



ENCAPSULATION OF PLASMID DNA INTO  
COLLOIDAL CARRIER OF POLY(D,L-LACTIDE-CO-  
GLYCOLIDE)/CHITOSAN/*NIGELLA SATIVA* INTENDED  
FOR GENE DELIVERY TO CENTRAL NERVOUS  
SYSTEM

BY

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## ABSTRACT

Delivery of plasmid DNA (pDNA) to the central nervous system (CNS) is challenging in gene therapy due to the presence of cellular and biochemical barriers, which restrict the passage of most substances into the brain. *Nigella Sativa* oil (NSO) is a lipophilic material and has been reported to have neurotherapeutics effects such as neuroprotective and neuroregenerative. Owing to its lipophilicity, NSO-incorporated carrier system could enhance penetration of the gene into the brain. Here, we aimed to amplify and isolate pDNA from *Escherichia coli* (*E. coli*) DH5 $\alpha$ . We had attempted to co-encapsulate the pDNA and NSO into biodegradable poly(D,L-lactide-co-glycolide) (PLGA) and chitosan using diffusion-solvent evaporation technique. Firstly, commercial pDNA was amplified and isolated using conventional isolated/purification of pDNA, namely 'IUM Concentrated Alkaline Lysis' (iCALL); a modified Sambrook and Russell's protocol in order to obtain high quality and plasmid yield for our study. Secondly, the pDNA and NSO were co-encapsulated into PLGA/chitosan by manipulating several variables i.e. fabrication techniques (single or double emulsion solvent evaporation); volume ratio of solvent to co-solvent, concentration of chitosan and NSO; and mixing rate/type (homogenizer and sonicator processor) in order to investigate their effects on the particles' characteristics and to obtain optimized formulations. The optimized formula that was prepared by single emulsion (o/w), volume ratio of solvent to co-solvent at 1:3 and sonicated at 15 sec time was selected. To improve the encapsulation efficiency of pDNA using single emulsion, pre-complexation between pDNA and cetyltrimethylammonium bromide (CTAB) prior encapsulation was introduced. Further optimization of co-encapsulation of NSO and pDNA in PLGA NPs were carried out by employing two independent variables namely, molecular weights (MW) of PLGA 50:50 (14 and 34 kDa) and chitosan (50-190 and 190-310 kDa). These NPs were thereafter called "Neurobionanoparticles" (NBPs). Size, surface morphology, zeta potential, pDNA in vitro release profile, cell viability and transfectibility were characterized as a function of those multiple variables. The selected NBPs were subjected to stability studies by investigating the effect of excipients, namely human serum albumin, glycine and potassium chloride with different NBP/excipients weight ratio systems on stability of lyophilized NBP upon three months' storage. Our data revealed that iCALL method gave the highest value for the pDNA yield compared with other commercial methods. The resultant NBPs showed an encapsulation efficiency of ~99%, particle sizes around 400 nm and positive zeta potential. These optimized preparations showed sustained release rate of pDNA over 5 weeks and was capable of expressing pGL3 gene in Neuro-2A (N2a) cell line. Interestingly, preparation with PLGA 50:50 (14 kDa) and chitosan (190 – 310 kDa) showed higher gene expressions in N2a cells. None of these NPs were toxic for N2a cells after incubation for 48 h in the tested dose ranges (0.01, 0.1 and 0.2 mg/ml). Furthermore, the co-lyophilized of NBP with HSA at ratios 10:1 and 2:1 abled to stabilize lyophilized particles for three months storage and exhibit better enhancement in the transfection activity of gene delivery when compared with NBP alone and combination of NBP with other excipients. In conclusion, the fabricated NBPs may be used as promising non-viral gene delivery to the CNS.

