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)* DH5α. 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 'IIUM 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 تحديا في العلاج الجيني نظرا 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 تهدف لتضخيم وعزل pDNA من الإشريكية القولونية DH5α . كنا قد حاولنا أن نشارك في تغليف pDNA تسمى IIUM Concentrated' Alkaline Lysis' (iCALL)سامبروك ورسل بروتوكول تعديلي نأمل الحصول على جودة عالية والعائد البلازميد لدراستنا. ثانيا، تم تغليف pDNA وزيت الحبة السوداءPLGA/ 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، وpDNA الجزيئات PLGA من خلال توظيف متغيرين مستقلين هما، والأوزان الجزيئية لل (34 PLGAو 14 كيلو دالتون) والشيتوزان (50-190 و190-310 كيلو دالتون). وهذه الجسيمات صغيرة تسمى بعد ذلك "Neurobionanoparticles" (NBP) حجم، مورفولوجيا السطح، وإمكانات زيتا، 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 وكلوريد البوتاسيوم مع نسبة مختلفة من NBP الوزن سواغ / نظم على استقرار مجفف بالتجميدNBP 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 زيتا إيجابية. وأظهرت هذه الاستعدادات الأمثل معدلا لإستمرار في اطلاق سراحأكثر من5 أسابيع، وكانت قادرة على التعبير عن pDNA الجينات في Neuro-2a (N2a) خط الخلية. ومن المثيرللاهتمام، تم الحصول على تعبيرات الجين أعلىفي N2a في إعداد (PLGA14 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 جرعةاختبار (0.01، 0.1 و 0.2 ملغ/ مل). وعلاوة على ذلك، تجميد دريد ل NBP مع HSA في نسب 10: 1 و 2: 1 قادرة على تحقيق استقرار الجزيئات لمدة ثلاثة أشهر وعرض أفضل في تعزيز النشاط ترنسفكأيشن مقارنة مع NBP وحده، ومزيج من NBP تخزين مع سواغ أخرى. في الختام، ملفقة NBP يمكن استخدامها اعدة كما توصيل الجينات غير الفيروسي إلىCNS.

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.

Nur ‘Izzati binti Mansor

Signature……. Date….....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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Affirmed by Nur 'Izzati binti Mansor

……..……..…… ……..

Signature Date

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.

I praise Allah for sending His blessings through His chosen messenger, who is the most virtuous of all creatures, the best in characteristics, the most excellent in thoughts; our most beloved Prophet Muhammad, may mercy and peace be upon him and upon his family and companions.

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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	*Nigella sativa* 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

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; Sarmento, 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 μm in diameter. Therefore, the size of particles to be fitted into those capillaries should be smaller than 5 μm 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.