行政院國家科學委員會補助專題研究計畫成果報告

研究 DNA 於細胞中轉染與輸送基因載體結構間之關聯性

A correlative study of DNA transfection and polycation-mediated transfectants

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一. 中文計畫摘要:

本計劃專注於高分子載體系統的探討。高分子系統以利用陽離子性高分子和帶負電伸展之 DNA 形成緊縮之複合體，並利用多餘之淨正電荷與負電荷細胞膜互相結合，進而達成基因傳送效果。目前高分子以 poly(L-lysine), poly(2-dimethylamino)ethyl methacrylate) (P(DMAEMA)) , polyethylenimine(PEI)等最常使用。目標細胞選擇，高分子分子量，高分子/DNA 複合混合比例，pH，複合體強度，粒徑大小，分佈範圍，結構型態，均勻性，穩定度，DNA 均足以影響 DNA 轉染效率，而目前文獻對於此關連性仍待建立，而其中最關鍵之角色，即為多聚陽離子本身之材料特性。故本計劃評估高分子載體系統，尋求製備之最適化條件，以設計安全且有效率之基因傳送系統。本研究成果在於複合體物理/化學特性量測分析及以熱分析 DNA 與高分子之間的交互作用和結構的變化。

二. 英文計畫摘要:

This project focus on polymeric carrier systems. In polymer systems, negatively extended structure of DNA is attached to the surfaces of polycations and formed small, tightly packed complexes. The synthetic carrier/DNA complexes are positively charged leading to cell binding and furthermore gene transfer. Poly(L-lysine) , poly(2-dimethylamino)ethyl methacrylate)(P(DMAEMA) , polyethylenimine(PEI) are the commonly used polymers for gene carrier systems. Target cell moiety choice, molecular weight of polymers, mixing ratio of carrier and DNA, pH, ionic strength, particle size and distribution, morphology, homogeneity and stability of complexes, and DNA type and size are considerable factors affecting gene transfection efficiency. At this moment, literatures still cannot provide these correlations for gene delivery efficiency. Among these factors, the most important key role will be material characteristic of polymers. The accomplished efforts of this project are: physical / chemical characterization of DNA/ polymer complexes and thermodynamic study of cationic polymer-plasmid DNA complexes by high-sensitive differential scanning calorimetry and FTIR.

三. 計畫緣由與目的:

伴隨著基因科學近年快速進展，基因療法被寄以厚望。[1] 基因治療(gene therapy)是指使用分子生物學中 DNA 重組以及轉殖(染)(transfection)的技術，將重組後的 DNA 分子傳遞至一個生物體的細胞內，把帶有遺傳性、新陳代謝或癌症等的致病基因來加以修補或置換，使其恢復正常功能；或藉輸入重組的正常基因來替代已喪失功能的基因以製造必要之產物。[2-7] DNA 是一個大分子，其攜帶的質體(plasmid)(包含治療性的基因以及在細胞中此基因的調控序列)大小約為 0.4 μm 以上，此大小的分子要進入細胞本屬不易，而且 DNA 上攜帶的負電荷使得其更難以通過同為負電荷之細胞膜。 所以尋找一個具有安全性而且有效率(efficient)的基因載體(gene vectors；transfectant)來能夠將治療性重組 DNA 送至標的細胞。 尋找具有安全性、效率性的載體是目前發展基因治療的關鍵。 基因載體可分為：病

