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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

評估眼角膜專一啟動子之超微粒載體傳送表現

計畫類別：■ 個別型計畫　　□ 整合型計畫
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計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：■精簡報告 □完整報告

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
■涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：台北醫學大學藥學系

中　華　民　國　92 年　10 月　7 日

中文摘要

一般維持脊椎動物眼角膜(cornea)之完整性時，角質素(Keratin)與圓椎形角膜素(katakana)，這二種分別位在眼角膜上皮(epithelium)、與實質(stoma)細胞組織中，為其主要成分；而其功能在維持眼角膜的組織與細胞內之架構，並影響眼角膜之明晰、彈性、脆硬度功能，例如 Merman's 先天性 Keratin K12 基因缺失失養症，或實質失養症。然而這些疾病，大多屬於先天性基因不完整或缺失之疾病。雖然，先前我們曾利用 PEO 聚合載體與非專一性之啟動子(cytomegalovirus, CMV)基因，並結合 *Lac*k (reporter gene)報告基因，進行兔子與老鼠的體內眼藥水投與給藥方式，發現在角膜、鞏膜、虹彩、及肌肉層中，均可以發現有此基因之蛋白質表現 *(Gene There* 2001, 8, 999)，但是，發現此基因，卻缺乏眼角膜組織之特異與專一性。因此，本研究目的，結合最近二個被發現具有眼角膜上皮與實質組織之專一性啟動子(promoters)：Keratin (K12)與 katakana (*Ketch, K3.2)*基因質體與 PEO 聚合載體製備，進行動物眼藥水體內給藥方式，評估其基因質體在角膜組織部位上之表現與影響穿透機制。結果發現製備三個不同大小基因質體 pCMV-*LacZ* (7.2 Kb)，pK12-*LacZ* (8.6 kb)，pK3.2-*LacZ* (10.9 kb)與 PEO 聚合載體後，粒徑呈現隨著基因質體大小，而呈現製備後有變大特性(142, 182, and 187 nm)；同時，測量其擴散係數時，亦呈現下降趨勢(2.7，2.3，1.9 x10^{-8} cm^2/s)。在 DNA 酵素中，則發現 PEO 聚合載體之保護這三種基因質體能力時，至少均可以在維持在 45 分鐘內；但隨著質體大小越小而其保護能力可以增加至 105 分鐘。而評估這三種專一啟動子之基因質體，在體內眼角膜組織部位中之專一性分佈時，發現除了 pCMV-*LacZ* 基因質體表現與先前實驗中，在角膜、鞏膜、虹彩、及肌肉層中，均可發現有其半乳糖苷基因表現之無專一性外，pK12-*LacZ*，pK3.2-*LacZ* 均可以在眼角膜上皮、與實質細胞組織中，分別有半乳糖苷基因專一表現與分佈。同時，利用先前投與 EDTA 後，發現只有實質專一啟動子之基因質體(pK3.2-*LacZ*)，可以利用此添加方法而增強基因表現量。再利用反轉錄酶聚合連鎖反應(RT-PCR)方式，萃取出角膜中之半乳糖苷 mRNA，亦發現與其基因表現量有相同印證。

