ELECTROSPRAYED CRISPR PLASMID DNA LOADED ALGINATE NANOPARTICLES: PREPARATION, CHARACTERISATION, AND GENE EDITING

BY

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ABSTRACT

Background: CRISPR/ Cas9 is one of the most powerful among the approaches being developed to rescue fundamental causes of gene-based inheritable diseases. Several strategies for delivering such genome editing materials have been developed, but the safety, efficacy over time, cost of production, and gene size limitations are still under debate and must be addressed to further improve applications. Sodium alginate is frequently used as the model encapsulation matrix for bioactive ingredients in the field of drug and gene delivery due to its safety. **Objective**: To encapsulate CRISPR/Cas9 plasmid DNA in alginate nanocarrier to perform genome editing. **Methodology**: alginate nanoparticles loaded with two CRISPR pDNA were fabricated using electrospray method. Both formulation and process were optimised. Chitosan-, Arabic gum- and PEG-coated CRISPR-loaded alginate nanoparticles were fabricated and characterised. CRISPR-loaded alginate nanoparticles physicochemical properties were compared to the surface-modified nanoparticle properties. The influence of surface modification of nanoparticles on their interaction with cell was studied in regard to cellular uptake, cytotoxicity, transfection efficiency, and genome editing. **Results**: Using electrospray, a nanoparticle carrier was developed to deliver CRISPR pDNA into HepG2 cells. The nanoparticles size was approximately 230 nm, with an encapsulation efficiency of 99%. Release study revealed that over one-third of the pDNA was released within the first 24 h. *In vitro* experiments conducted with HepG2 cells demonstrated that after 48 h of treatment with the CRISPR-loaded alginate nanoparticles, the particles were not toxic. CRISPR-loaded alginate nanoparticles mediated 1.5-folds more efficient transfection than a commercially available cationic liposome-based transfection reagent. However, their indel efficiency was 3.4-folds lower than the transfection reagent. The surface coating highly affected the nanoparticles physicochemical properties, consequently, their safety and efficiency in delivering the plasmid. CS CRISPR ALG NPs showed mean size and zeta potential of 377 nm and 33.67 mV, respectively. Over 90% encapsulation efficiency was achieved while protection payload from serum. The tests revealed that the nanoparticles were cytocompatible and successfully introduced the Cas9 transgene in HepG2 cells. CS CRISPR ALG NPs-transfected HepG2 was able to edit its target plasmid by introducing double-strand break (DSB) in GFP gene, 18.26-folds higher than CRISPR ALG NPs. **Conclusions**: In this work, plasmids for the CRISPR/Cas9 system were encapsulated in alginate nanoparticles and were shown to induce expression of Cas9 and perform a genome editing in HepG2 cells *in vitro*. Chitosan-coated CRISPR-loaded alginate nanoparticles revealed the best results with high plasmid protection, sustained release and high indel efficiency. These results suggest that this nanoparticle-based plasmid delivery method can be effective for future *in vivo* applications of the CRISPR/Cas9 system.