毒(viral carrier)及非病毒性(nonviral carrier)。[8-16] 病毒性載體如反轉錄病毒(retrovirus)、腺病毒(adenovirus)等，雖然其體內(in vivo)傳送效率較佳，但可隨機嵌入染色體形成突變；與體內潛伏病毒重組轉成致病性病毒引發免疫反應(immunogenic reaction)；細胞培育(cell culture)可再製性(reproducibility)較難掌握以至於難以大規模製造等因素，使得許多研究轉向於非病毒性載體或"人工病毒"計劃。非病毒性載體系統乃根據基因原理以系統化藥物配方製造，以達成可再製、安全之基因製劑。質體或純化 DNA 與載體形成錯合體透過體內傳送方式轉染(transfection) DNA 於目標細胞，並在一段時間後表現出特定蛋白質。 除了擁有無毒、易製備，可傳送較大基因等優點外，其在細胞內非永久性表現(transient expression)之特性；故投藥劑量及頻率較為彈性，對於癌細胞、非繁殖性(nonproliferative)細胞（肝、中樞神經系統(CNS)等）之基因治療，非常適合此短時效之特性。 由藥物動力學觀點，DNA 複合體在體內之分布特性必須充分了解，才能設計出有效率、安全之非病毒性基因傳送系統。 考慮其較差之穿透性(permeability)，一般用藥途徑侷限於器官間隙內進行以逃避多重之屏障(barrier)效應。全身或局部注射為最常採用送藥方式。理想中，非病毒載體首先須擁有目標細胞性(cell-targeting)，與細胞表面接合，繼而行核內體釋放(endosomal release)，並親近細胞核(nuclear localization)，最後展現基因。除了考慮傳統之藥物動力分布，基因在體內之內在動力(intrinsic kinetics)特性如 DNA 之半生期，細胞內/外分布、細胞攝取複合體速率、核內體 DNA 釋放速率、轉錄、轉譯速率亦影響基因傳送速率。[17] 一般以體外轉染細胞試驗作為基礎，以進一步設計體內傳送特性。早期 DNA 轉染方式有直接注射(direct injection)、電極法(electroporation)，$CaPO_4$ 沈澱法，Diethyl aminoethyl-dextran(DEAE-dextran)等物理/化學方式，但轉染效率非常低，對細胞傷害大。[18-21] 目前則應用"人工病毒"理念，發展出微脂粒(liposomes)及高分子(polymers)載體系統，利用其與負電荷 DNA 形成離子作用力來傳送基因。初步顯示傳送效率有顯著改善。 微脂粒載體系統 DNA 被包覆於微脂粒中，以避免酵素分解，並利用帶正電荷表面與負電荷細胞膜接近，並避免被清除接受器(scavenger receptor)確認。通常在體液中之穩定性及網狀內皮(reticuloendothelia)系統(RES)吞噬為必須克服之問題。[22-25] 本計劃將專注於高分子載體系統之探討。高分子系統乃利用多聚陽離子(polycation)和帶負電伸展之 DNA 分子形成緊縮之複合體(complexes)(大小約 100nm)，並利用多聚陽離子之淨正電荷和細胞膜表面互相結合，Endocytosis，進而造成傳送效果。目前常用之多聚陽離子為 poly(L-lysine)(PLL)，poly(2-dimethylamino)ethyl methacrylate)(P(DMAEMA), polyethyenimine(PEI)。 目標細胞選擇，高分子分子量，高分子配位體結合比率，高分子/DNA 混合比，pH，DNA 複合體離子強度，粒徑大小，分佈範圍，結構型態(morphology)，均勻性，穩定度、DNA 種類、大小等為控制變數。[26-32] 截至目前，複合體進入細胞膜、細胞核的可能機轉及攜帶 DNA 在細胞核內表現與上述變數間之關連性仍待建立，而體內傳送效率之障礙因素如 DNA 緊密度、複合體粒徑大小、用藥用途、對核酵素(nuclease)之穩定度、目標細胞、體內分布、接受器運作功能、細胞內交流(intracelluar trafficking)仍有改善空間，最重要之差異性應由載體本身材料特性所引起，故本計劃擬開發多聚陽離子載體系統，以期建立較佳之基因傳遞效率。

參考文獻：

[1]. N. Wivel. Human gene transfer trials. *Adv. Drug Del. Rev.* **17**, 211-212 (1995).

[2]. D. L. Gill, K. W. Southern, K. A. Mofford et al. A placebo-controlled study of liposome -mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **4**, 199-209 (1997).

[3]. A. Porgador, R. Bannerji, Y. Watanabe et al. Antimetastatic vaccination of tumor-bearing mice with two types of IFN-gamma gene-inserted tumor cells. *J. Immunol.* **150**, 1458-1470 (1993).

[4]. F. M. Rosenthal, K. Cronin, R. Bannerji et al. Augmentation of antitumor immunity by tumor cells transduced with a retroviral vector carrying the interleukin-2 and interferon-gamma cDNAs. *Blood* **83**, 1289-1298 (1994).