ABSTRACT

Keratin (K12) and keratocan (Ktcn, K3.2) are major components of extracellular keratocyte-specific keratin sulfate proteoglycan (KSPG) in vertebrate cornea epithelium and stroma. It has been suggested that cornea KSPGs modulate intermediate filament that form cytoskeletal networks in epithelial cells and stroma collagen fibrilogenesis. These two gene, thus, contribute to the corneal function and transparency. Although, previous our studied have found that efficient and stable transfer of the functional *LacZ* reporter gene with non-specific early promoter cytomegalovirus (CMV), could be achieved with PEO polymeric micelles as a carrier for eye drop gene delivery of plasmid DNA *in vivo (Gene Ther* 2001, 8, 999), it was lack of specific-tissues localization of gene expression. Therefore, these studies are to provide two specific-tissue promoters of keratin K12, and keratocan (*Ktcn*) with topical eye drop delivery into corneal epithelium and stroma areas. Our results found that three different promoters with *LacZ* gene: pCMV-*LacZ* (7.2 kb), pK12-*LacZ* (8.6 kb), and pK3.2-*LacZ*(10.9 kb) were able to formulate with PEO polymeric micelles. The formulated particle size of three plasmids with PEO polymeric micelles was increased with increasing plasmids size, 142, 182, 187 nm, respectively. In the meantime, the diffusion coefficients of three plasmids with PEO polymeric micelles were all consistently decreased with increasing particle size (2.7, 2.3, and 1.9 $x10^{-8}$ cm^2/s). Under DNase treatment, the PEO polymeric micelles were able to protect three plasmids from degradation within 45 min. However, the protective ability of pCMV-*LacZ* was found to increase up to 105 min. Using eye drops delivery of three plasmids all containing β-*gal* cDNA driven by pCMV, K12 and K3.2 promoters with PEO polymeric micelles in nude mice, we found that the specific *LacZ* gene expression of K12 and K3.2 were specific localized in the epithelium and stroma of cornea, though the gene expression with pCMV promoter still observed randomly distribution in whole eye. Furthermore, prior EDTA delivery on the cornea, the enhancement of *LacZ* gene expression could only found in the K3.2 promoter of plasmid. In the meantime, the mRNA of *LacZ* gene was extracted from each eye and able to correlate with quantitative *LacZ* gene expression of plasmid delivery as well as prior EDTA treatment.

INTRODUCTION

Two genes of keratin (K12) and keratocan (*Ktcn K3.2*), are major components of extracellular keratocyte-specific keratin sulfate proteoglycan (KSPG) in vertebrate cornea epithelium and stroma, which contribute to the corneal function and transparency. In general, these hereditary cornea dystrophies are caused by mutations of gene expression in the cornea. Gene delivery to the specific cornea would be one choice of treatment to stop progressive degeneration and restore its function. However, the integrity of cornea can be approaches only complicated surgical interventions as putting the gene into the epithelial and stroma. Eye drops of non-viral delivery systems are gaining as an alternative delivery with the hope of overcoming some of the problems associated with invasive delivery. However, in developing non-viral gene carriers, those that are efficient *in vitro* often fail to show the same efficiency when applied *in vivo*. The reasons for poor efficacy *in vivo* could be the sensitivity of carrier to serum, stability of complex formation between DNA and carrier, and the unknown mechanisms of cellular uptake and intracellular trafficking of the complex. Little is known concerning the individual parameters that influence complex formation and the characteristics of the complex that are relevant to transfection efficiencies in gene therapy.

Recently, a PEO-PBLA copolymer with an average molecular weight of 120,000 has been used in cancer medical, pharmaceutical, and transdermal systems as solubilizing, surfactant and carriers with its relatively low toxicity. In addition, these types of nano-polymeric-micelle drug systems, which having hydrophilic PEO chains as palisade regions, can prohibit protein/serum absorption *in vitro*, liver cellular interaction, and increase stability in the blood stream. Thus, PEO type of block copolymer carrier not only leads to enhanced passive transport, but also can avoid liver degradation. Evaluation of transfection efficiency usually involves comparison of different stable formulations using transgene expression as the endpoint. In our previous study showed that efficient and stable transfer of the functional *LacZ* reporter gene with non-specific early promoter cytomegalovirus (CMV), could be achieved with PEO polymeric micelles as a carrier for eye drop gene delivery of plasmid DNA *in vivo.* Thus, these PEO of copolymer micelles-carrier appear to have a good potential for use as specific topical drug delivery carriers. Thus, the aim of the present study was to evaluate the ability of PEO block copolymeric micelles to improve target-specific gene expression. Finally, on the basis of these finding, we proposed a mechanism to explain the effects of polymeric micelles upon the process of gene transfer.