## خلاصة البحث

إنتقال البلازميد الحمض النووي (pDNA) إلى الجهاز العصبي المركزي (CNS) يمثل تحدياً في العلاج الجيني نظراً لوجود الحواجز الخلوية والكيمياء الحيوية، والتي تحد من مرور معظم المواد في الدماغ. زيت الحبة السوداء هو مادة محبة للدهون وتم الإبلاغ عن أن يكون العلاجات التأثيرات على الخلايا العصبية مثل الحماية والتجدد. لما له من خصائص، أدرج النفط الاسود، في نظام الناقل لتعزيز تغلغل هذا الجين في الدماغ. هنا، نحن نهدف لتضخيم وعزل pDNA من الإشريكية القولونية DH5 $\alpha$ . كنا قد حاولنا أن نشارك في تغليف pDNA تسمى 'IIUM Concentrated Alkaline Lysis (iCALL) سامبروك ورسول بروتوكول تعديلي نأمل الحصول على جودة عالية والعائد البلازميد لدراستنا. ثانياً، تم تغليف pDNA وزيت الحبة السوداء/PLGA/الشيتوزان عن طريق التلاع بعدة متغيرات أي تقنيات تصنيع مستحلب ( واحد أو مزدوج تبخر المذيبات)، نسبة حجم المذيب إلى المذيبات، تركيز الشيتوزان وزيت الحبة السوداء، ومعدل خلط / نوع (الخالط وsonicator المعالج) من أجل التحقيق آثارها على خصائص الجسيمات والحصول على تركيبات الأمثل. الصيغة الأمثل التي كتبها أعد تواحد مستحلب، إلى المذيب في 1: 3 نسبة حجم المذيبو sonicated في 15 مرة الثانية تم اختيار. لتحسين كفاءة التغليف من pDNA و cetyltrimethyl ammonium bromide (CTAB) التغليف مسبق. أجريت تنفيذ المزيد من التحسين من التعبئة والتغليف من زيت الحبة السوداء، و pDNA السجزيات PLGA من خلال توظيف متغيرين مستقلين هما، والأوزان الجزئية لل (34 PLGA و 14 كيلو دالتون) والشيتوزان (50-190 و 190-310 كيلو دالتون). وهذه الجسيمات صغيرة تسمى بعد ذلك "Neurobionanoparticles" (NBP) حجم، مورفولوجيا السطح، وإمكانات زيتنا، pDNA في الملف الشخصي الإفراج المختبر، تميزت بقاء الخلية وtransfectibility. بوصفها وظيفة من تلك المتغيرات متعددة. أفضل NBPs تعرض للدراسات الاستقرار من خلال التحقيق في تأثير سواغ مثل الألبومين البشري في الدم، والجلايسين وكوريد البوتاسيوم مع نسبة مختلفة من NBP الوزن سواغ / نظم على استقرار مجفف بالتجميد NBP لتخزين ثلاثة أشهر. وكشفت البيانات التي تقوم iCALL طريقة أعطى أعلى قيمة للالعائد pDNA بالمقارنة مع الطرق التجارية الأخرى. أظهرت NBPs الناتجة كفاءة التغليف من ~ 99%، وأحجام الجسيمات حوالي 400 نانومتر، وإمكانات زيتنا إيجابية. وأظهرت هذه الاستعدادات الأمثل معدلاً لإستمرار في إطلاق سراح أكثر من 5 أسابيع، وكانت قادرة على التعبير عن pDNA الجينات في Neuro-2a (N2a) خط الخلية. ومن المثير للاهتمام، تم الحصول على تعبيرات الجين أعلفي N2a في إعداد (PLGA 14 كيلو دالتون) والشيتوزان (190-310 كيلو دالتون) ولم تكن أي من هذه مصادر القدرة النووية السامة للخلايا N2a بعد الحضانة لمدة 48 ساعة في نطاقات جرعة اختبار (0.01، 0.1 و 0.2 ملغ/مل). وعلاوة على ذلك، تجميد دريد ل NBP مع HSA في نسب 1: 10 و 1: 2 قدرة على تحقيق استقرار الجزئيات لمدة ثلاثة أشهر وعرض أفضل في تعزيز النشاط ترنسفاكيشن مقارنة مع NBP وحده، ومزيج من NBP تخزين مع سواغ أخرى. في الختام، ملفقة NBP يمكن استخدامها اعدة كما توصيل الجينات غير الفيروسي إلى CNS.

## APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Science (Pharmaceutical Technology)

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## DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature.....

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*This thesis is dedicated to my beloved family.*

*'What does not kill us makes us stronger.' - Friedrich Nietzsche*

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In the name of Allah, The Most Gracious, The Most Merciful.

