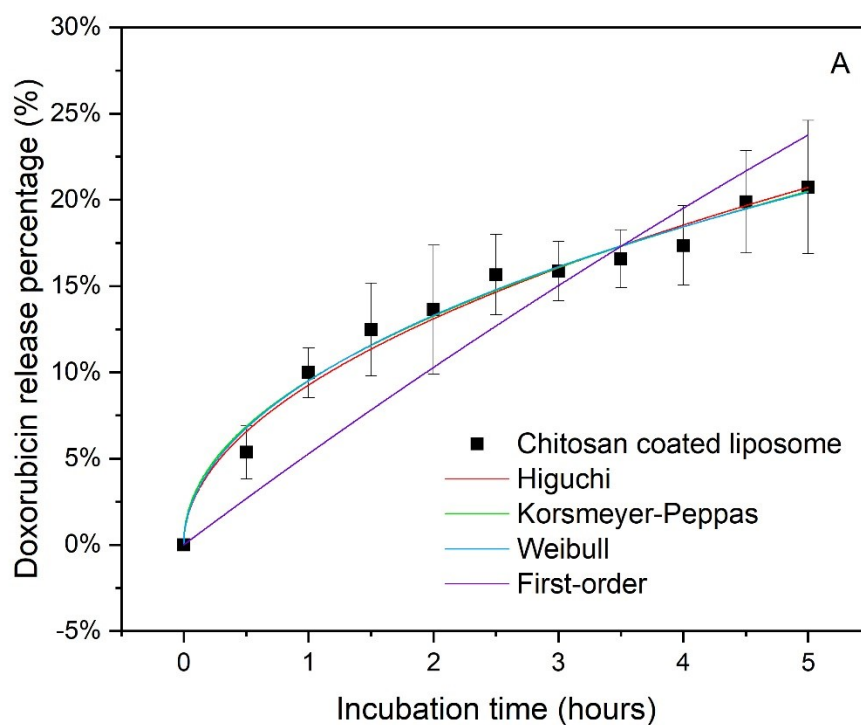


Figure 4.15 Higuchi, Korsmeyer-Peppas, Weibull and First-order models are utilised to fit doxorubicin release from plain liposomes (■) at 37 °C (A), 40 °C (B) and 42 °C (C). Data represents mean $\pm$ SD. $n=3$ independent batches.

As can be seen from the Figure 4.15, First- order model can not fit the release curve well obviously at 37 °C. Whereas, Higuchi model does not fit the doxorubicin release curves from plain liposomes at both 40 °C and 42 °C. Weibull and Korsmeyer-Peppas models have better goodness-of-fits, but it is hard to determine the best one by a rough comparison.