EXPERIMENTAL DESIGN AND METHODS

Formulation of PEO Copolymeric Micelles: PEO-PBLA

The synthesis of PEO-PBLA block copolymer will be based on PEO and PBLA has been described in our previous studies (1). Briefly, PEO-PBLA block copolymers will be obtained by the ring opening polymerization of β-benzyle L-aspartate N-carboxyl anhydride using α-methoxy-w-amino-poly(ethylene oxide) as an initiator. The block copolymer will be

precipitated in diethyl ether, collected and dried under vacuum. ^{1}H NMR spectra of PEO-PBLA will be examined in 1 % dimethylsulfoxide (DMSO). Molecular weights of the copolymers and their polydispersity will be determined by gel exclusion chromatography on two TSK gel G3000H and G4000H columns using dimethyformaide for the mobile phase at a flow rate of 1 ml/min. Both refractive index and UV will detect the peaks with a wavelength of 280 nm. Data for aqueous gel permeation chromatography will be derived from preparative runs on a column of TSK column. Copolymers will be injected and eluted at 1 ml/min with double distilled water. Peaks will be detected using a refractive index and UV detectors.

Preparation of Polymeric Micelles with Three Different Plasmids in Aqueous Medium.

The K3.2, K12, and pCMV plasmids, which encodes the *LacZ* gene for the β̃-galactosidase (β-gal) protein, were driven by K3.2, K12, and cytomegalovirus (CMV) promoters to assess expression. K3.2 and K12 two specific promoters were gifts from Dr. Liu. Plasmids DNA were amplified in the *Escherichia coli* host strains TG-1 and purified by equilibrium centrifugation in CsCl-ethidium bromide gradient (2). The purity and stability of plasmid preparations was investigated by electrophoresis on agarose gel following by ethidium bromide staining and the DNA concentration also will be measured by UV absorption at 260 nm. Briefly, plasmid preparations was applied in the slots of a 0.8% agarose gel containing 40 mM Tris/TBE buffer solution (pH 7.6), 1 mM EDTA and 0.6 g/ml ethidium bromide.

Aqueous dispersion of the PEO copolymers with three different promoters were prepared by dissolving the different amounts of copolymers in methanol solution then adding double distilled water containing different concentration of plasmids. The system was generally heated for several hours until the required volume was obtained and Hank's buffer solution was used to adjust the concentration of plasmid.

Characterization of Physicochemical Properties of PEO Polymeric Micelles/Plasmid by: Particle size, and Zeta potential

Dispersions of polymeric micelles/plasmid was analyzed by dynamic light scattering with a helium laser light source operating at a wavelength of 632 nm with an assumed refractive index ration of 1.33 and viscosity of 0.88. The carrier of polymeric micelles/plasmid was also examined the surface Zeta potential by Malvern Zetasizer.

***In Vivo* Eye Drops of Gene Delivery in Cornea Tissues.**

For the *in vivo* study, the eyes of nude mice was treated with PEO polymeric micelles with three plasmids by simple instillation of drops as previous our studies (2). The right eyes were received treated formulation and the left eyes received plasmid only or DNA/polymeric micelles as control /comparison. The animals were scarified by cervical dislocation of the neck. The enucleated eyes were immediately removed after 48 h topical administration of formulation.

Evaluation of Transfected Efficacy of PEO Polymeric Micelles with three Plasmids.

The presence of β-gal activity was determined by histochemical analysis using X-gal substrate following the previous method (2). Enucleated eyes were fixed with 4 % paraformaldehyde at 4 °C for 90 min. Eyes were next exposed to 10 mM $K_4Fe(CN)_6$, 10 mM $K_3Fe(CN)_6$, 0.01 % sodium deoxycholate, 0.02% NP40, and 2 mM $MgCl_2$ in PBS solution containing 1 mg/ml of the X-gal substrate. The *LacZ* gene was considered to be expressed when the tissue areas show a green-blue color under an operating microscope at two constant magnifications. Quantification of β-gal expression was be assayed by using the substrated chlorophenol red galactopyranoside at 1 mg/ml to induce color development, which measured at 580 nm with a microtube. Total tissue protein was determined using the DC protein assay reagent kit, which subsequently used to normalize β-gal activities of each transfection.