خلاصة البحث

خلفية البحث: تعد تقنية CRISPR / Cas9 من أقوى الأساليب التي يتم تطويرها لتخليص الأسباب الأساسية للأمراض الوراثية الجينية. تم تطوير العديد من الاستراتيجيات لايصال مواد التعديل الجيني هذه ، لكن السلامة والفعالية بمرور الوقت وتكلفة الإنتاج والقيود على حجم الجين لا تزال قيد المناقشة ويجب معالجتها لتحسين التطبيقات. كثيرا ما تستخدم ألجينات الصوديوم كقالب تغليف نموذجي للمكونات النشطة بيولوجيا في مجال توصيل الأدوية والجينات بسبب أمنها. **هدف البحث:** لتغليف بلاسميد CRISPR / Cas9 في جسيمات نانوية من الألجينات لإجراء التعديل الجيني. **طريقة البحث:** تم تصنيع الجسيمات الألجينات النانوية المحَملة باثنين من بلاسيدات CRISPR باستخدام طريقة النشر الكهربائي. تم تحسين كل من الصيغة و العملية. تم تصنيع و تمييز جسيمات الألجينات النانوية الملبسة بالشيتوزان والصمغ العربي والبولي اثيلين غليكول. تمت مقارنة الخصائص الخصائص الفيزيائية و الكيميائية للجسيمات النانوية المحملة بـ CRISPR مع خصائص الجسيمات النانوية المعدلة السطح. تمت دراسة تأثير تعديل سطح الجسيمات النانوية على تفاعلها مع الخلية فيما يتعلق بالقبض الخلوي والسمية الخلوية وكفاءة التعداء والتعديل الجيني. **النتائج:** باستخدام النشر الكهربائي ، تم تطوير حامل جسيمات نانوية لتوصيل بلاسميدات CRISPR إلى خلايا HepG2. كان حجم الجسيمات النانوية حوالي 230 نانومتر ، بكفاءة تغليف 99٪. كشفت دراسة التحرر أن أكثر من ثلث الدنا تم إطلاقه خلال الـ 24 ساعة الأولى. أظهرت التجارب المختبرية التي أجريت على خلايا HepG2 أنه بعد 48 ساعة من العلاج بجزيئات الألجينات النانوية المحملة بـ CRISPR ، لم تكن الجسيمات سامة. توسطت جزيئات الألجينات النانوية المحملة بـ CRISPR تعداء أكثر كفاءة ب 1.5 مرة من كاشف التعداء الشحمي الموجب المتاح تجاريًا. على أية حال ، كانت كفاءتهم للتعديل الجيني أقل بمقدار 3.4 أضعاف من كاشف التعداء. أثر تلبيس السطح بشكل كبير على الخصائص الفيزيائية و الكيميائية للجسيمات النانوية ، وبالتالي على سلامتها وكفاءتها في توصيل البلاسميد. أظهر جسيمات الألجينات النانوية الملبسة بالشيتوزان حجم وسطي و قيمة زيتا 377 نانومتر و 33.67 مللي فولت ، على التوالي. تم تحقيق أكثر من 90٪ من كفاءة التغليف بالإضافة لحماية الحمولة من المصل. كشفت الاختبارات أن الجسيمات النانوية متوافقة مع الخلايا ونجحت في إدخال الجين المعدل Cas9 إلى خلايا HepG2. كان خلايا HepG2 الحاوية على جسيمات الألجينات النانوية الملبسة بالشيتوزان قادرة على تعديل البلاسميد المستهدف من خلال إدخال كسر مزدوج الجديلة (DSB) في جين GFP ، أعلى بمقدار 18.26 ضعفًا من جسيمات الألجينات النانوية. **الخلاصة:** في هذا العمل ، تم تغليف البلاسميدات لنظام CRISPR / Cas9 في جسيمات ألجينات نانوية وتبين أنها تحفز التعبير عن Cas9 وتقوم بتحرير الجينوم في خلايا HepG2 في المختبر. كشفت جسيمات الألجينات النانوية الملبسة بالشيتوزان و المحملة بـ CRISPR أفضل النتائج مع حماية عالية للبلاسميد ، وإطلاق مستمر وكفاءة عالية للتعديل الجيني.تشير هذه النتائج إلى أن طريقة توصيل البلاسميد القائمة على الجسيمات النانوية يمكن أن تكون فعالة للتطبيقات المستقبلية في الجسم الحي لنظام CRISPR / Cas9.

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 in 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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“If I were to have kids someday, this is for them. I want them to realise that once, their mum had a dream like they do”

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LIST OF ABBREVIATIONS

AGE	Agarose gel electrophoresis
ALG	Alginate
ANOVA	Analysis of variance
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
bp	Base bair
Cas	CRISPR Associated protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPR ALG NPs	Alginate naoparticles loaded with two CRISPR plasmid DNA
crRNA	CRISPR RNA
CS	Chitosan
CS CRISPR ALG NPs	Alginate naoparticles loaded with two CRISPR plasmid DNA and coated with chitosan
DHDA	Electrohydrodynamic atomisation
Di 50	Median diameter
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOTAP	1,2-dioleoyl-3-trimethylammoniumpropane
DSB	Double Strand Break
E coli	*Escherichia coli*
EE	Encapsulation Efficiency
FBS	Fetal bovine serum
G	α-L-guluronic acid
GA	Arabic gum
GA CRISPR ALG NPs	Alginate naoparticles loaded with two CRISPR plasmid DNA and coated with arabic gum
GFP	Green Fluorescent Protein
gRNA	Guide Ribonucleic Acid
h	Hour
HepG2	Hepatocellular carcinoma
In vitro	Research or work is done in the glass
In vivo	Research or work is done with or within an entire, living organism.
INDELs	Insertions/Deletions
kb	kilobases
kDa	Kilo Datlton
Kg	Kilo gram
kV	Kilovolt
LB	Luria-Bertani
M	β-D-mannuronic acid
m^3	Meter cube
min	Minute
mPa.	Mili pascal
mRNA	Massenger Ribonuclic Acid
ms	Mili second

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
N	Newton
NHEJ	Non-Houmolgous End Joinung
nm	Nanometer
NPs	Nanoparticles
o/w	Oil-in-water
PAM	Protospacer Adjacent Motif
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PDI	Poly dispersity index
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEG CRISPR ALG NPs	Alginate naoparticles loaded with two CRISPR plasmid DNA and coated with polyethelene glycol
PLGA	Poly(lactic-co-glycolic acid)
RFP	Red Fluorescent Protein
RNA	Ribonuclic Acid
s	Second
S	Solution
*Sa*Cas9	Cas9 derived from *Staphylococcus aureus*
SD	Standard Deviation
*Sp*Cas9	Cas9 derived from *Streptococcus pyrogene*
T7EI	T7 endonuclease I
TAE	Tris acetate edetate
TEM	Transmission Electron Microscope
TR	Transfection Reagent
tracerRNA	Trans CRISPR RNA
V	Volt
v/v	The amount by volume of a solid substance dissolved in a known amount (by volume) of liquid
w/o	Water-in-oil
w/v	The amount by weight of a solid substance dissolved in a known amount (by volume) of liquid