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# TABLE OF CONTENTS

Abstract .....	ii
Abstract in Arabic .....	iii
Approval page .....	iv
Declaration .....	v
Copyright Page.....	vi
Dedication .....	vii
Acknowledgements.....	viii
List of Tables .....	xiv
List of Figures .....	xvi
List of Abbreviations .....	xx
List of Equations .....	xxii
<b>CHAPTER 1: INTRODUCTION.....</b>	<b>1</b>
1.1 Research Background .....	3
1.2 Objectives and Scope of the study.....	5
<b>CHAPTER 2: LITERATURE REVIEW.....</b>	<b>7</b>
2.1 Drug Delivery to Central Nervous System.....	7
2.2 Gene Therapy.....	10
2.2.1 Overview .....	10
2.2.2 Viral Vector.....	13
2.2.3 Non-viral Vector .....	15
2.2.4 Plasmid Deoxyribonucleic Acid (pDNA) .....	19
2.3 Nanoencapsulation and Nanoparticulate Delivery System.....	21
2.3.1 Overview .....	21
2.3.2 Poly (D,L-Lactic-Co-Glycolic Acid) (PLGA) .....	22
2.3.3 Chitosan .....	25
2.3.4 <i>Nigella sativa</i> .....	30
2.4 Parenteral Formulation for Nanoparticles .....	34
2.4.1 Excipients Used in the Current Study .....	35
2.4.1.1 Human Serum Albumin.....	35
2.4.1.2 Glycine .....	40
2.4.1.3 Potassium Chloride.....	44
<b>CHAPTER 3: AMPLIFICATION, EXTRACTION AND PURIFICATION OF SUPERCOILED PLASMID DNA (PDNA).....</b>	<b>45</b>
3.1 Introduction.....	45
3.2 Materials and Method .....	46
3.2.1 Materials.....	46
3.2.2 Methodology .....	46
3.2.2.2 Amplification, Extraction and Purification of Supercoiled pDNA .....	46
3.2.2.2.1 Preparation of Competent <i>E. coli</i> DH5- $\alpha$ Cells.....	46

3.2.2.2.2 Preparation and Transformation of Competent <i>E. coli</i> DH5- $\alpha$ Cells.....	47
3.2.2.2.3 pgl3-control amplification in <i>E. Coli</i> .....	47
3.2.2.3 pDNA Extraction and Purification .....	48
3.2.2.3.1 iCALL: A Modified of Sambrook and Russell.....	50
3.2.2.3.2 PureYield™ Plasmid Maxiprep system.....	51
3.2.2.3.3 PureLink® Hipure Plasmid Gigaprep.....	52
3.2.2.3.4 Effect of Different Culture Media Volume on pDNA Yield by Three Different Purification Protocols.....	53
3.2.2.4 Effect of Various Variables in iCALL Method.....	54
3.2.2.4.1 Effect of Concentrations of Lysate on pDNA Yield by iCALL Method.....	54
3.2.2.4.2 Fermentation of <i>E. coli</i> Using Bioreactor .....	54
3.2.2.4.3 Effect of Types of Culture Media on Cell Biomass Production and pDNA Yield by iCALL .....	56
3.2.2.5 Analysis of pDNA .....	57
3.2.2.5.1 Agarose Gel Electrophoresis of pDNA.....	57
3.2.2.5.2 UV Absorbance.....	57
3.3 Result and Discussion.....	58
3.3.1 Amplification and Purification of Supercoiled pDNA.....	58
3.3.1.1 Effect of Different Culture Media Volumes on pDNA Yield by Three Different Purification Protocols. ....	58
3.3.1.2 Effect of Concentration of Lysate on pDNA Yield.....	65
3.3.2 Amplification of <i>E. Coli</i> Using Bioreactor and Extraction and Purification of pDNA by iCALL Method.....	68
3.3.2.1 Effect of Types of Culture Media on Production of Cell Biomass and pDNA Yield .....	68
3.4 Conclusion .....	72