[5]. E. M. Elder, M. T. Lotze, and T. L. Whiteside. Successful culture and selection of cytokine gene-modified human dermal fibroblasts for the biologic therapy of patients with cancer. *Hum. Gene Ther.* 7, 479-487 (1996).

[6]. Y. Tan, M. Xu, W. Wang et al. IL-2 gene therapy of advanced lung cancer patients. *Anticancer Res.* **16**, 1993-1998 (1996).

[7]. W. Z. Abdel, C. Weltz, D. Hester et al. A phase I clinical trial of immunotherapy with interferon-gamma gene-modified autologous melanoma cells: Monitoring the humoral immune response. *Cancer* **80**, 401-412 (1997).

[8]. J. -Y. Cherng, P. van de Wetering, H. Talsma et al. Effect of size and serum proteins on transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. *Pharm. Res.* **13**, 1038-1042 (1996).

[9]. S. P. Squinto, S. A. Rollins, J. P. Springhorn et al. Injectable retroviral particles for human gene therapy. *Adv. Drug Del. Rev.* 17, 213-226 (1995).

[10]. D. Jolly. Viral systems for gene therapy. *Cancer Gene Ther.* **1**, 51-64 (1994).

[11]. S. Yei, N. Mittereder, K. Tang et al. Adenovirus-mediated gene transfer for cystic fibrosis: Quantitative evaluation of repeated in vivo vector administration to the lung. *Gene Ther.* **1**, 192-200 (1994).

[12]. S. Yei, N. Mittereder, S. Wert et al. In vivo evaluation of the safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lung. *Hum. Gene. Ther.* **5**, 731-744 (1994).

[13]. P. Lehn, S. Fabrega, N. Oudrhiri et al. Gene delivery systems: Bridging the gap between recombinant viruses and artificial vectors. *Adv. Drug Del. Rev.* **30**, 5-11 (1998).

[14]. J. -P. Behr. Gene transfer with synthetic cationic amphiphiles: Prospects for gene therapy. *Bioconjugate Chem.* **5**, 382-389 (1994).

[15]. C. P. Hodgson. The vector void in gene therapy. *Bio/Technology* **13**, 222-225 (1995).

[16]. J. Zabner, A. J. Fasbender, T. Moninger, K. A. Poellinger, and M. J. Welsh. Cellular

and molecular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270**, 18997-19007 (1995).

[17]. M. J. Poznanski, and R. L. Juliano. Biological approaches to the controlled delivery of drugs: A critical review. *Pharmacol. Rev.* **36**, 277-336 (1984).

[18]. M. R. Capecchi. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**, 479-488 (1980).

[19]. G. Chu, H. Hayakawa, and P. Berg. Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* **15**, 1311-1326 (1987).

[20]. N. S. Yang, J. Burkholder, B. Roberts et al. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc. Natl. Acad. Sci. USA* **87**, 9568-9572 (1990).

[21]. A. Loyter, G. Scangos, D. Juricek et al. Mechanisms of DNA entry into mammalian cells. II. Phagocytosis of calcium phosphate DNA co-precipitate visualized by electron microscopy. *Exp. Cell Res.* **139**, 223-234 (1982).

[22]. X. Gao, and L. Huang. Cationic liposomes and polymers for gene transfer. *J. Liposome Res.* **3**, 17-30 (1993).

[23]. A. P. Rupprecht, and D. L. Coleman. Transfection of adherent murine peritoneal macrophages with a reporter gene using DEAE-dextran. *J. Immunol. Methods* **144**, 157-163 (1991).

[24]. J.-P. Leonetti, G. Degols, and B. Lebleu. Biological activity of oligonucleotide-poly-L-lysine conjugates: Mechanism of cell uptake. *Bioconjugate Chem.* **1**, 149-153 (1990).

[25]. R. L. Page, S. P. Butler, A. Subramanian et al. Transgenesis in mice by cytoplasmic injection of polylysine/DNA complexes. *Transgenic Res.* **4**, 353-360 (1995).

