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GENE DELIVERY

Ethanol enhanced *in vivo* gene delivery with non-ionic polymeric micelles inhalation

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Abstract

Modifications of both carriers and host barriers have been investigated for efficient inhalation gene delivery to lung. Here we used a biocompatible, non-ionic poly(ethyleneoxide)-poly(propyleneoxide)-poly(ethyleneoxide) (PEO-PPO-PEO) polymeric micelles (PM) as a carrier and combined it with ethanol to enhance membrane penetration of delivered DNA. The inhalation delivery with six 100 μg doses of pCMV-*Lac Z* with PM co-formulated with 10%–40% ethanol to nude mice in 2 days at 8 h interval was performed. The β-galatosidase (β-Gal) activity was assessed using chlorophenol red-β-D galactopyranoside (CPRG) and X-gal staining for quantitative and qualitative analysis in tissues. The results showed that β-Gal activity was significantly increased by 38% in lung around bronchioles when inhalation with PM and 10% ethanol was given. The 10% ethanol also increased the intracellular apparent permeability by 42% in stomach and by 141% in intestine at 48 h after the first dosage of delivery. Also delivery of DNA encoding a functional human cystic fibrosis transmembrane protein (CFTR) using the same inhalation delivery method co-formulated with 10% ethanol, an increased expression of CFTR in lung was detected by immunostaining. We concluded that 10% ethanol co-formulated with the PM system could enhance inhaled gene delivery to airway and gastrointestinal (GI) tract.

Keywords: Inhalation; Ethanol; Gene; Polymeric micelles; CFTR

1. Introduction

The delivery of genes by inhalation holds promise for the treatment of a wide range of pulmonary (cystic fibrosis, asthma, etc.) [1,2] and non-pulmonary systemic disorders (insulin aerosol, etc.) [3,4]. It offers non-invasive, simple and large absorption lung surface with numerous advantages over more invasive modes of delivery. Various non-viral carriers have been developed for inhalation gene transfer such as, cationic polyethyleneimine (PEI) polymer, liposome, and non-ionic polymers [3–6]. PEI has been shown to be one of the most effective DNA delivery systems, and is a very effective gene delivery vehicle for lung transfection [7,8]. However, the use of PEI as a gene delivery system in humans is hampered by its well-known toxicity and its immune-stimulatory activity [9]. In addition, many genes involved in apoptosis, stress responses and oncogenesis were activated by PEI polymer [9], further draw back the potential application of PEI being the delivery carrier.

Several low immunogenicitic cationic lipids liposome have been used to delivery gene into lung, including DOTMA, EDMPC, and DOPE [10–12]. However, poor efficiency is often observed when non-viral vectors applied *in vivo* [13], which may be explained by the sensitivity of carrier to serum, the stability of complex formed between DNA and carrier, and possibly the cellular uptake and intracellular trafficking pathways [6,14]. Moreover, using phospholipids to form a liposome, it will cost more for repeated administration. In general, synthesized polymers are thought to have the potential for repeated administration and large-scale production. Among non-ionic

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polymeric carriers, a non-ionic PEO-PPO-PEO triblock copolymer has been approved non-immunogenic, resistant to protein absorption, and stable in blood stream in the medical, pharmaceutical, and cosmetic fields [5,6,15–17]. Previously studies using intramuscular injection, eye drop or oral delivery of pCMV-*Lac Z* formulated with PEO-PPO-PEO PM, have achieved the purpose of gene delivery reflecting by the increased β-Gal activity in muscular, ocular and GI tissues [5,6,14,18]. The non-ionic polymeric vectors with biocompatible characters may be appropriate carriers to develop for gene therapeutics in pulmonary diseases. Recently, Desigaux et al. [19] have shown that improved transgene expression in the lung only up to 60% after directly aerosolization with microsprayer compared with PEI-DNA intratracheal delivery. Unfortunately, inefficient penetration through mucous barriers and inhibitory effects of surfactant and other lung-specific features have generally resulted in a lack of therapeutic effect [20]. Thus, further progress in lung delivery clearly requires other more safer and efficient gene delivery systems [21].

Disruption of these natural barriers by organic solvent and ethanol could improve adenovirus-mediated gene transfer and expression to the bladder epithelium of rodents [22]. In addition, ethanol has been reported to inhibit synthesis, transport, and processing of gastric mucus glycoprotein [23], alter the morphology and function of GI mucosal surface [24,25], and disrupt tight junction in Caco-2 cell [26], also has been recognized as an immunosuppressive agent [27]. Thus, ethanol would be a good candidate to be an enhancer for gene delivery. This study was aimed to explore the enhanced ability of ethanol with non-ionic PM to deliver plasmid DNA by inhalation method. We assessed the expression of reporter gene in lung and other tissues.

2. Materials and methods

2.1. Materials

Non-ionic block copolymer, a poly(ethyleneoxide)$_{120}$-poly (propyleneoxide)$_{76}$-poly (ethyleneoxide)$_{120}$ (PEO-PPO-PEO) block copolymer with an average molecular mass of 15,000 Da, was obtained from BASF (Ludwigshafen, Germany) and ethanol was purchased from Merck (Darimstadt, Germany). All other chemicals were analytical reagent grade and used without further purification.

2.2. Animals

Male nude mice, (BALB/cAnN.Cg-*Foxn1nu*/CrlNarl) 6–8 weeks old, were maintained under specific pathogen-free (SPF) conditions for inhalation delivery and were purchased from National Laboratory Animal Center (Taipei, Taiwan).

2.3. Plasmid DNA preparation

The pCMV-*Lac Z* containing the *Lac Z*, driven by a cytomegalovirus (CMV) promoter and the pcDNA3-CFTR containing the CFTR cDNA sequences, driven by a CMV promoter, were used [5,28]. These plasmids were amplified in *Escherichia coli* host strain TG1 and purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradient as previous study [5]. The purity of plasmid was checked by electrophoresis on 0.8% agarose gel followed by ethidium bromide staining, and the DNA concentration was measured by ultraviolet (UV) at 260 nm.