**CHAPTER 4: FABRICATION AND EVALUATION OF *NIGELLA SATIVA*/CHITOSAN/PLGA NANOPARTICLES .....**

4.1 Introduction.....	72
4.2 Materials and method .....	75
4.2.1 Materials.....	75
4.2.2 Preliminary Study of PLGA/NSO Nanoparticles: Effect of Fabrication Technique on the Surface Morphology and Particle Size.....	76
4.2.2.1 Double-Emulsion Solvent Evaporation(w/o/w) Technique ...	78
4.2.2.2 Single-Emulsion Solvent Evaporation(w/o/w) Technique .....	78
4.2.3 Preliminary Study of PLGA/NSO Nanoparticles on the Effect of <i>Nigella Sativa</i> oil (NSO) on the Surface Morphology .....	79
4.2.4 Preliminary Study of PLGA/Chitosan/NSO Nanoparticles: Effect of Types and Concentrations of Chitosan on Zeta Potential and Stability of Colloidal System .....	80
4.2.5 Fabrication of pDNA-loaded PLGA/Chitosan/ <i>N. Sativa</i> Oil Nanoparticles.....	82
4.2.5.1 Preparation of pDNA-CTAB Complexes.....	82
4.2.5.2 Fabrication of pDNA-loaded PLGA/Chitosan/ <i>N. sativa</i> Oil Nanoparticles .....	82
4.2.5.3 Characterization of NBP.....	84

4.2.5.3.1 Particle Morphology .....	83
4.3 Result and Discussion.....	84
4.3.1 Preliminary Study of PLGA/NSO Nanoparticles: Effect of Fabrication Technique on the Surface Morphology and Particle Size.....	84
4.3.2 Preliminary Study of PLGA/NSO Nanoparticles on the Effect of <i>N. Sativa</i> oil (NSO) on the Surface Morphology.....	88
4.3.3 Preliminary Study of PLGA/Chitosan/NSO Nanoparticles: Effect of Types and Concentrations of Chitosan on Zeta Potential and Stability of Colloidal System .....	91
4.3.4 Fabrication of pDNA-loaded PLGA/Chitosan/ <i>N. Sativa</i> Oil Nanoparticles.....	94
4.4 Conclusion .....	97

**CHAPTER 5: DEVELOPMENT OF ‘NEUROBIONANOPARTICLE’ (NBP): EFFECT OF POLY(D,L-LACTIC-CO-GLYCOLIC ACID) AND CHITOSAN MOLECULAR WEIGHT ON FORMULATION ..... 99**

5.1 Introduction.....	99
5.2 Materials and Method .....	101
5.2.1 Materials.....	101
5.2.2 pDNA Amplification, Purification and Evaluation.....	101
5.2.3 Preparation of pDNA-CTAB Complexes .....	101
5.2.4 Fabrication of ‘Neurobionanoparticles’ (NBP).....	102
5.2.5 Characterization of NBP .....	104
5.2.5.1 Particle Morphology .....	104
5.2.5.2 Particle Size Analysis .....	105
5.2.5.3 Zeta Potential Measurement .....	105
5.2.5.4 Agarose Gel Electrophoresis .....	106
5.2.5.5 Thermal Analysis.....	106
5.2.5.6 pDNA <i>In vitro</i> Release Profiles.....	106
5.2.5.7 Cell Viability Assay .....	107
5.2.5.8 <i>In vitro</i> Transfection Studies .....	108
5.2.5.9 Luciferase Assay .....	108
5.2.5.10 Statistical Analysis .....	108
5.3 Result and Discussion.....	109
5.3.1 Surface Morphology, Particle Size and Zeta Potential Analysis ....	109
5.3.2 Agarose Gel Electrophoresis Analysis and Encapsulation Efficiency .....	120
5.3.3 Determination of Glass Transition.....	122
5.3.4 pDNA <i>In vitro</i> Profiles.....	124
5.3.5 Cell Viability Assay .....	128
5.3.5 <i>In vitro</i> Transfection Studies.....	131
5.4 Conclusion .....	134

**CHAPTER 6: STABILITY STUDIES OF LYOPHILIZED NBP ..... 135**

6.1 Introduction.....	136
6.2 Materials and Method .....	138
6.2.1 Materials.....	138
6.2.2 Methodology .....	138
6.2.2.1 Fabrication of ‘Neurobionanoparticles’ (NBP).....	138

6.2.2.2 Addition of Excipients to the NBP prior Lyophilization.....	138
6.2.2.3 Characterization of Particle Size and Zeta Potential .....	138
6.2.2.4 Thermal Analysis.....	140
6.2.2.5 <i>In vitro</i> Transfection Studies .....	140
6.2.2.6 Stability Studies.....	140
6.3 Result and Discussion.....	141
6.3.1 Characterization of NBP .....	141
6.3.1.1 Effect of Different Types and Concentrations of Excipients on Particle Size of NBPs .....	141
6.3.1.2 Effect of Different Types and Concentrations of Excipients on Zeta Potential of NBPs .....	144
6.3.2 Thermal Analysis .....	147
6.3.3 Effect of Different Types and Concentration of Excipients on Transfection Efficiency of NBPs .....	158
6.3.4 Stability Study After Three Months.....	160
6.4 Conclusion .....	164
<b>CHAPTER 7: CONCLUSION AND FUTURE STUDY .....</b>	<b>165</b>
<b>REFERENCES.....</b>	<b>168</b>
APPENDIX A.....	192
APPENDIX B .....	194
APPENDIX C .....	195
APPENDIX D.....	196
APPENDIX E .....	197