[26]. S. Kawai, and M. Nishizawa. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell Biol.* **4**, 1172-1174 (1984).

[27]. Y. Dong, A. I. Skoultchi, and J. W. Polland. Efficient DNA transfection of quiescent mammalian cells using poly-L-ornithine. *Nucleic Acids Res.* **21**, 771-772 (1993).

[28]. J. Haensler, and F. C. Szoka, Jr. Polyaminoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem.* **4**, 372-379 (1993).

[29]. O. Boussif, F. Lezoualc'h, M. A. Zanta et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92**, 7297-7301 (1995).

[30]. S. Walker, M. J. Sofia, and H. R. Axelrod. Chemistry and cellular aspects of cationic facial amphiphiles. *Adv. Drug Del. Rev.* **30**, 61-71 (1998).

[31]. D. Balicki, and E. Beutler. Histone H2A significantly enhances in vitro DNA transfection. *Mol. Med.* **3**, 782-787 (1997).

[32]. M. S. Wadhwa, W. T. Collard, R. C. Adami et al. Peptide-mediated gene delivery: Influence of peptide structure on gene expression. *Bioconjugate Chem.* **8**, 81-88 (1997).

四. 結果與討論:

1. 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 Fig. 1. MicroDSC thermal diagrams of 150μg DNA (A), 450μg pDMAEMA (B) and their formed pDMAEMA-DNA complexes (C) in the first heating process (at a heating rate 1 ℃/min from 20 to 110 ℃).
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 thermal study was performed at the polymer/DNA ratio which shows the best transfection efficiency。如圖 2
3. 討論：For the formation of the cationic polymer-plasmid DNA complexes, conformational changes of plasmid DNA were constrained by the presence of electrostatic interactions. From the judgment of no alternation of thermodynamic data, the cationic polymer can be identified as reversible. The plasmid DNA was not thermally reversible for smaller denaturation peak and disappearance of conformational changes which are corresponded to the mismatched between DNA base-pairs and change in packing behavior, respectively. As for the cationic polymer-plasmid DNA complexes, electrostatic forces led to higher denaturation temperature of plasmid DNA. The thermal behavior of polymer-DNA complexes is not reversible because the changed DNA topology results in the change in complexation capacity. Consequently, electrostatic interactions between the cationic polymer and plasmid DNA had dramatically influences on the thermal processes.

五. 計劃成果自評:

本研究提供對非病毒性載體高分子如何與 DNA 分子結合而成的複合體的了解及非病毒性載體的結構和載體/DNA 之間組成對於形成複合體時作用力的關係；如此不但可以 (1) 進一步了解非病毒載體之化學結構對其 DNA 轉染效率之間的關聯性，在(2) 設計安全且具好的基因轉染效果之載體提供了一項快速且有效的分析方式； (3) 改善目前在設計、製備非病毒性載體上採取的 trial and error 策略以及 DNA/載體複合體結構改變。