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

The emergence of the clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR associated protein (Cas) genome editing system has brought much excitement to the field of gene therapy and the larger scientific community. The application of CRISPR-based technologies seems to be endless, ranging from increasing crop resistance to curing genetic diseases previously considered untreatable, providing a flexible approach to high-fidelity gene editing. Nevertheless, in order for CRISPR-based therapies to be translated to *in vivo* application, there is an urgent need to develop optimised vectors for their delivery. The delivery vector is a crucial determinant of the therapeutic efficacy of gene editing and should be designed to accommodate various factors including the form of the payload, and the physiological environment. The large majority of CRISPR plasmid therapy has been based on the use of viral vectors (Ding *et al*., 2014; Ran *et al*., 2015; Kaminski *et al*., 2016; Long *et al*., 2016; Nelson *et al*., 2016). However, several drawbacks have been associated with their delivery, among these: immunogenicity (Wang *et al*., 2015), carcinogenesis (Donahue *et al*., 1992), and limited packaging size (Ran *et al*., 2015). Non-viral gene delivery systems were developed with potential to overcome these limitations; nevertheless, nanoparticle delivery is still challenged with difficulty in scale-up manufacturing and low gene delivery efficiency compared to viral delivery. Recently, biomaterials have become an attractive option for the delivery of Cas9 due to their tunability, biocompatibility and increasing efficacy at drug delivery. Biomaterials offer a unique

solution for creating tailored vectors to meet the demands of various applications that cannot be easily met by other delivery methods. In this study, we optimise a delivery carrier for CRISPR/Cas9 system in its plasmid form. Therefore, two CRISPR plasmid DNA were encapsulated into alginate nanoparticles using electrospray method. The optimisation of the formulation and process variables were done using a statistical approach. The nanoparticles were characterised in terms of their physicochemical characteristics. In addition, functionalisation of the surface of CRISPR plasmid DNA-loaded alginate nanoparticles (CRISPR ALG NPs) was done using three polymers (chitosan, Arabic gum, and polyethylene glycol) to understand their roles in carrier properties. Finally, the influence of surface functionalisation in the safety and efficiency of the carrier in CRISPR/Cas9 delivery was evaluated.

This project will help to understand the relationship between the nanoparticle formulation and the efficacy of gene editing and ultimately guide the field in designing new delivery systems that can lead to develop the best formulation serves as a carrier for the clinical applications of CRISPR/Cas9.

1.2 PROBLEM STATEMENT

In recent decades, gene editing CRISPR/Cas systems have become a platform in biomedical research due to its high potential for treating viral infections (Schwank *et al*., 2013). Many benefits were utilised from CRISPR/Cas systems *in vitro*. However, the delivery system remains the main challenge for robust implementation of CRISPR system *in vivo*. Multiple methods are seen for delivering *in vitro*, which are usually not compatible or unsafe for *in vivo* use (Wang *et al*., 2017). Polymeric nanoparticles are alternative delivery vector that can be prepared from biocompatible materials. Alginate nanoparticles appeared to be an excellent candidate' for polymeric carrier to deliver

CRISPR plasmids. Nucleic acid and protein have been successfully encapsulated in alginate micro and nanoparticle (You & Peng, 2005; Tahtat *et al*., 2013; Mukhopadhyay *et al*., 2015; Zhang *et al*., 2016). However, to translate the gene editing into a therapeutic benefit, the maximum gene editing efficacy should be attempted. Up until this day, there is still a lack of research that investigates the effect of the nanoparticle properties onto their efficacy of the gene editing using CRISPR system (Tao *et al*., 2019).

1.3 RESEARCH AIM AND OBJECTIVES

This research aims to develop and optimise a carrier for two CRISPR plasmid DNA by an easy and applicable method with special emphasis on safety and efficiency of the carrier. Based on this aim, the objectives of the proposed project are summarised as follows:

i. To optimise and characterise alginate nanoparticles to encapsulate CRISPR plasmid DNA using electrospray technique.
ii. To optimise the surface coating of CRISPR-loaded alginate nanoparticles *via* applying different coating molecule
iii. To investigate the effect of surface coating on the nanoparticle's physicochemical properties.
iv. To evaluate *in vitro* cellular uptake, cytotoxicity, transfection efficiency, and genome editing efficiency of the CRISPR-loaded alginate nanoparticles.
v. To investigate how different surface coating of the nanoparticles affects their genome editing efficiency.