RESULTS AND DISCUSSION

By dynamic light scattering of particle size measurement, the size of PEO polymeric micelles with pCMV-*LacZ* (7.2 kb), pK12-*LacZ* (8.6 kb), and pK3.2-*LacZ*(10.9 kb) complexes shows that from 394 ± 38 nm decreased to be with the range 142.9 ± 8.7 nm, 182.1 ± 6.1 nm, and 187.3 ± 9.1 nm, respectively (**Table 1**). The surface charge of three plasmids DNA only, complexes with PEO polymeric micelles solution were fall from –26.2 ± 8.4, –27.9 ± 13.0, –24.7 ± 11.0 mV to –8.9 ± 3.8, –12.1 ± 1.2, and –12.1 ± 1.1 mV, respectively (**Table 1**). In the meantime, the diffusion coefficients of three plasmids with PEO polymeric micelles were all consistently decreased with increasing particle size (2.7, 2.3, and 1.9 $x10^{-8}$ cm^2/s). In order to test the protection of plasmid with PEO polymeric micelle in nuclease digestion, DNase I was incubated with time-profiles. Three plasmids alone under DNase I treatment, the degradation of was found within 1 min by electrophoresis measurements. The three plasmids complexes with polymeric micelles still could remain the size of integrity at 105 min, 90 min and 45 min, respectively (**Fig. 1**).

After forty-eight hours after topical eye drop delivery with pCMV-*LacZ* in mouse *in vivo*, the whole-mount preparations of mice' eyes reacted with β-Gal substrate, X-Gal, still revealed a dark blue spots corresponding to specked staining with choroid sclera, conjunctiva, and tendon of lateral rectus muscle etc. (**Fig. 2 B**). No β-Gal activity was detected in the ocular tissues of control eyes or delivery DNA plasmid only (**Fig. 2A**). Delivery with epithelium specific promoter of keratin K12 (pK12-*LacZ*), it could specific localize in the epithelial tissues after section (**Fig. 3A**). In the meantime, delivery of stroma specific promoter of keratocan K3.2 (pK3.2-*LacZ*), it also could observe the gene expression in the stroma area (**Fig. 3B**). The quantitative expression levels of six doses of *in vivo* eye drops delivery into cornea, β-Gal gene expression activity was detected under pK12-*LacZ* with polymeric micelle delivery from 3.09 ± 0.39 to 3.58 ± 0.80 mu/mg protein with statistical significance ($p < 0.05$) (**Table 2**). In addition, pCMV-*LacZ* with polymeric micelle delivery was enhanced 26 % (from 3.10 ± 0.42 to 3.91 ± 0.61 mu/mg

protein, $p< 0.01$). The pK3.2-*LacZ* with polymeric micelle delivery can't observe significantly β-Gal gene expression activity (3.27 ± 0.27 mu/mg protein, compare with control 3.27 ± 0.37 mu/mg protein). For EDTA enhance study, 10 μl of 5 mM EDTA was prior applied to eyes 10 min before. There was no influence of polymeric micelle delivery with pK12-*LacZ*(**Table 2**). However, using complexes of pK3.2-*LacZ* with polymeric micelles, the EDTA enhancement of gene expression was achieved and up to 3.88 ± 1.08 mu/mg protein ($p< 0.05$) (**Table 2**).

The mRNA of *LacZ* gene was extracted from each eye as well as mRNA of β-actin for control quantization by polyacrylamide gel electrophoresis (**Fig. 4**). The results of β-Gal extracts were appeared in the pCMV-*LacZ* pK12-*LacZ* delivery with polymeric micelles, not in pK3.2-*LacZ* (**Fig. 4**). With EDTA pretreatment of pK12-*LacZ* still appear similar β-Gal band. Furthermore, delivery of pK3.2-*LacZ* with polymeric micelles show a weak β-Gal band (**Fig. 4**). The ratios of β-Gal/β-actin of pCMV-*LacZ*, pK12-*LacZ*, and pK3.2-*LacZ* with polymeric micelles delivery were 109, 108, and 1.12 respectively. Prior treatment of EDTA, the enhancement of ratio of β-Gal/β-actin was found only in pK3.2-*LacZ* delivery with polymeric micelles and increased to 35.55. These results were similar to the results of *in vivo* gene delivery.