2.4. Preparation of pCMV-Lac Z/PM mixture

A pyrene fluorescence probe was used to analyze the critical micelles concentration (CMC) of PM as described previously [14,29,30]. The non-ionic block copolymers were freshly prepared on a weight percentage basis with a range from 0.001% to 10% (w/v) in water or co-formulated with 10%–40% ethanol. The spectral data of pyrene were acquired using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) and all fluorescence experiments were carried out at 25 °C. Above the CMC concentration of non-ionic polymer was used to form PM for plasmid delivery. The pCMV-*Lac Z*/PM or pcDNA3-CFTR/PM mixture was formulated in a way that plasmid was gently mixed with PM in a vial for 2 h at 25 °C.

2.5. Size and Zeta-potential measurements

The size and *Zeta*-potential of pCMV-*Lac Z*, PM alone, pCMV-*Lac Z*/PM mixture in water, and co-formulated with various concentrations of ethanol (10%–40%) were determined by quasielastic laser dynamic light scattering (DLS) (Zetasizer 3000; Malvern Instruments, Malvern, UK). All measurements were performed at 25 °C, at an angle of 90° as described in previous study [5,14,29] and results were presented as mean ± SD.

2.6. Atomic force microscope observation

Ten microliters of 6% (w/v) PM alone, pCMV-*Lac Z*/PM mixture in water or co-formulated with ethanol (10%–40%) was placed on a mica surface with no further treatment. The atomic force microscope (AFM) (diCP-II; Digital Instruments/Veeco Metrology Group, Santa Barbara, CA) was operated in constant tapping mode. The cantilevers were standard NSC15/AIBS silicon single-rectangular cantilever (230 μm) (MikroMasch, Estonia) and the constant force mode was used with a resonant scan frequency of 328 kHz. All images were collected within 5×5 μm^2 areas.

2.7. DNase I digestion

Protection of pCMV-*Lac Z* by PM against DNase I was carried out with modified methods as other reports [31,32]. Briefly, the reaction mixture contained 200 μl of digestion buffer or with ethanol (10%–40%), 6.5 units of RQ1 RNase-free DNase I (Promega Biotech Co., Ltd, Madison, WI) and 100 μg of pCMV-*Lac Z* with or without PM. The reaction was carried out for 2 h at 37 °C. This mixture was sampled in 10 μl volume at fixed time interval and was then stopped by the immediate

addition of 1 μl of RQ1 DNase I stop solution (Promega Biotech Co., Ltd, Madison, WI). The resulting solutions were loaded onto a 0.8% agarose gel for electrophoresis and then the gel was stained with ethidium bromide. Qualification of band intensities was performed with a Kodak EDAS290 Analysis system (Kodak Scientific Imaging System, New Haven, CT).

2.8. In vivo inhaled gene delivery

Nude mice were fasted for 24 h before the experiments, and then treated with an unstrained whole-body inhaled exposure cage (12.5×10×9.5 cm) for delivery. Six doses of PM containing 100 μg of pCMV-*Lac Z* in water or co-formulated with 10%–40% ethanol were inhaled to mice for 15 min, using a DeVilbiss nebulizer (Pulmo-Aide 5610 D, Somerset, PA, USA) with a particle size range of 0.5–5 μm. The mice received pCMV-*Lac Z* or PM alone was presented as control groups. All formulations were administered with six dosages at 8 h interval for 2 days as described in previous study [5]. Also, the negative control tests of inhaled 10% ethanol alone, PM alone or plasmid co-formulated with 10% ethanol were performed. In order to further understand the ethanol-enhanced permeation of delivered DNA/PM formulation, pretreatment of PM co-formulated with 10% or 40% ethanol before pCMV-*Lac Z*/PM gene delivery was also evaluated.

2.9. Determination of delivered gene expression

To evaluate the delivered gene expression, mice were sacrificed by cervical dislocation at 48 h after the first dosage delivered. The trachea, lung, stomach, duodenum, heart, liver, kidney, testis, nasal and skin tissues were removed immediately for further analysis. All tissues were gently rinsed three times in phosphate buffered saline (PBS, pH 7.4), which was pre-warmed at 37 °C. The β-Gal activity was quantified with the enzyme substrate, chlorophenol red-β-D galactopyranoside (CPRG, 1 mg/ml, Gene Therapy Systems, San Diego, CA). Induced color development was measured at 580 nm as previous described [5]. Total protein measured with a DC protein assay reagent kit (Bio-Rad, Hercules, CA) was to normalize the β-Gal activity for each sample. Statistical comparisons were made with Student-*t* and ANOVA test (Dunnett's multiple comparison tests) with a 95% confidence level. All results were presented as mean±SEM. To evaluate gene expression with time duration study *in vivo*, inhalation delivery mice with pCMV-*Lac Z*/PM in 10% ethanol mixture were scarified at 24, 48, 72, and 96 h time points and all major organs and tissues were removed and processed immediately for individual analysis.

2.10. β-Gal enzyme histochemistry

The tissues were prepared as previously described [5]. Briefly, tissues were rinsed in ice-cold PBS, and immersed in ice-cold fixation solution (4% paraformaldehyde, Merck, Darimstadt, Germany) for 1.5 h, and then stained for 48 h at 37 °C in PBS buffer containing 10 mM $K_4Fe(CN)_6$ (Sigma Chemical Co., St. Louis, MO), 10 mM $K_3Fe(CN)_6$ (Sigma Chemical Co., St. Louis, MO), 0.01% sodium deoxycholate (Sigma Chemical Co., St. Louis, MO), 0.02% Nonidet P-40 (NP-40, Sigma Chemical Co., St. Louis, MO), 2 mM $MgCl_2$ (Merck, Darimstadt, Germany), 5 mM EDTA (Amresco, Solon, OH) and 1 mg/ml X-gal substrate (Gibco BRL, Grand Island, NY, USA). Cryosections (10 μm) of the O.C.T. (Sakura Finetek USA, Torrance, CA)-embedded stained tissues were washed by acetone-methanol (1:1) solution, and then stained with hematoxylin-eosin for histological assessment. The β-Gal enzyme was considered to be expressed when the tissue was blue-green in color under an operating microscope (Olympus BX-40, Japan) at magnifications of ×40, ×100 and ×400.