## LIST OF TABLES

<u>Table No.</u>		<u>Page No.</u>
2.1	Formulation for DNA carriers	18
2.2	The fatty acids composition of NSO	34
3.1	Culture volumes using three different purification protocols	54
3.2	Manufacturing cost (USD) for Amplification and Purification of plasmid from 1 L culture volume using three different techniques	68
3.3	Cell biomass of different culture media following 12 hours incubation (n=1)	69
3.4	pDNA quality from different culture media (n=3)	72
4.1	Summary of experiments performed	78
4.2	Composition of difference formulations varying concentration of NSO in the organic phase	81
4.3	Summary of experiments performed	82
4.4	Contents of different NP formulation	84
5.1	List of NBP formulations	105
5.2	The ingredients for all formulations but varying MW of chitosan and PLGA	105
5.3	Particle size distribution of NBP synthesized with selected molecular weight of PLGA and chitosan	114
5.4	Zeta potentials of all NBPs at pH7	121
5.5	The onset and midpoint of PLGA glass transition for NBPs	124
5.6	The correlation between transfection efficiency and pDNA released profile for 72 h	134
6.1	Ingredients of all formulations with excipients	139
6.2	Glass transition temperature ( $T_g$ ) of all the formulations	149

6.3	The enthalpy of fusion of NBP/Gly mixtures and percentage of crystallinity of the sample	156
-----	--	-----

## LIST OF FIGURES

<u>Figure No.</u>		<u>Page No.</u>
2.1	Illustration of the neurovascular unit/cell association forming the BBB	8
2.2	A schematic diagram illustrating the extracellular and intracellular barriers of gene (i.e naked DNA) delivery	12
2.3	Schematic diagram of converting virus into a vector	14
2.4	Illustration of the gene transfer using chemical vectors	17
2.5	Map of a pGL3 control vector.	20
2.6	Synthetic pathway of poly(D,L-lactide-co-glycolide) (PLGA)	24
2.7	Degradation of poly(D,L-lactide-co-glycolide) (PLGA) chain by hydrolysis	24
2.8	Structure of chitin	26
2.9	Structure of chitosan	26
2.10	A) Schematic representation of the brush-type conformation of adsorbed chitosan chains on PLGA NP; B) Schematic representation of conformations of adsorbed chitosan chains	29
2.11	<i>Nigella sativa</i> plant, flower and seeds	31
2.12	Chemical structure of Thymoquinone, Thymol and Dithymoquinone	32
2.13	HSA is composed of 3 homologous domains, I–III and every domains is divided into subdomains A and B. N and C represent the N-terminal and C-terminal ends, respectively	37
2.14	Steps involved in lyophilisation	40
2.15	(A) Good cake structure ensures proper pore formation which provides the channels for removal; (B) whereas in case of cake collapse, a poor structure is obtained, leading to increased resistance in removal of vapor	41



3.1	A simplified schematic diagram of the amplification process of <i>E. coli</i> DH5- $\alpha$ with pGL3 plasmid	49
3.2	A simplified schematic diagram of the lysate preparation	50
3.3	A simplified schematic diagram of the extraction/purification method of plasmid using PureYield™ Plasmid Maxiprep system and PureLink® HiPure Plasmid Gigaprep	53
3.4	A simplified schematic diagram of the amplification process of <i>E. coli</i> DH5- $\alpha$ with pCMV-Gaussia Luc plasmid using bioreactor	56
3.5	Graph shows the plasmid yield extracted by iCALL method as a function of media volumes, (n=1)	59
3.6	Graph shows pDNA yield extracted by PureYield™ Plasmid Maxiprep system method (150 ml, 850 ml, 1 L and 3.5 L culture volume), (n=1)	60
3.7	Graph shows pDNA extracted by PureLink® HiPure Plasmid Gigaprep (4L and 5 L culture volume), (n=1)	60
3.8	Agarose gel analysis to check integrity (relaxed or supercoiled) of pDNA isolated/purified from three different purification protocols	61
3.9	Graph of pDNA samples from different concentration of lysate extracted/purified using iCALL method	66
3.10	Gel electrophoresis images for pDNA samples from different concentration of lysate extracted/purified using iCALL method	67
3.11	Graph of pDNA extracted from three different culture media and purified either with or without lyophilisation step	70
3.12	Agarose gel analysis of pDNA extracted from three different culture media and purified either with (A) or without lyophilisation (B) steps	71
4.1	The SEM images of PLGA nanoparticles labeled with (A) T1, (B) T2, (C) T3, (D) T4 and (E) T5	88
4.2	The SEM images of PLGA/NSO/CS nanoparticles fabricated by using different concentration of NSO and chitosan in primary emulsion labeled with (A) N1, (B) N2, (C) N3, (D) N4, (E) N5 and (F) N6	91

