

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※

※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※

※※ 超音波對於提昇非病毒性輸送基因載體於細胞中轉染效率之研究 ※※

※※ **Transfection enhancement of non-viral-based gene delivery systems by ultrasound** ※※

※※ **mediated transfectants** ※※

※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：個別型計畫 整合型計畫

計畫編號：NSC 90-2320-B-041-006-

執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：程中玉

共同主持人：蕭明達

計畫參與人員：陳曉恩，王佩琪

本成果報告包括以下應繳交之附件：

- 赴國外出差或研習心得報告一份
- 赴大陸地區出差或研習心得報告一份
- 出席國際學術會議心得報告及發表之論文各一份
- 國際合作研究計畫國外研究報告書一份

執行單位：嘉南藥理科技大學醫藥化學系

中華民國九十一年十月二十三日

行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 90-2320-B-041-006-

執行期限：90 年 8 月 1 日至 91 年 7 月 31 日

主持人：程中玉 嘉南藥理科技大學醫藥化學系

共同主持人：蕭明達 嘉南藥理科技大學

計畫參與人員：陳曉恩，王佩琪 嘉南藥理科技大學醫藥化學系

中文摘要

微脂粒及高分子聚合物-DNA 複合體可提高其轉染效率，但仍有改善空間，超音波之孔洞化效應可促使細胞膜穿透性提高，外來之大分子可穿過此障礙進入細胞內，並且超音波能量有助於外來之複合體在進入細胞中能脫離 endosomes，到達細胞核進而提高轉染效率，超音波在臨床之實用性比起其他物理性方法(基因槍、電穿孔)較佳，並且其對細胞之衝擊損壞性較小。超音波之操作變數(如頻率、波形、功率、施加時間等)之控制為重要之影響轉染效率因素，過高之能量會降低細胞之存活率。在超音波最適化操作範圍下，觀察超音波對”naked” DNA 之物理化學完整性及生物活性之影響，並與高分子-DNA 複合體、微脂粒-DNA 複合體比較及進行”naked”DNA 對體外細胞之轉染，測量轉染效率及細胞存活率。再進行高分子-DNA 複合體(polylysine, PEI, or chitosan)、微脂粒-DNA 複合體對體外細胞轉染，測量轉染效率及細胞存活率。結果發現超音波改善目前以非病毒性載體輸送 DNA 之較低基因轉染效果，亦可以應用於未來在臨床實驗上合併醫用超音波來進行基因治療。

關鍵詞：DNA 轉染；非病毒載體；高分子聚合物；微脂粒；超音波

Abstract

The improvement of transfection efficiency for nonviral vectors will be the key role to achieve successful gene therapy. The widely used nonviral vectors are polymers and liposomes. The entry into cell membrane by plasmid DNA alone is difficult due to its size and electrical repulsion. In principles, the cationic polymers or liposomes will interact with negatively charged plasmid DNA and condensed to net positively small complexes. The resulting complexes can be attracted by negative cell membrane , entered the cell via endocytosis ,and transported into nucleus for gene expression. The transfection efficiency of nonviral vectors may be further improved by exposure to ultrasound. The cavitation created by ultrasound can induce transient permeabilization of cell membranes, which leads to the uptake of complexes into the cell. Also, the ultrasound energy may release endosomally trapped complexes and forced it into the nucleus. The little cell damages and clinical practicality make ultrasound technique more promising than other physical methods such as electroporation and gene gun. The transfection efficiency will be affected by ultrasound operating factors such as frequency, wave mode, intensity, and

treatment time. The higher ultrasound energy will reduce cell survival. In this project, for the first time the relationship between cell transfection efficiency of polymer/DNA complexes and ultrasound operating factors was studied. The synergistic effect of optimal ultrasound conditions and transfection efficiency of polymer/DNA complexes was demonstrated

Keywords: DNA transfection ; non-viral ;
polymer ; liposome ; ultrasound,

Introduction

In 1992, Ray and Gage [1] 比較 calcium phosphate precipitation, lipofection 及 electroporation 對 rate skin 取出之 primary fibroblast 細胞對 40 μ g/ml plasmid DNA 之體外轉染效率，上述三種方法之穩定轉染效率均小於 0.1%。然而，Kim et. [2] 使用超音波在 1 MHz, 4 bars, 30 sec 條件下，獲得 0.34% 穩定轉染效率，較 Ray and Gage 之方法為高，並且無論是懸浮或附著式細胞，均可在施加超音波條件下，獲得轉染。Bao et al.,[3] 觀察到在超音波(2.55 MHz, 0.1 MPa, 1 min)作用於體外之 Chinese hamster ovary 細胞，高分子量 fluorescent dextran 分子(Mw=580,000 Da)可穿透細胞膜，並保持原有細胞膜構造，然而壓力強度升至 0.2 MPa 以上，不可逆之 cell lysis 出現，並伴隨壓力強度增加而升高。當超音波施加於有添加 serum 條件下轉染 plasmid DNA 所得到之轉染效率較無添加 serum 條件下為高，一般文獻中提到 serum 之存在會降低 DNA 轉染效率，而當 serum 存在下，超音波可對轉染效率反而有增進作用。超音波本身波動變數，如波形方式 continuous wave (CW) 或 pulse mode 頻率、功率等，對不同細胞之轉染效率有顯著影響。Tata et al.,[4] 觀察在總能量固定(328.5J)下，改變頻率、功率及波形方式下 green fluorescent protein(GFP) reporter 基因在體外對兩種人類 prostate cancer 細胞(LnCap 及 pc-3)之轉染表現效率不同，低頻