2.11. In vitro stomach and intestinal permeability studies after in vivo inhaled or oral delivery

To determine the ethanol effect on enhancing the penetration of delivered plasmid in GI tract, the permeabilities of stomach and intestine tissues were examined at 48 h after the first dosage of inhaled (following the procedure described in Section 2.8) or oral (see the Section 2.13) delivery by flux measurements using the intracellular tracer, [^{14}C]-estradiol, and the intercellular tracer, [^{14}C]-mannitol [33]. The mice received no treatment or PM alone was served as the control groups. These tissues were gently rinsed three times in glutathione bicarbonate reagent (GBR), which was pre-warmed at 37 °C, and then mounted in Ussing chamber as previous study [33]. Briefly, both surfaces of the tissues were bathed in GBR solution. All experiments were performed at 37 °C with a mixture of 95% O_2/5% CO_2 supplied. The permeating ^{14}C-radioactive compound was sampled from the receiver compartment at fixed intervals and replaced with an equal volume of 37 °C pre-warmed GBR solution. The penetrated radioactive compound was counted as the total number of disintegrations per minutes (dpm) with a liquid scintillation counter (Beckman LS6500, Beckman Coulter Inc., CA). *In vitro* apparent permeability coefficients (*P*) were calculated from the equation:

$$P = V/A \times 1/C \times \mathrm{d}C/\mathrm{d}t$$
$$= \text{Fraction of dose transported}/\mathrm{d}t \times V/A$$

Where the fraction of the dose transported through the tissues can be calculated after correction for sampling and solution replacement at each time point. These values were then plotted versus time (*t*). The apparent permeability coefficient (*P*, cm/s), was calculated from the slope by using the receiver compartment volume (*V*, 7 ml), and surface area (*A*, 0.02 cm^2) of the tissue. Statistical comparisons were made by ANOVA test (Dunnett's multiple comparison tests) with a 95% confidence level.

2.12. Electrical resistance measurement

Electrodes were prepared from silver wire and resistance was measured using an Ag-AgCl four-electrode system as previous report [33]. Variable current pulses were given and corresponding potential difference was measured by a digital

multimeter. Resistance was calculated from the slope of the applied current and potential difference plot.

2.13. In vivo oral gene delivery

The oral gene delivery was followed as previously described [5]. The six 150 μl doses of PM containing 40 μg of pCMV-*Lac Z* in water or co-formulated with 10%–40% ethanol were orally administered to nude mice with a stomach feeding needle (KN-342, Nature Seisakusho). The mice received pCMV-*Lac Z* or PM alone served as the control groups. The negative control groups receiving 10% ethanol alone, PM alone or plasmid co-formulated with 10% ethanol, and ethanol pretreatment tests were also performed. The CPRG method was used to determine the delivered gene expression at various tissues, including trachea, lung, stomach, duodenum, heart, liver, kidney, testis, nasal and skin tissues following the procedure described in Section 2.9.

2.14. In vivo inhaled CFTR gene delivery

Six doses of PM co-formulated with 10% ethanol containing 100 μg of pcDNA3-CFTR were inhaled to mice as the experimental group and the mice received no further treatment was served as the control group. Two days after inhaled delivery at 8 h interval, the lung tissues for cryosection analysis were immediately removed and rinsed in ice-cold PBS, then immersed in ice-cold fixation solution (4% paraformaldehyde, Merck, Darimstadt, Germany) for 1.5 h. Cryosections (10 μm) of the O.C.T.-embedded lung tissue were fixed with acetone/methanol (1:1) on ice for 10 min, and blocked by 1% bovine serum albumin (BSA) for 30 min at room temperature. The cryosection was hybridized with goat anti-CFTR antibody (1:200, Santa Cruz Biotechnology Inc., CA) and incubated in a moisture condition at 4 °C overnight, and then washed by PBS and hybridized with donkey anti-goat IgG-FITC (1:200, Santa Cruz Biotechnology Inc., CA) in the dark for 1 h at room temperature. The section was washed with PBS and stained with propidium iodide [(PI), 0.005 mg/ml, Roche Diagnostic Corp., IN] for localization of nucleus, and then sealed with PBS: glycerol (1:1). The FITC and PI filters were used to observe the green signal (CFTR) and red signal (cell nucleus) using immunofluorescence microscope (Olympus BX40, Japan). For assessing the intensity of green signal, the control and experimental groups were adjusted to a fixed exposure time, and the *SimplePCI* image software (Compix Inc., PA) was used to evaluate the intensity level of CFTR gene expression.

3. Results

3.1. Characterization of PM formations

The CMC of PM formation in water or co-formulated with 10%–40% ethanol was measured by a fluorescence probe, pyrene, and the partitioning of pyrene into the hydrophobic micellar phase was determined using the peak ratio of peak I_1/peak I_3 of the pyrene spectrum as previous report [14,29,30]. We found that the I_1/I_3 peak height ratio decreased when the concentration of PM was above 0.01% (w/v) for polymer alone (Fig. 1A). When pyrene interacted with this non-ionic polymer with different concentrations of ethanol, the ratios of peaks I_1/I_3 decreased at 0.01%, 0.1%, and 1% (w/v) with increasing ethanol concentrations (10%–40%), respectively (Fig. 1A). In this study, the 6% (w/v) of polymer was used in all formulations to form PM.

3.2. Characterization of pCMV-Lac Z/PM mixture

AFM was used to visualize the morphology of pCMV-*Lac Z*/PM mixture. The results showed that PM alone, pCMV-*Lac Z*/PM mixture in water, or co-formulated with 10%–40% ethanol readily attached and remained bounded to mica, and could be imaged to exist as round micelles with an AFM tip (Fig. 1B–F). Using DLS measurement, PM alone and pCMV-*Lac Z*/PM mixture showed an average diameter of 182.2±10.3 nm and 236.9±10.2 nm, respectively (Table 1). Co-formulated with 10%, 20%, and 40% ethanol, the sizes of the resulting pCMV-*Lac Z*/PM mixtures were increased to 512.4±10.3, 645.8±15.7, and 488.3±15.9 nm, respectively (Table 1). The *Zeta*-potential was −11.7±1.7 mV, −40.3±2.2 mV, and −11.4±1.3 mV for PM alone, pCMV-*Lac Z* only, and pCMV-*Lac Z*/PM mixture, respectively (Table 1). The presence of 10%–40% ethanol did not alter the *Zeta*-potential of the pCMV-*Lac Z*/PM mixture (Table 1).