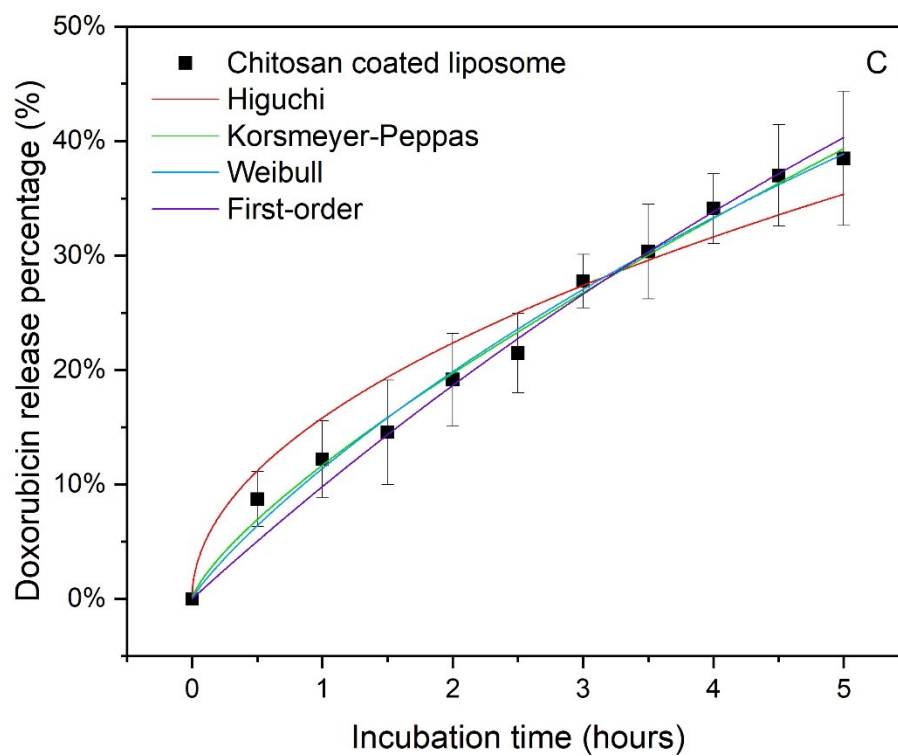
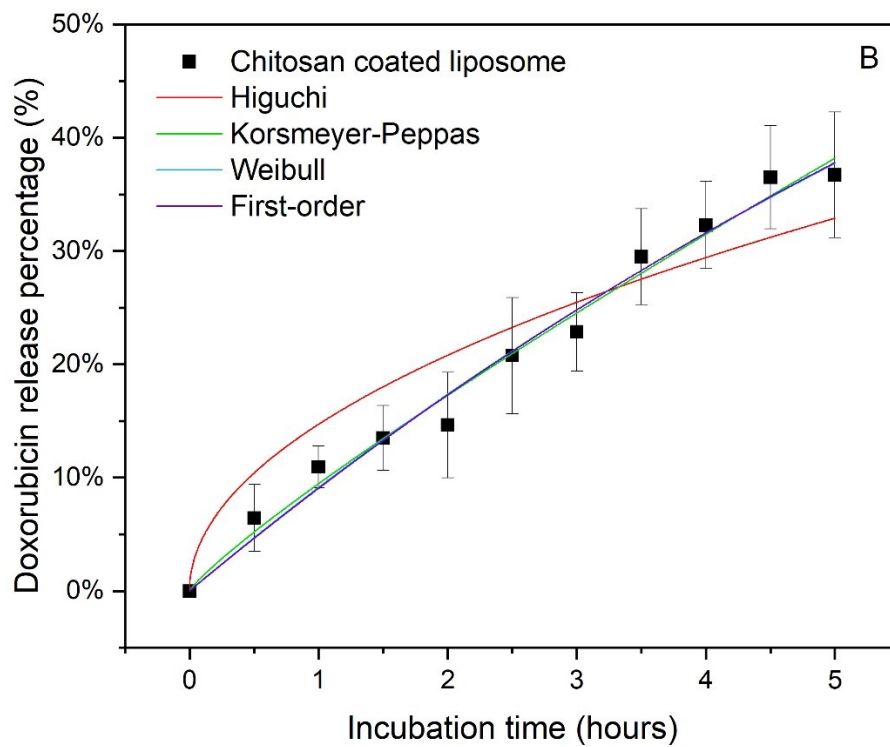


Figure 4.16 Higuchi, Korsmeyer-Peppas, Weibull and First-order models are utilised to fit doxorubicin release from liposomes coated with 0.1% (w/w) medium molecular weight chitosan (■) at 37 °C (A), 40 °C (B) and 42 °C (C). Data represents mean $\pm$ SD. n=3 independent batches.

Figure 4.16 depicts the fitting results of four models at three temperatures. Apparently, the First-order model does not suitable for the fitting of doxorubicin release curve from chitosan coated liposome at 37 °C. Besides, the Higuchi model does not fit those well at 40 °C and 42 °C. Korsmeyer-Peppas and Weibull model are still the best two models for the doxorubicin release curves of chitosan coated liposomes. In that case, it is essential to compare them with fitting parameters.

4.3.6 Comparison of fitting parameters

Table 4.1 The R^2 (COD) of each fitted curves using Higuchi, Korsmeyer-Peppas, Weibull and First-order models. Abbreviations: DPL, plain liposomes encapsulated with doxorubicin; DCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with doxorubicin.

Model	37 °C DPL	40 °C DPL	42 °C DPL	37 °C DCL	40 °C DCL	42 °C DCL
Higuchi	98.8%	94.6%	95.4%	98.3%	89.8%	94.0%
Korsmeyer-Peppas	99.3%	98.4%	98.8%	98.4%	98.6%	99.2%
Weibull	99.4%	97.9%	98.1%	98.5%	98.5%	99.1%
First-Order	76.6%	96.8%	97.1%	77.1%	98.4%	98.3%

Table 4.1 compares the R^2 of each fitted curves using four models. It is manifest that R^2 values of each kind of liposomes simulated by First-order model are the minimum, among which '37 °C DPL' and 37 °C DCL' have extremely low values of 76.6% and 77.1%, respectively. Apart from the First-order model, Higuchi model is the second smallest since all R^2 values of Higuchi model are lower than that of Korsmeyer-Peppas and Weibull model. Korsmeyer-Peppas and Weibull model are considered to be good models to fit these release curves because all R^2 of them are higher than 97.0%, between which the R^2 values of Korsmeyer-Peppas model are relatively higher than those of Weibull model. Therefore, the most suitable model to fit these release curves is Korsmeyer-Peppas model based on the analysis of R^2 .