Fig. 1A: MicroDSC thermal diagrams

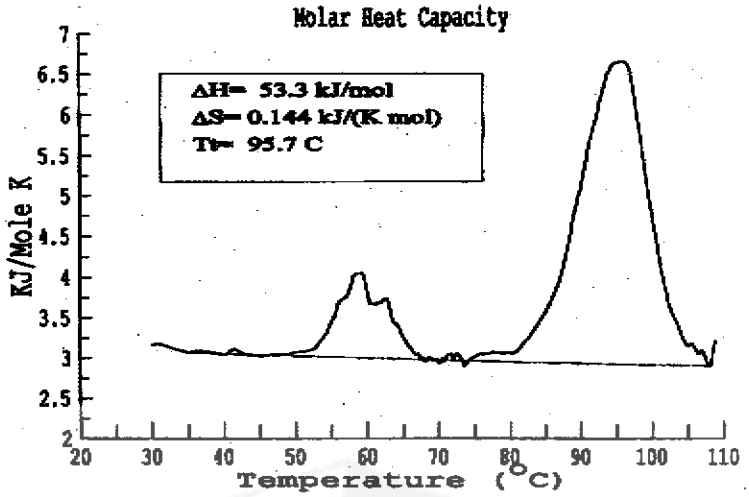


Fig. 1B

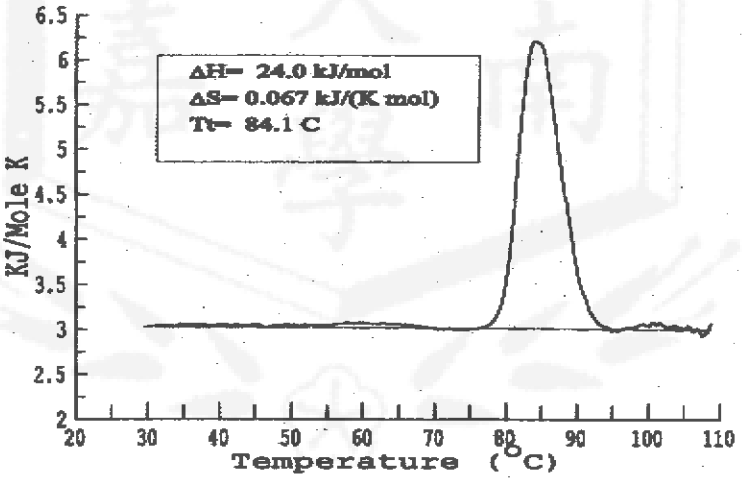
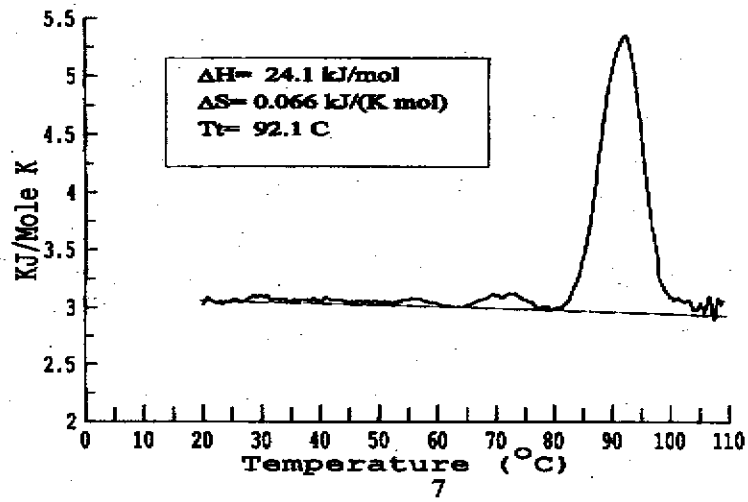


Fig. 1C



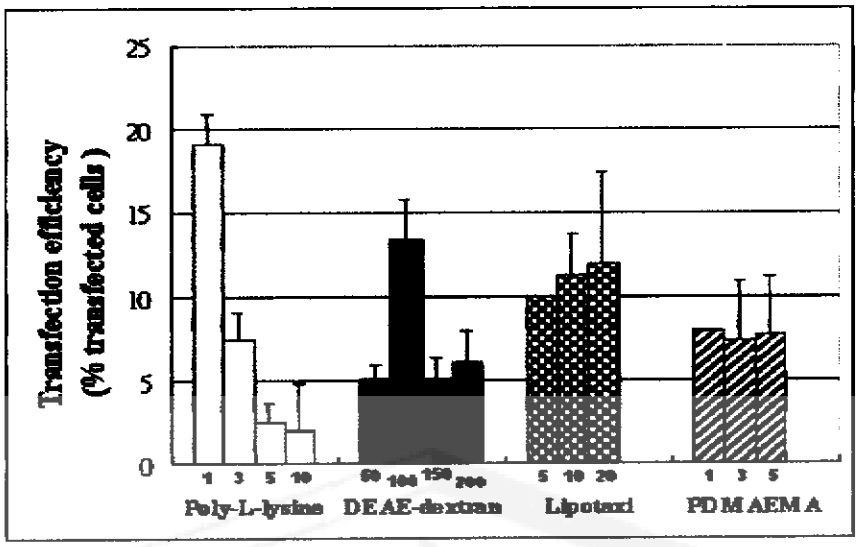


圖 2 : Effect of polymer/DNA ratios on transfection efficiency