3.3. Stability of pCMV-Lac Z/PM

RNase-free DNase I, an endonuclease, is active for all free forms of single and double-stranded DNA, and hydrolyzes DNA molecules to a mixture of short oligo- and mononucleotides. To determine whether the PM formulation would enhance the stability of DNA against enzymatic degradation by DNase I, *in vitro* DNase I digestion assay was performed. Similar electrophoretic mobility of supercoiled, open circular, and multi-mer forms of plasmid DNA was observed for all samples before treated with DNase I. (Fig. 2A–D), indicating that neither PM nor ethanol would interfere the electrophoretic mobility of plasmid. However, exposure of pCMV-*Lac Z*/PM or pCMV-*Lac Z*/PM co-formulated with ethanol to DNase I, at the ratio of 0.13 unit DNase I per 1 μg DNA, resulted in a delayed complete DNA degradation during the experimental period of up to 15–30 min, as shown by the absence of visible bands or smearing on the last lane in all four gels (Fig. 2A–C) except in 40% ethanol (Fig. 2D). For plasmids alone, the supercoiled forms in all conditions tested were completely diminished after 5 min of DNase I-digestion (Fig. 2A–C), except co-formulated with 40% ethanol in that a partial digestion reflected by the presence of minute amount of DNA in the open circular form along with the smeared DNA pieces was observed up to 120 min incubation (Fig. 2D). In addition, we have observed similar pattern of DNase I less-sensitivity of either pCMV-*Lac Z* plasmid alone or plasmid/PM co-formulated with 50% ethanol, but not occurred when plasmid/PM co-formulated with 30% ethanol was analyzed (data not shown).

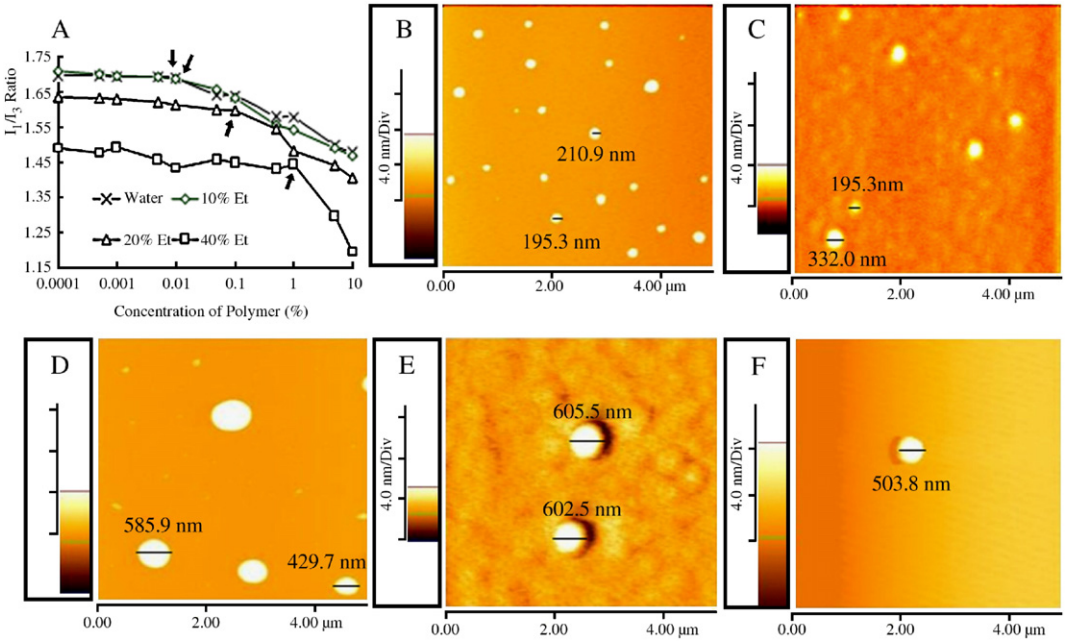

Fig. 1. (A) The I_1/I_3 ratio of vibrational bands in the pyrene fluorescence spectrum were as a function of polymer concentrations for polymeric micelles (PM) in water only, PM co-formulated with 10%, 20%, and 40% of ethanol (Et) in pyrene solution. The arrows indicate the critical micelles concentration. AFM images of (B) PM in water only, pCMV-*Lac Z*/PM mixture co-formulated with (C) water, (D) 10%, (E) 20%, and (F) 40% ethanol were presented on a mica surface within 5×5 μm^2 areas.

3.4. β-Gal activity evaluated by CPRG

After delivery the pCMV-*Lac Z*/PM, CPRG was used to evaluate the β-Gal activities in trachea, lung, stomach, duodenum, heart, liver, kidney, testis, nasal and skin tissues. For those mice receiving pCMV-*Lac Z* alone, PM alone, and pCMV-*Lac Z*/PM mixture 48 h after the first dose, the results showed that no significantly increased *Lac Z* gene expression could be detected in all tissues (Table 2). After delivery the pCMV-*Lac Z*/PM co-formulated with 10% ethanol, the β-Gal activities were analyzed in various tissues collected at 24, 48, 72, and 96 h after the first dose. Results showed that the β-Gal activity in lung was most significant increased (38%) at 48 h, but returned to the background level at 96 h post-initial delivery, compared with the plasmid alone inhalation group (Table 2). This indicates that the transferred *Lac* Z gene could be achieved to lung tissues. On the other hand, after 6 doses delivery the pCMV-*Lac Z*/PM co-formulated with 10% ethanol, the β-Gal activities were also significantly increased in other tissues such as trachea (23–56%), stomach (22–37%) and duodenum (21–26%) within 24–96 h and similar decreasing pattern of the transgene expression was detected at 96 h (Table 2). In order to confirm that 10% ethanol alone or PM alone did not influence β-Gal expression, inhalation deliveries with PM alone, 10% ethanol alone and PM or plasmid co-formulated with 10% ethanol were analyzed. All these control experiments showed no significant activities of β-Gal activity in the tissues (Table 2).