Table 4.2 The BIC value of each fitted curves using Higuchi, Korsmeyer-Peppas, Weibull and First-order models. Abbreviations: DPL, plain liposomes encapsulated with doxorubicin; DCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsualted with doxorubicin.

<i>Model</i>	<i>37 °C DPL</i>	<i>40 °C DPL</i>	<i>42 °C DPL</i>	<i>37 °C DCL</i>	<i>40 °C DCL</i>	<i>42 °C DCL</i>
<i>Higuchi</i>	-97.9	-70.5	-70.3	-102.2	-67.3	-73.0
<i>Korsmeyer-Peppas</i>	-101.2	-81.2	-83.0	-100.4	-87.0	-93.2
<i>Weibull</i>	-102.8	-78.3	-77.8	-101.0	-85.6	-91.0
<i>First-Order</i>	-65.1	-76.5	-75.4	-73.5	-88.0	-87.0

Table 4.2 outlines the BIC values of each release profiled simulated by four pharmaceutical models. As discussed in the previous chapter, the lower BIC value indicates the better goodness-of-fit. Thus, Higuchi model obviously is not appropriate to fit these release curves,

because BIC values of curves fitted by Higuchi model are the highest in most cases, expect the group of '37 °C DPL' and '37 °C DCL'. Besides, even though the lowest BIC value is observed at the column of First-order model in the group of '40 °C DCL', most BIC values of First-order model are relatively higher than Korsmeyer-Peppas and Weibull model. Overall, the distinctions of BIC values of each model are not as clear as R^2 and RSS. Nevertheless, it still can be recognised that Korsmeyer-Peppas model has the lowest values of BIC in three categories ('40 °C DPL' at -81.2, '42 °C DPL' at -83.0 and '42 °C DCL' at -93.2). As a contrast, Higuchi model, Weibull model and First-order model merely has one smallest BIC value in one group, respectively. Overall, it can be concluded that Korsmeyer-Peppas model can be utilised to simulate these release curves with a maximum goodness-of-fit, whose conclusion is consistent with that of R^2 .

Table 4.3 The RSS of each fitted curves using Higuchi, Korsmeyer-Peppas, Weibull and First-order models. Abbreviations: DPL, plain liposomes encapsulated with doxorubicin; DCL, liposomes coated with 0.1% (w/w) medium molecular weight chitosan and encapsulated with doxorubicin.

<i>Model</i>	<i>37 °C DPL</i>	<i>40 °C DPL</i>	<i>42 °C DPL</i>	<i>37 °C DCL</i>	<i>40 °C DCL</i>	<i>42 °C DCL</i>
<i>Higuchi</i>	9.68E-04	1.17E-02	1.19E-02	6.54E-04	1.56E-02	9.36E-03
<i>Korsmeyer-Peppas</i>	5.76E-04	3.55E-03	3.04E-03	6.19E-04	2.11E-03	1.20E-03
<i>Weibull</i>	4.99E-04	4.61E-03	4.87E-03	5.90E-04	2.38E-03	1.46E-03
<i>First-Order</i>	1.91E-02	6.81E-03	7.47E-03	8.92E-03	2.39E-03	2.60E-03

Table 4.3 features the comparison of RSS of each release profiles fitted by four models. Apparently, there are massive differences among RSS of each model. The smaller the RSS

is, the better the goodness-of-fit is. It is obvious that the RSSs of Higuchi model and First-order model are much greater than those of Korsmeyer-Peppas and Weibull model in most cases. To be specific, in the group '37 °C DPL', the RSS of First-order model is almost 38 times as much as that of Weibull model. Besides, it can be found that the RSS of Higuchi model is around 7.5 times more than that of Korsmeyer-Peppas model in '40 °C DCL'. In terms of the contrast of Korsmeyer-Peppas and Weibull model, it can be observed there is only a marginal difference on RSS between these two models. Nevertheless, the RSSs of Korsmeyer-Peppas model are slightly lower than that of Weibull model in most release curves, which makes it a superior model in this database.