4.3	Effect of type and concentration of chitosan on zeta potentials of nanoparticles	93
4.4	Effect of PLGA MW and hydrophilicity on zeta potentials of nanoparticles	94
4.5	Effect of chitosan MW on zeta potentials of nanoparticles	94
4.6	Effect of chitosan concentration on zeta potential of nanoparticles	95
4.7	The SEM images of pDNA-Loaded PLGA/Chitosan- <i>N. sativa</i> nanoparticles fabricated	96
4.8	Electrophoresis mobility analyses of pDNA-loaded NPs	97
5.1	Schematic diagrams of diffusion-solvent-evaporation method of NBP synthesis	104
5.2	The SEM images of pDNA-loaded nanospheres fabricated by using different molecular weight of PLGA in primary emulsion labeled with (A) NBP1, (B) NBP2 and (C) NBP3	110
5.3	Illustrations of “Neurobionanoparticles” (NBP)	113
5.4	(A) Particle size distribution of NBPs suspended in deionized water; (B) size distribution of NBP	115
5.5	Comparison of zeta potentials of pDNA-loaded nanospheres	118
5.6	Effect of pH values on zeta potentials for all NBP formulations before lyophilization	119
5.7	Effect of pH values on zeta potentials for all NBP formulations after lyophilization	120
5.8	Electrophoresis mobility analyses of pDNA-loaded nanospheres	122
5.9	DSC thermograms of NBPs	125
5.10	Cumulative data (%) for the release profile of pDNA-loaded nanoparticles plotted as mean values with error bars with standard deviation; n=3	128
5.11	Agarose gel electrophoresis for the particles at 0 hour and after 5 weeks (840 h) release	129

5.12	Cell viability of N2a loaded with NBPs at various concentrations	131
5.13	Transfection of N2a with pGL-3 encapsulated in NBPs	133
6.1	Effect of different types and concentrations of excipients on particle size of NBPs	144
6.2	Effect of different types and concentrations of excipients on zeta potential of NBPs	147
6.3	DSC thermograms of NBP fabricated compared to raw PLGA in second heating	150
6.4	DSC thermograms of excipients: Glycine, Human serum albumin (HAS) and potassium chloride	151
6.5	DSC thermograms of NBP/HSA containing three different weight ratio w/w	153
6.6	DSC thermograms of NBP/Gly containing three different weight ratio w/w	154
6.7	DSC thermograms of NBP/KCl containing three different weight ratio w/w	157
6.8	Effect of different types and concentrations of excipients on transfection efficiency of NBPs	160
6.9	Particle size distribution of NBP-excipients formulation three month post-storage measured	161
6.10	Comparison of zeta potentials of NBP-excipients formulation three month post-storage	162
6.11	Transfection of N2a with pGL-3 encapsulated in NBPs before three (3) months of storage	163
6.12	Agarose gel electrophoresis for the particles three (3) month post-storage	164

## LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
CTAB	Cetyl trimethylammonium bromide
DSC	Differential Scanning Calorimeter
DCM	Dichloromethane
EA	Ethyl acetate
Gly	Glycine
HSA	Human Serum Albumin
iCALL	IIUM Concentrated Alkaline Lysis
KCl	Potassium chloride
MW	Molecular Weight
LMW	Low Molecular Weight
MMW	Medium Molecular Weight
NBP	Neurobionanoparticle
NP	Nanoparticle
NSO	<i>Nigella sativa</i> oil
O.D	Optical density
PBS	Phosphate Buffer Saline
pDNA	Plasmid DNA
PLGA	Poly (D,L-lactide-co-glycolide) acid
PVA	Poly vinyl alcohol
RES	Reticuloendothelial system
SDS	Sodium dodecyl sulphate