(<20kHz)，低功率(0.33W/cm^2)，pulse mode 對 LnCap 及 pc-3 之轉染效率較佳，而 LnCap 較 pc-3 細胞對超音波之反應靈感性較高，一般低頻較高頻對體內軟組織之穿透深度較深，在臨床體內之目標鎖定上較具彈性，另外，由超音波引發之孔洞化產生對轉染效率亦有顯著效應。Greenleaf et. [5] 報告外加人工孔洞引發劑(albumin 小顆粒)於超音波體外，lipofection 轉染於人類 chondrocytes 細胞過程中，存活之細胞可高達 45% 轉染效率相對於 15% 在無添加孔洞引發劑轉染過程。過高之 Albumin 濃度，會造成細胞存活率過低。文獻中提到微脂粒轉染過程中，微脂粒進入細胞膜後大部分被 endosomes 包圍困住，並被分解，因而導致較低的轉染效率[6]，而超音波除了幫助微脂粒通過細胞膜外，有可能超音波的能量有助於把困在 endosomes 脂粒釋放，進而到達細胞核表現基因。[7] 最近 Koch et al.[8] 使用醫療用 2 MHz pulsed Dopper US 儀器，在 60 sec 操作下可增進微脂粒-DNA 複合體對體外人類腦瘤細胞之轉染效率，並無任何細胞死亡。超音波之臨床實用性更進一步被確認。

我們在先前之國科會計劃中(1999-2000)，各項高分子聚合物-DNA 之結構因素對細胞轉染之影響已充分被了解，，非病毒性載體之 DNA 轉染效率遠較病毒性載體為低，雖然微脂粒及高分子聚合物-DNA 複合體可提高其轉染效率，但仍有改善空間，超音波之孔洞化效可促使細胞膜穿透性提高，外來之大分子可穿過此障礙進入細胞內，並且超音波能量有助於外來之複合體在進入細胞中能脫離 endosomes，到達細胞核進而提高轉染效率，超音波在臨床之實用性比起其他物理性方法(基因槍、電穿孔)較佳，並且其對細胞之衝擊損壞性較小。超音波之操作變數(如頻率、波形、功率、施加時間等)之控制為重要之影響轉染效率因素，過高之能量會降低細胞之存活率。，故本專題擬就超音波對高分子聚合物-DNA 複合體細胞轉染之各項控制變數(頻率、功率、施加時間等)作更進一步之研究，以期掌握最適化之操作條件，使得超音波與高分子聚合物-DNA 複合體之結合對細胞轉染效率達到”

相乘”(synergetic)之提高效果。

Results and Discussion

1. 陽電性聚合物高分子的存在對 DNA 結構的影響：Cationic polymer affects both the packing behavior and the topological state of plasmid DNA. On the basis of the results, the interaction in formation of cationic polymer-plasmid DNA complexes constrains the molecular motions for both cationic polymer and plasmid DNA. 如圖 1

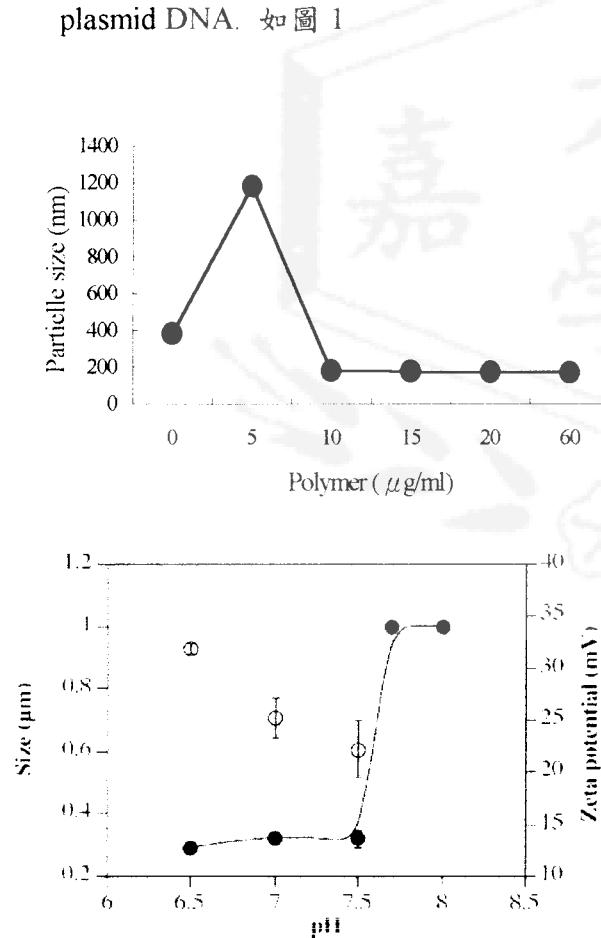


圖 1: Effect of pH on the size (upper) and zeta-potential of polymer/DNA complexes