In addition, the β-Gal activities were also significantly increased in trachea (17%), stomach (17%) and duodenum (17%) of mice inhaled the pCMV-*Lac Z*/PM co-formulated with 20% ethanol (Table 2). Nevertheless, there was no significant increase in β-Gal activity in lung tissues of mice receiving pCMV-*Lac Z*/PM co-formulated with 20% ethanol as well as with 40% ethanol. Furthermore, in order to understand the enhanced permeation effect of ethanol, we have pretreated mice with PM co-formulated with 10% ethanol (PM in 10% Et) for 15 min followed by the administration of pCMV-*Lac Z*/PM (P/PM) mixture to access the ethanol effect. These are similar levels of the β-Gal activities detected in trachea (28%), lung (38%), stomach (24%) and duodenum (18%) with those administered with pCMV-*Lac Z*/PM co-formulated with 10% ethanol (Table 2) ($p > 0.05$). However, no enhancement of β-Gal activity was found in mice pretreated with PM co-formulated with 40% ethanol for 15 min and then administered with pCMV-*Lac Z*/PM (Table 2). The co-administration of ethanol and pretreatment with ethanol experiments showed the similar

Table 1
The size and *Zeta* potential of pCMV-*Lac-Z* (P) formulated by polymeric micelles (PM) in water and co-formulated with ethanol (Et) (10%–40%)

Formulations	Size[a] (nm)	*Zeta* potential[a] (mV)
P alone[b]	61.6±7.9	−40.3±2.2
PM alone[c]	182.2±10.3*	−11.7±1.7[#]
P/PM	236.9±10.2*	−11.4±1.3[#]
P/PM in 10% Et[d]	512.4±10.3*	−11.3±0.7[#]
P/PM in 20% Et[d]	645.8±15.7*	−10.6±0.5[#]
P/PM in 40% Et[d]	488.3±15.9*	−10.7±1.3[#]

[a] Results were expressed as mean values with standard deviation (SD) of five experiments.
[b] 0.1 μg/μl of plasmid.
[c] 6% (w/v) polymer.
[d] plasmid/PM co-formulated with 10%–40% ethanol (Et).
* Indicated a significant difference ($p < 0.05$) compared with the size of P alone.
[#] Indicated a significant difference ($p < 0.05$) compared with the *Zeta* potential of P alone.

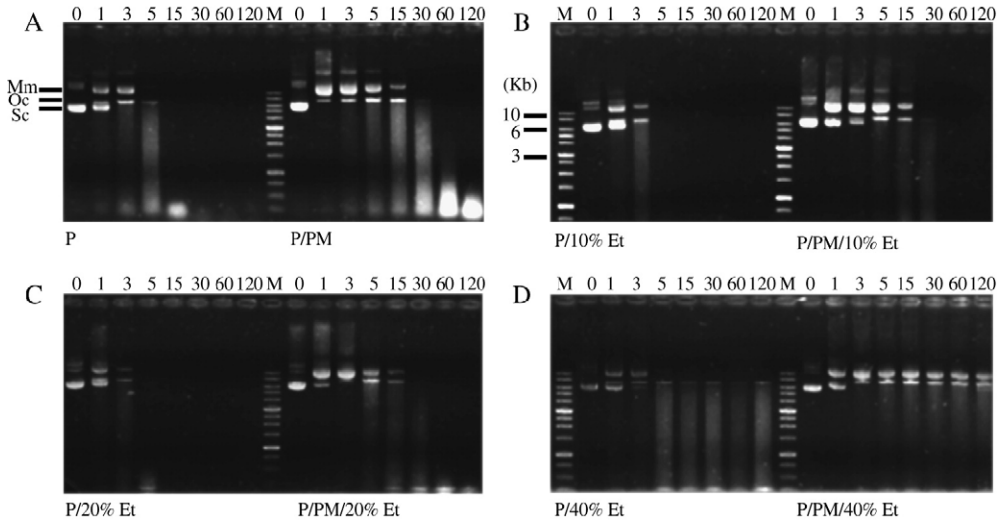

Fig. 2. Stability of pCMV-Lac Z (7.2 kb) with polymeric micelles (P/PM) analyzed by DNase I digestion. Agarose gel electrophoresis showed that P or P/PM mixture co-formulated with (A) water, (B) 10%, (C) 20% and (D) 40% of ethanol (Et) with 120 min. The markers (M) were represented 10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.75, 0.5 and 0.25 kb. At time 0 min could observe the supercoiled (Sc), open circular (Oc), multi-mers (Mm) forms and indicated that reaction performed at formulations without DNase I added.

levels of β-Gal activities pattern, emphasizing the potential enhancer role of ethanol in gene transfer through inhalation.

3.5. β-Gal enzyme histochemistry

To further localize the *Lac Z* expression in both of airway and GI tract, histochemical analysis of the β-Gal enzymatic activity in whole-mount tissues and cryosections including trachea (T), lung (L), stomach (S) and duodenum (D) from control mice and mice administered with pCMV-*Lac Z*/PM co-formulated with 10% ethanol were performed. Cryosections were counter-stained with hematoxylin-eosin for histological analysis. As shown in Fig. 3, the β-Gal activity in tracheal tissue was mostly found in tracheal gland (t) (Fig 3). In lung tissues,

Table 2
The β-Gal activities in nude mice tissues inhaled delivery with pCMV-*LacZ* (P)/polymeric micelles (PM)