S.D	Standard Deviation
SEM	Scanning Electron Microscopy
US FDA	United State Food Drug Administration
w/o	Water-in-oil
w/o/w	Water-in-oil-in-water
IC50	Minimum concentration can inhibit 50% of cell growth
LB Broth	Luria Bertani Broth
TB-P Broth	Terrific with Peptone broth
TB-T Broth	Terrific with Tryptone broth

## LIST OF EQUATIONS

<u>Equation No.</u>		<u>Page No.</u>
Equation 3.1	DNA Concentration ( $\mu\text{g/ml}$ )	62
Equation 3.2	DNA Yield ( $\mu\text{g}$ )	62
Equation 6.1	Percentage of crystallinity	155

## **CHAPTER ONE**

### **INTRODUCTION**

In gene therapy technology nowadays, the significance of the high quality, purified and high yield of the plasmid DNA cannot be underrated (Prazerez, Ferreira, Monteiro, Cooney, and Cabral, 1999). DNA amplification, known as a type of nucleic acid amplification is defined as an increase in the number of copies of a specific DNA fragment into millions of copies through replication process (Mosby's Medical Dictionary, 2009).

Formulation of the drug into a particulate vehicle in drug delivery application is a method to protect the active ingredients from pre-mature degradation when administered in the body. Besides, it can improve the targeting and enhance the therapeutic effect by maximizing the biological activities, controlling the drug release rate and reducing the frequency of administration (Papadimitriou, Papageorgiou, Kanaze, Georgarakis and Bikiaris, 2009). In today's world, development of nano-encapsulation technology has emerged in response to broad medical needs in terms of targeting and therapeutic efficacy (Sundar, Kundu and Kundu, 2010).

Nano-encapsulation is a formulation process in which small materials are enclosed by a coating layer with the size in the range of 10 to 1000 nm, known as nanoparticle. Nanoparticles are made from synthetic or natural polymers (Amiji, 2006; Mohanraj and Chen, 2006) and can be in the form of nanospheres or nanocapsules depending on the preparation process. Nanosphere is one of the colloidal systems that encapsulate the drugs within the matrix of the carrier in which the drugs are dispersed physically and uniformly in the particle. Whereas nanocapsules are systems in which

drug is confined in a cavity surrounded by a polymeric membrane (Tiyaboonchai, 2003; Chia, 2005; Mohanraj and Chen, 2006). The size of the nanoparticles is influenced by various preparation techniques with parameters such as therapeutic agents' concentration, pH, charge ratios and temperatures (Lai and Lin, 2009).

Nanoparticulate delivery systems can improve drug stability and efficiency, prolong the period of therapeutic effect and allow for various routes of administration, which may protect the drug from degradation and metabolism as well as cellular efflux (Mohanraj and Chen, 2006; Sarmiento, Ribeiro, Veiga, Ferreira and Neufeld, 2007; Sundar et. al., 2010). Compared to microparticles, nanoparticles are better suited for intravenous (IV) delivery. The small size of nanoparticles is fundamental for systemic circulation since the smallest capillaries are 5 to 6  $\mu\text{m}$  in diameter. Therefore, the size of particles to be fitted into those capillaries should be smaller than 5  $\mu\text{m}$  so that the formation of an embolism or obstruction of the microvasculature can be reduced during the distribution of particles into the bloodstream (Hans, 2005; Khademhosseini and Langer, 2006).

The aims in designing nanoparticulate system are to obtain therapeutically optimal rate and dose regimen for the action of drugs by controlling particle size, surface properties and release of pharmacologically active agents (Mohanraj and Chen, 2006). From the previous studies, 100 nm polymeric nanoparticles of polyvinylpyrrolidone can be sustained in bloodstream for eight hours after intravenous injection (Gaur et al., 2000). It was also reported that, the action of nanoparticles in vivo is influenced by morphological characteristics, surface chemistry and molecular weight (Bala, Hariharan and Kumar, 2004).

Briefly, here we had attempted to encapsulate the pDNA into biodegradable poly(D,L-lactide-co-glycolide) (PLGA) using diffusion-solvent evaporation technique.