2. 複合體的粒子大小與攜帶 DNA 之轉染效率成正比，而與攜帶之正電荷成正比，但有一最適當值(optimal value)。The transfection study with ultrasonics was performed at the polymer/DNA ratio which shows the best transfection efficiency。如圖 2

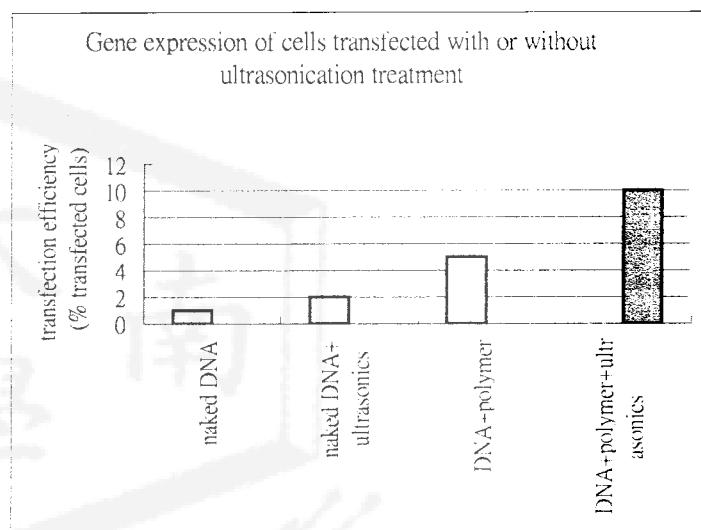


圖 2: Gene expression of cells transfected with pCMV-lacZ gene in absence or presence of ultrasonic treatments.

3. 討論：This study is aimed to investigate experimentally that ultrasound whether or not is effective in enhancing the gene expression of plasmid DNA. The results confirm that after treatment of ultrasonics, transfection efficiency of nonviral vectors was further improved by exposure to ultrasound. The cavitation created by ultrasound can induce transient permeabilization of cell membranes, which leads to the uptake of complexes

into the cell. Also, the ultrasound energy may release endosomally trapped complexes and forced it into the nucleus. The little cell damages and clinical practicality make ultrasound technique more promising than other physical methods such as electroporation and gene gun. The transfection efficiency was affected by ultrasound operating factors such as frequency, wave mode, intensity, and treatment time. The higher ultrasound energy will reduce cell survival. The synergistic effect of optimal ultrasound conditions and transfection efficiency of polymer/DNA complexes may be very useful for clinical gene delivery.

4. 在超音波最適化操作範圍下，超音波對 naked DNA 之物理化學完整性及生物活性有少許之影響。進行”naked”DNA 對體外細胞之轉染，測量轉染效率及細胞存活率得到效率並不高。但再進行高分子聚合物-DNA 複合體(polylysine, PEI, or chitosan)、微脂粒-DNA 複合體(liposome)及超音波處理則對體外細胞轉染，測量轉染效率及細胞存活率有提升。如此不但可以期望改善目前以非病毒性載體輸送 DNA 之較低基因轉染效果，亦可以應用於未來在臨床實驗上合併醫用超音波來進行基因治療

References

- [1]. J. Ray and F. H. Gage, Gene transfer into established and primary fibroblast cell lines: comparison of transfection methods and promoters, *BioTechniques* 13, 598-603, 1992
- [2]. H. J. Kim, J.F. greenleaf, et al., Ultrasound-mediated transfection of mammalian cells, *Human gene therapy* 7, 1339-1346, (1996)
- [3]. S. Bao, B.D. Thrall, et al., Transfection of a reporter plasmid into cultured cells by sonoporation in vitro, *Ultrasound in Med. & Biol.* 23, No. 6, 953-959, 1997.
- [4]. D.B. Tata, F. Dunn, et al., Selective clinical ultrasound signals mediate differential gene transfer and expression in two human prostate cancer cell lines: LnCap and PC-3, *Biochemical and biophysical research communications* 234, 64-67, 1997
- [5]. W.J. Greenleaf, M.E. Bolander, et al., Artificial cavitation nuclei significantly enhance acoustically induced cell transfection, *Ultrasound in Med. & Biol.* 24, No.4, 587-595, 1998
- [6]. J. Zabner , A.J. Fasbender, et al., Cellular and molecular barries to gene transfer by a cationic lipid, *J. Biol. Chem.* 270, 18997-19007, 1995
- [7]. E.C. Unger, T.P. McCreery, et al., Ultrasound enhances gene expression of liposomal transfection, *Invest. Radiol.* 32, 723-727, 1997.
- [8]. S. Koch, P. Pohl, et al., Ultrasound enhancement of liposome-mediated cell transfection is caused by cavitation effects, *Ultrasound in Med. & Biol.* 26, No.5, 897-903, 2000