Inhalation[a]	β-Gal activity (mU/mg protein) in tissues[b]									
	Trachea	Lung	Stomach	Duodenum	Heart	Liver	Kidney	Testis	Nasal	Skin
Formulations										
P alone[c]	(*n*=12)	0.78 (0.06)	0.39 (0.04)	0.46 (0.02)	5.47 (0.27)	0.16 (0.02)	0.38 (0.03)	1.22 (0.04)	0.66 (0.03)	1.43 (0.14)	2.40 (0.16)
PM[d]	(*n*=10)	0.76 (0.09)	0.40 (0.01)	0.47 (0.02)	5.51 (0.38)	0.17 (0.01)	0.38 (0.02)	1.23 (0.03)	0.67 (0.05)	1.45 (0.13)	2.36 (0.21)
P/PM	(*n*=10)	0.76 (0.14)	0.36 (0.04)	0.53 (0.02)	5.43 (0.50)	0.15 (0.03)	0.36 (0.05)	1.25 (0.11)	0.71 (0.07)	ND[f]	ND[f]
Ethanol effect										
10% Et	(*n*=8)	0.77 (0.04)	0.33 (0.06)	0.49 (0.03)	4.55 (0.72)	0.17 (0.01)	0.34 (0.05)	1.27 (0.10)	0.56 (0.03)	1.47 (0.11)	2.26 (0.03)
P in 10% Et	(*n*=8)	0.82 (0.04)	0.36 (0.03)	0.48 (0.03)	5.38 (0.33)	0.16 (0.01)	0.34 (0.01)	1.18 (0.15)	0.58 (0.03)	1.37 (0.23)	2.54 (0.36)
PM in 10% Et	(*n*=8)	0.79 (0.08)	0.40 (0.03)	0.51 (0.04)	5.50 (0.40)	0.17 (0.01)	0.42 (0.03)	1.20 (0.03)	0.70 (0.05)	1.43 (0.14)	2.36 (0.17)
P/PM in 10% Et											
24 h	(*n*=8)	1.16* (0.23)	0.38 (0.05)	0.56* (0.01)	6.17* (0.23)	0.15 (0.01)	0.31 (0.03)	1.18 (0.03)	0.69 (0.03)	1.46 (0.18)	2.56 (0.09)
48 h	(*n*=10)	1.03* (0.08)	0.54* (0.02)	0.56* (0.04)	6.91* (0.21)	0.18 (0.01)	0.42 (0.02)	1.27 (0.06)	0.68 (0.03)	1.44 (0.33)	2.44 (0.14)
72 h	(*n*=8)	1.23* (0.15)	0.49* (0.02)	0.61* (0.07)	6.43* (0.38)	0.16 (0.01)	0.32 (0.04)	1.23 (0.04)	0.68 (0.02)	1.40 (0.11)	2.57 (0.12)
96 h	(*n*=8)	0.96* (0.10)	0.42 (0.03)	0.63* (0.05)	5.99 (0.28)	0.17 (0.01)	0.35 (0.05)	1.35 (0.08)	0.57 (0.07)	1.51 (0.03)	2.43 (0.24)
P/PM in 20% Et	(*n*=12)	0.91* (0.05)	0.39 (0.03)	0.54* (0.03)	6.42* (0.51)	0.14 (0.02)	0.42 (0.03)	1.22 (0.07)	0.69 (0.04)	1.41 (0.19)	2.41 (0.18)
P/PM in 40% Et	(*n*=10)	0.82 (0.09)	0.40 (0.04)	0.47 (0.06)	5.95 (0.28)	0.16 (0.01)	0.41 (0.02)	1.27 (0.05)	0.68 (0.03)	1.45 (0.11)	2.42 (0.19)
PM in 10% Et, then P/PM[e]	(*n*=10)	0.98* (0.04)	0.54* (0.03)	0.57* (0.03)	6.44* (0.18)	0.16 (0.02)	0.37 (0.02)	1.23 (0.07)	0.74 (0.06)	1.49 (0.09)	2.32 (0.40)
PM in 40% Et, then P/PM[e]	(*n*=10)	0.78 (0.07)	0.40 (0.02)	0.48 (0.04)	5.72 (0.37)	0.18 (0.02)	0.36 (0.01)	1.21 (0.11)	0.66 (0.02)	1.48 (0.18)	2.45 (0.16)

[a] BALB/cAnN.Cg-*Foxn1nu*/CrlNarl nude mice were inhaled with six 100 μg doses of formulations for 15 min at 8 h interval for 2 days. Except time duration study, organs were collected at 48 h after the first dosage delivered and the β-Gal activity was measured.
[b] The β-Gal activity was represented by mean with (SEM).
[c] Mice were inhaled with six 100 μg doses of P alone in water for 15 min at 8 h interval for 48 h after the first dosage delivered.
[d] 6% w/v of polymeric micelles (PM).
[e] Mice were inhaled with six doses of PM co-formulated with 10% or 40% ethanol (Et) for 15 min following inhaled with P/PM for 15 min for 48 h.
[f] Not determined (ND).
* Indicated a significant difference ($p<0.05$) compared with the same tissue of the control group.

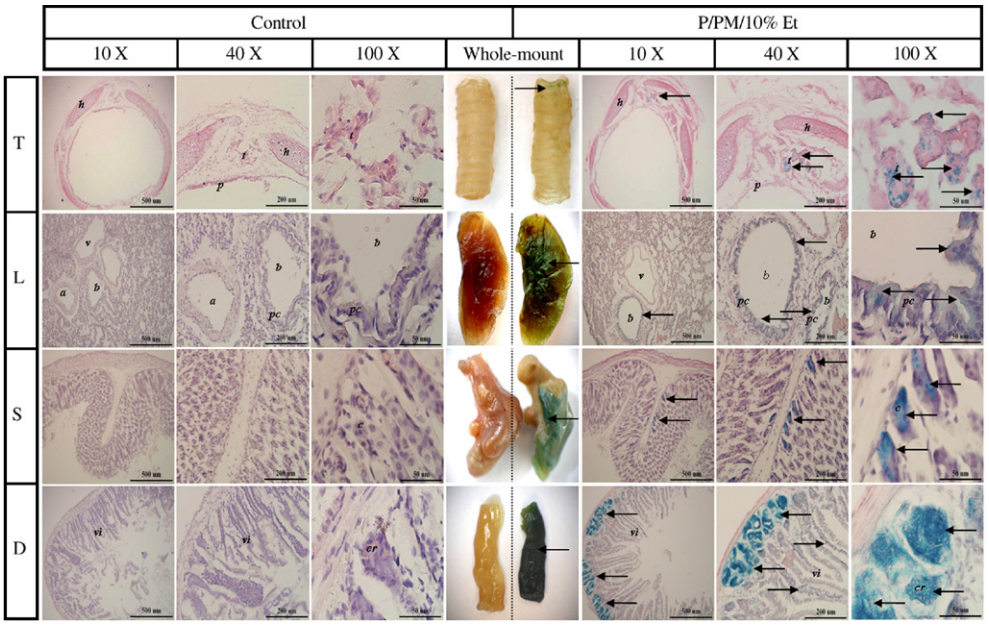

Fig. 3. The β-Gal whole mount and histological analysis on tissues of nude mice at 48 h after inhalation with six doses PM in 10% ethanol (Et) containing 100 μg of pCMV-*Lac Z* (P) (P/PM/10% Et) at 8 h intervals. The expression of β-Gal enzymes was indicated as blue-green color. All the images of histological cryosections were counter-stained with hematoxylin-eosin on trachea (T), lung (L), stomach (S) and duodenum (D). Note the expression of β-Gal enzyme indicated by the arrows and observed in the tracheal gland (t), pseudostratified columnar epithelium (pc), chief cell (c) and crypt cells (cr) of villi (vi). No inflammatory reaction was noted after P/PM co-formulated with 10% ethanol delivery. The other abbreviations were described as hyaline cartilage (h); pseudostratified epithelium (p); bronchiole (b); artery (a); and vein (v). Original magnification is indicated on each column. The whole mount original magnification in four tissues is ×10.

the β-Gal activity was mainly found in pseudostratified columnar epithelium (pc) around bronchioles. In GI tract, the β-Gal activity was considerably strong at chief cell (c) of gastric mucosa (Fig 3), and crypt cell (cr) and villi (vi) in duodenum (Fig. 3). No inflammatory reaction was noted in all images of cryosections.