Therefore, based on the analysis of all three fitting parameters (R^2 , BIC and RSS), Korsmeyer- Peppas model and Weibull model both fit doxorubicin release curves well, however, Korsmeyer-Peppas model can be treated as the best model to simulate doxorubicin release curve from plain HaT liposome and chitosan coated liposome. This is because both First-order model ($Q/Q_0 = 1 - e^{(-k*t)}$) and Higuchi model ($Q/Q_0 = k * t^{0.5}$) have only one constant 'k'. On the contrary, Weibull model ($Q/Q_0 = 1 - e^{(-b*t^a)}$) and Korsmeyer-Peppas model ($Q/Q_0 = k * t^n$) both have two constants which can be adjusted to the optimum number for the best fitting results. The Weibull model has a similar equation structure with the First-order model, with a more constant 'a' on the exponent, which can be regarded as an 'upgraded' model based on the First-order model. Similarly, the Korsmeyer-Peppas model can be treated as a modified version of Higuchi model, which replace the exponent '0.5' into a variable constant 'n', allowing the Origin software to adjust it to suitable number for the best goodness-of-fit. It is hard to distinguish the best model in this scenario between Korsmeyer- Peppas model and Weibull model. This is because the shapes of doxorubicin release curves don't have obvious "Γ" shapes as calcein release curves in Chapter 3, because the lower encapsulation efficiency of doxorubicin compared to that of

calcein. Nevertheless, according to the overall analysis of three fitting parameters, Korsmeyer-Peppas model is slightly better than Weibull model.

4.4 Conclusion

1. This chapter plotted two fluorescence intensity standard curves of doxorubicin using two instruments. The results obtained from Horiba FluoroMax-4 spectrofluorometer proved the fluorescence intensity of doxorubicin is influenced by pH and an acidic environment enhanced fluorescence intensity of doxorubicin marginally. The data from Microplate reader was applied to plot the fluorescence intensity standard curve as a reference for the following measurements of doxorubicin encapsulation efficiency, release property and shelf life.
2. 3 mg of initial doxorubicin added in the preparation process was shown to have the highest encapsulation efficiency of 14% and this concentration was used in all subsequent release studies.
3. At 37 °C, 40 °C and 42 °C, all 0.1% (w/w) medium molecular weight chitosan coated liposome samples experienced released less than plain HaT liposome. Especially at 37 °C, the coating of chitosan enhanced the stability of liposome dramatically (only 5% released in the first half hour and 20% in 5 hours). Above 40 °C, chitosan coated liposomes were still able to release a significant amount of encapsulated doxorubicin after 5 hours incubation (39% at 42 °C and 35% at 40 °C).
4. At 4 °C, both plain liposome and chitosan coated liposome experienced slow leakages, with the chitosan coated lipid able to reduce the liposome leakage from 6% to 4.5% after one-month storage. Moreover, at room temperature, the plain liposome leaked 14% of encapsulated doxorubicin which is much higher than at 4 °C after 30

days. Nevertheless, the coating of chitosan prevented this leakage significantly to around 7% over 30 days.

5. Finally, Higuchi, Korsmeyer-Peppas, Weibull and First-order models were chosen from pharmaceutical models database since other models are obviously not suitable to fit doxorubicin release curves in this chapter. Fitting procedure was conducted by Origin software, and three fitting parameters such as R^2 , BIC and RSS were calculated as well. Based on the analysis of these parameters and observation of the differences between each fitting curve and original release curve, Korsmeyer-Peppas is recognised as the optimal solution for the fitting of doxorubicin release curves from plain HaT liposome and chitosan coated liposome at all three temperatures.

Chapter 5 Conclusion

5.1 Conclusion

Firstly, both empty and drug loaded liposomes with and without chitosan coating were prepared in this study. Liposomes with a unified 100 nm diameter can be achieved by the application of 8 minutes of ultrasonication. Zeta potentials of DPPC, Brij S20, low, medium, and high MW chitosan were measured at different pH to calculate their IP points. It can be also found that DPPC and Brij S20 are negatively charged at pH 7, which provides condition for cationic chitosan attaching on the liposome composed of DPPC and Brij S20 due to electrostatic force. In addition, the zeta potentials and sizes of liposomes composed of various percentages of DPPC and Brij S20 (freshly made ones and stored ones) were investigated. The higher ratio of Brij S20 in the liposome formulation can lead to the higher zeta potentials of liposomes and smaller sizes. Besides, the TEM images of plain liposome and chitosan coated liposomes were taken. The results demonstrated that the chitosan coating can successfully prevent liposome from aggregation and fusion after one month storage. Different MW chitosan coated liposomes showed various shapes, from 'flower' shape of low MW chitosan coated liposomes to hexagon or quadrangle shape of high MW chitosan coated liposomes. The higher MW of chitosan resulted in thicker coating with better protection from aggregation and fusion. In another study, the curcumin-encapsulated liposomes coated with low and high MW chitosan were prepared [245]. The results demonstrated that high MW chitosan coated liposome exhibited better stability in storage and centrifugal tests compared to low MW chitosan. Furthermore, the high MW chitosan has made liposome membranes more compact and rigid, which diminished the membrane disruption and the unwanted drug leakage efficaciously [245]. However, the large size of liposome is not suitable for intravenous injection. Therefore, the high MW chitosan is not the