REFERENCES:

1. Liaw J, Aoyagi T, Kataoka K, Sakurai Y, Okano T. Visualization of PEO-PBLA-Pyrene polymeric micelles by atomic force microscopy. *Pharm Res* 1998, 15, 1721-1726.
2. Liaw J, Chang SF, Hsiao FC. In vivo gene delivery into ocular tissues by eye drops of polymeric micelles. *Gene Ther* 2001, 8, 999-1004.

Table 1. The physicochemical properties of three plasmids with polymeric micelles.

	Plasmid alone	Plasmid with polymeric micelles	Polymer alone
Zeta potential **(mV)**			
pCMV-*LacZ* (7.2 kb)	-26.2 ± 8.4	-8.9 ± 3.8 [a.]	
pK12-*LacZ* (8.6 kb)	-27.9 ± 13.0	-12.1 ± 1.2 [a.]	-5.5 ± 0.7
pK3.2-*LacZ* (10.9 kb)	-24.7 ± 11.0	-12.1 ± 1.1 [a.]	
Particle size (nm)			
pCMV-*LacZ* (7.2 kb)	69.7 ± 36.1	142.9 ± 8.7 [a, b]	
pK12-*LacZ* (8.6 kb)	70.0 ± 31.0	182.1 ± 6.1 [a]	36.7 ± 20.8
pK3.2-*LacZ* (10.9 kb)	72.9 ± 28.6	187.3 ± 9.1 [a]	

[a.] Significant difference at $p < 0.01$ as compared without polymeric micelles.
[b.] The size of significant difference between pCMV-*LacZ* and pK12-*LacZ* or pK3.2-*LacZ* with polymeric micelles was found $p < 0.01$.

Table 2 β-Gal expression activity of three plasmids with polymeric micelle by eye drops delivery in nude mice [a].

Treatment	Expression (mu/mg protein)	
Control	3.09 ± 0.39	(n=17)
pK12-*LacZ*	3.58 ± 0.80[b]	(n=17)
Control	3.17 ± 0.27	(n=12)
EDTA + pK12-*LacZ*	3.58 ± 0.39[c]	(n=14)
Control	3.27 ± 0.27	(n=14)
pK3.2-*LacZ*	3.27 ± 0.37 [e]	(n=18)
Control	3.21 ± 0.33	(n=18)
EDTA + pK3.2-*LacZ*	3.88 ± 1.08[b d]	(n=18)
Control	3.10 ± 0.42	(n=16)
pCMV-*LacZ*	3.91 ± 0.61[c]	(n=18)

[a.] Values are the average of the β-gal activity ± standard error of the mean per specimens (mu/mg proteins). Numbers in parentheses are the numbers of specimens examined.
[b]. Significantly increased activity compared with the control plasmids only ($p < 0.05$).
[c.] Significant increased activity compared with the control plasmids only ($p < 0.01$).
[d.] Significant increased activity compared with the pK3.2-*LacZ* with polymeric micelles ($p < 0.05$).
[e.] No significant difference between delivered polymeric micelles only and DNA only ($p > 0.1$).