3.6. Effect of ethanol on apparent permeability and resistance of stomach and intestine

Due to the absorption taking place from multiple sites of inhaled deposition and the fact that major absorption site during inhalation delivery was reported through GI tract (63.3%), lung (24.2%), and nasal (12.5%) [34], the ethanol effect on the enhancement of gene transfer in GI tract was evaluated. After 2 days, 8 h interval *in vivo* inhaled delivery of pCMV-*Lac Z*/PM co-formulated with 10%–40% ethanol, the apparent intracellular (measured by ^{14}C-estradiol) and intercellular (measured by ^{14}C-mannitol) permeability coefficients of both stomach and intestine were determined by *in vitro* diffusion assay. The results showed that in both stomach and intestine, PM alone delivery did not influence either the intracellular or the intercellular permeability (Fig. 4A and B). Using inhaled formulation containing 10% ethanol, the apparent intracellular permeability coefficient ($P_{estradiol}$) was increased by 42% in stomach and by 141% in intestine tissues, compared to the control groups (Fig. 4A). However, when the content of ethanol in the formulation increased to 20%, there was no significant difference on the apparent intracellular permeability coefficient ($P_{estradiol}$) between the test and control groups. Furthermore, the intracellular apparent permeability coefficient ($P_{estradiol}$) was significantly decreased in stomach (38%) and intestine (38%) tissues when mice inhaled with pCMV-*Lac Z*/PM co-formulated with 40% ethanol (Fig. 4A). On the other hand, when mice inhaled with pCMV-*Lac Z*/PM co-formulated with 10%–40% ethanol, the $P_{mannitol}$ values detected in all treatments were all significantly increased in stomach (54%–83%) and intestine (41%–97%) tissues with increasing ethanol concentration, compared to the control groups (Fig. 4B). The results indicated that the presence of ethanol in inhaled delivery directly affected the membrane penetration in both stomach and duodenum.

In order to further directly elucidate the mechanisms of ethanol effect on the permeation of GI tissues, we orally delivered pCMV-*Lac Z*/PM co-formulated with 10%–40% ethanol and both $P_{estradiol}$ and $P_{mannitol}$ were determined. The results showed that a similar ethanol effect on both $P_{estradiol}$ and $P_{mannitol}$ were detected compared to those detected in mice with inhaled delivery (Fig. 4A and B). The $P_{estradiol}$ was increased in stomach (83%) and intestine (95%) tissues after orally delivery with pCMV-*Lac Z*/PM co-formulated with 10% ethanol, compared to the control groups. Furthermore, the $P_{estradiol}$ was significantly decreased in stomach (43%) and intestine (50%) tissues with formulation co-formulated with 40% ethanol (Fig. 4A). Similarly, the $P_{mannitol}$ values were significantly increased in stomach (28%–77%) and intestine (32%–51%) tissues along with increasing ethanol concentrations in the formulation, compared to the control groups (Fig. 4B). Thus, these results suggested that the enhancing effect of ethanol on gene transfer could be due to its influence on GI tissues in both inhaled and orally deliveries.