best choice in this case. Furthermore, the results from zeta potential and particle size showed that all three MW chitosan was successfully coated on the liposomes. The particle size of small MW chitosan coated liposome saw a dramatic rise after one month storage due to the thin chitosan coating, as demonstrated in TEM images. Among three concentration of medium molecular weight chitosan (0.1%, 0.2% and 0.3% (w/w)), 0.1% (w/w) chitosan had the best performance overall. It demonstrated the lowest increase in both zeta potential and particle size after one-month storage at 4 °C. Thus, it was not easy to be recognized and removed by the human immune system. Therefore, 0.1% medium molecular weight chitosan was selected for the following experiments.

Secondly, the calcein fluorescence intensity standard curve was plotted using Horiba FluoroMax-4 spectrofluorometer. The results showed that the fluorescence intensity of calcein is sensitive to pH. The acidic environment decreased the fluorescence intensity of calcein and changed the physical appearance of the calcein solution significantly. 7 mg of initially added calcein represented the highest encapsulation efficiency of 27%. In the experiments of calcein release property, both plain and chitosan coated liposomes released more calcein at 40 °C and 42 °C than liposomes at 37 °C. This could because the Brij S20 (a PEGylated single acyl chain) transformed from a gel to liquid crystalline state at T_c and form porous structures which cause the increased leakage of calcein from liposomes [99]. Besides, the liposomes coated with 0.1% (w/w) medium molecular weight chitosan released much less than plain liposomes at 37 °C and demonstrated enhanced stability. The release percentages at 40 and 42 °C of liposomes coated with 0.1% (w/w) medium molecular weight chitosan were still considerable with a slight decrease compared to plain liposomes. In addition, the release percentage of both plain liposomes and chitosan coated liposomes stored at 4 °C in fridge were much fewer than those reserved at room temperature for one month. This is because the long-time of exposure to light and oxygen can lead to the destabilization of liposome bilayer [246] [247]. The coating of chitosan can enhance the

stability of liposomes at room temperature significantly and at 4 °C slightly. Other studies have also found the slight leakage of encapsulated drugs from liposomes after the storage at 4 °C [248] [249]. Besides, the three kinetic models, Higuchi, Korsmeyer-Peppas and Weibull model, were used to stimulate the calcein release curves, among which the Weibull model was selected as the best model, because it has the highest R^2 and the smallest values of BIC and RSS.

Thirdly, the doxorubicin fluorescence intensity standard curve obtained from Horiba FluoroMax-4 spectrofluorometer represented acidic environment increased fluorescence intensity of doxorubicin marginally. The data obtained from the Microplate reader was utilized to plot the fluorescence intensity standard curve as a reference for the following experiments. The highest encapsulation efficiency of 14% was discovered in the group of 3 mg initial added doxorubicin. The release property and shelf life of doxorubicin encapsulated liposomes represented similar results with calcein encapsulated liposomes. The coating of 0.1% (w/w) medium molecular weight chitosan was also able to decrease the release of HaT liposomes at all three temperatures (37, 40 and 42 °C) and enhance the stability during the storage at both room temperature and 4 °C. Another research showed similar results of enhanced stability of chitosan coated liposomes. Mitoxantrone encapsulated liposomes coated with chitosan was prepared, which demonstrated longer retention time and higher plasma concentration after intravenous administration in rats [161]. In addition, four kinetic models (Higuchi, Korsmeyer-Peppas, Weibull and First-order model) were chosen for the comparison of fitting results on doxorubicin release curves. Based on the analysis of fitting parameters, the Korsmeyer-Peppas model was considered as the best model in this case.

5.2 Future work

Experiments regarding release property of calcein and doxorubicin encapsulated liposomes coated with chitosan should be conducted in the plasma *in vitro* at three temperatures (37, 40 and 42 °C) to investigate the influence of blood environment on its release behaviour.

Cytotoxicity test should be undertaken in human tumour cells by adding doxorubicin encapsulated HaT liposomes coated with chitosan in contrast with doxorubicin encapsulated HaT liposomes without chitosan coating.

Animal experiments should proceed in rabbits or rats. Rabbits or rats would need to be injected intravenously with doxorubicin encapsulated HaT liposomes coated with chitosan combined with hyperthermia. The control groups are injected with free doxorubicin and doxorubicin encapsulated HaT liposomes. Blood samples will be obtained periodically after administration from animals' bodies to monitor the doxorubicin release *in vivo*.

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