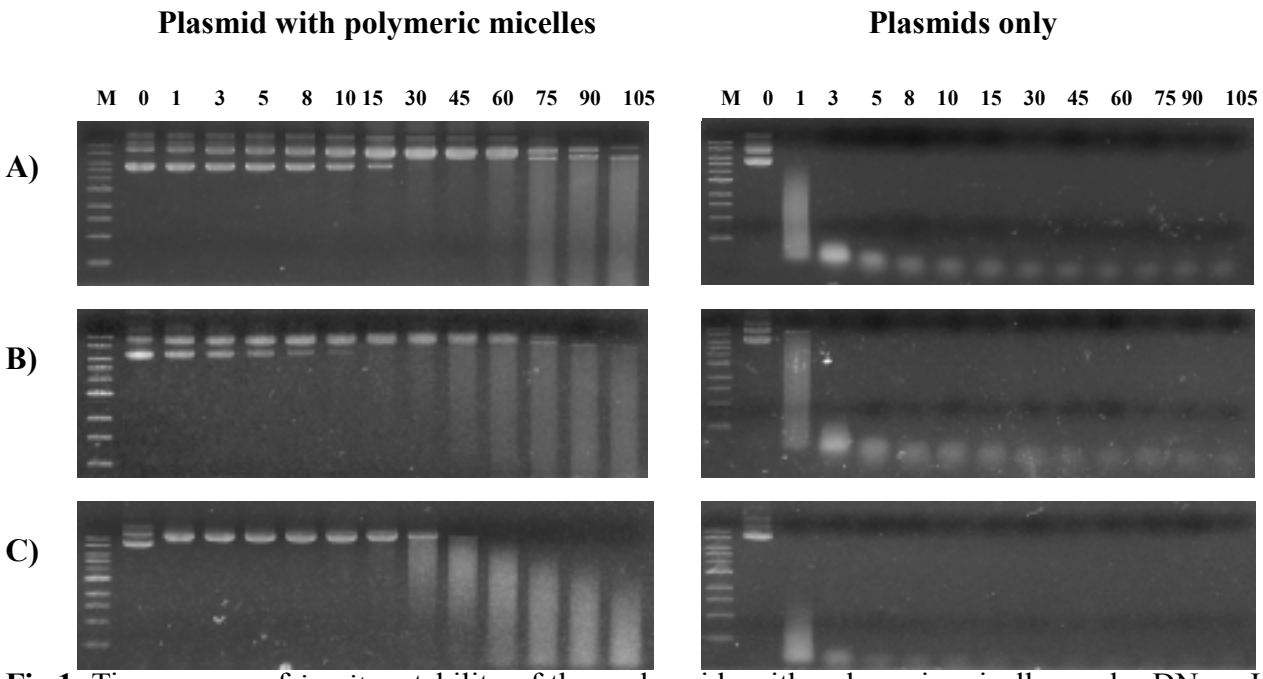

Fig.1. Time course of *in vitro* stability of three plasmids with polymeric micelles under DNase I treatments (unit as min): **A)** pCMV-*LacZ*, **B)** pK12-*LacZ*, **C)** pK3.2-*LacZ*. M represented 1kb ladder marker.

Fig. 2. Whole-mount eyes 2 days after eye drops delivery with pCMV-*LacZ* plasmids of polymeric micelles. The control group of delivery with plasmids alone into eyes was in (**A**). Note the speckled staining of the blue-green color corresponding to X-gal activity in the whole eyes tissues with pCMV-*LacZ* delivery (**B**).

Fig. 3. Histological analysis of delivery two plasmids (pK12-*LacZ* and pK3.2-*LacZ*) with polymeric micelles into the nude mice's eyes (**A-B**). Note the speckled staining of the blue-green color corresponding to X-gal activity in the whole eyes tissues. Light micrographs of 10-μm OCT-cryosections of the eyes.

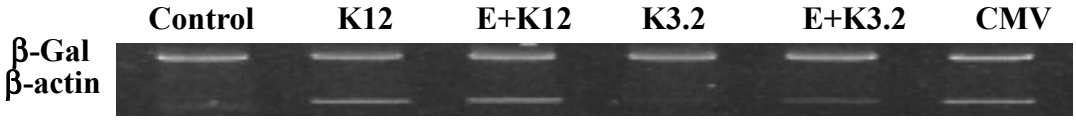

Fig. 4. RT-PCR analysis of *LacZ* gene transcription with three plasmids. Total RNA was prepared from eyes with plasmid only, samples with polymeric micelles/plasmid, and samples with pre-treatment of 10 mM EDTA before polymeric micelles/plasmid delivery. The samples were carried out to amplify the 249-bp sequence of the *LacZ* gene and the 659-bp sequence of the β-actin gene for RT-PCR.