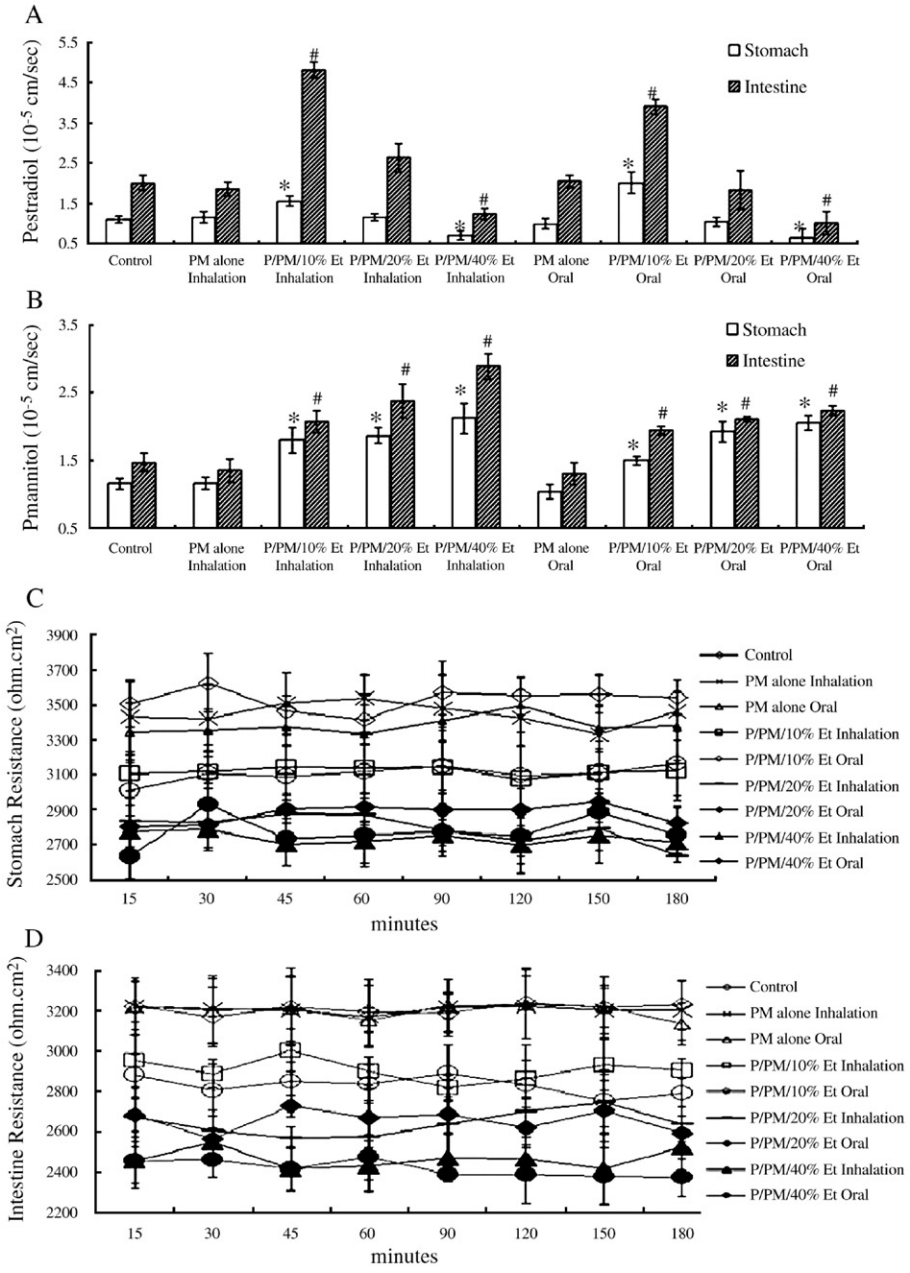

Fig. 4. The effect of ethanol (10%–40%) on apparent permeability coefficients of (A) $P_{estradiol}$ (intracellular indicator) and (B) $P_{mannitol}$ (intercellular indicator), and tissue resistance on (C) stomach and (D) intestine of nude mice. The *in vitro* diffusion studies of stomach and intestine were performed after inhaled and orally delivery with six doses of pCMV-*Lac Z* (P)/PM co-formulated with water, 10%–40% of ethanol (Et) at 8 h interval for 2 days. Nude mice received no further treatment or PM alone was as the control groups. All experimental groups were performed by 5 individuals. * and # showed significant difference in apparent permeability coefficients (*P*), compared to the control group in stomach and intestine, respectively.

To further confirm the ethanol effect on tissues permeation, the resistance was measured as an indicator for the integrity of tissues. For all control groups, the resistances of stomach and intestine tissues were at the range of 3300–3500 Ω cm^2 and 3100–3300 Ω cm^2, respectively (Fig. 4C and D). In the groups with gene delivery either by inhalation or by oral administration with formulation co-formulated with various concentrations of ethanol, the resistance measured for stomach and intestine were decreased to the range of 2600–3200 and 2300–3000 Ω cm^2, respectively (Fig. 4C and D). These data again confirmed that the membrane integrity was impaired with increasing concentration of ethanol in the formulations.

3.7. Determination of delivered gene expression after oral administration

Since the *in vitro* diffusion study demonstrated that oral administrated ethanol enhanced the permeation of stomach and duodenum tissues, further investigation of ethanol effect on orally delivered gene expression was examined. In mice orally

Table 3
The β-Gal activities in nude mice tissues orally delivery with pCMV-*Lac Z* (P)/polymeric micelles (PM)

Oral[a]	β-Gal activity (mU/mg protein) in tissues[b]									
	Trachea	Lung	Stomach	Duodenum	Heart	Liver	Kidney	Testis	Nasal	Skin
Formulations										
P alone[c]	(*n*=8)	0.77 (0.08)	0.38 (0.04)	0.46 (0.03)	5.43 (0.16)	0.15 (0.02)	0.38 (0.04)	1.23 (0.07)	0.67 (0.03)	1.44 (0.07)	2.47 (0.26)
PM[d]	(*n*=8)	0.72 (0.06)	0.35 (0.06)	0.44 (0.05)	5.25 (0.28)	0.17 (0.03)	0.40 (0.05)	1.20 (0.06)	0.64 (0.03)	1.45 (0.05)	2.46 (0.18)
P/PM	(*n*=8)	0.82 (0.09)	0.36 (0.05)	0.47 (0.08)	5.48 (0.22)	0.18 (0.03)	0.33 (0.04)	1.17 (0.11)	0.69 (0.07)	ND[f]	ND[f]
Ethanol effects										
10% Et	(*n*=8)	0.81 (0.05)	0.37 (0.02)	0.50 (0.09)	5.03 (0.16)	0.16 (0.02)	0.36 (0.05)	1.18 (0.20)	0.63 (0.04)	1.45 (0.17)	2.74 (0.38)
P in 10% Et	(*n*=8)	0.82 (0.02)	0.36 (0.01)	0.45 (0.02)	5.56 (0.59)	0.15 (0.03)	0.35 (0.03)	1.11 (0.11)	0.67 (0.10)	1.54 (0.30)	2.90 (0.16)
PM in 10% Et	(*n*=9)	0.82 (0.06)	0.42 (0.05)	0.49 (0.03)	5.46 (0.28)	0.14 (0.03)	0.38 (0.04)	1.23 (0.05)	0.64 (0.10)	1.39 (0.05)	2.38 (0.25)
P/PM in 10% Et	(*n*=10)	0.75 (0.09)	0.53* (0.05)	0.63* (0.03)	9.29* (0.32)	0.17 (0.01)	0.38 (0.02)	1.19 (0.11)	0.63 (0.04)	1.36 (0.16)	2.41 (0.09)
P/PM in 20% Et	(*n*=10)	0.83 (0.02)	0.40 (0.02)	0.59* (0.04)	7.00* (0.15)	0.16 (0.02)	0.37 (0.02)	1.22 (0.08)	0.66 (0.02)	1.37 (0.06)	2.44 (0.09)
P/PM in 40% Et	(*n*=8)	0.75 (0.06)	0.41 (0.02)	0.46 (0.05)	5.93 (0.17)	0.19 (0.05)	0.38 (0.03)	1.17 (0.06)	0.69 (0.03)	1.44 (0.08)	2.35 (0.32)
PM in 10% Et, then P/PM[e]	(*n*=8)	0.79 (0.03)	0.51* (0.03)	0.64* (0.04)	7.80* (0.56)	0.18 (0.01)	0.41 (0.02)	1.27 (0.03)	0.66 (0.04)	1.40 (0.04)	2.38 (0.31)
PM in 40% Et, then P/PM[e]	(*n*=10)	0.76 (0.07)	0.38 (0.02)	0.49 (0.02)	5.69 (0.45)	0.16 (0.02)	0.36 (0.01)	1.21 (0.10)	0.62 (0.05)	1.36 (0.06)	2.39 (0.20)

[a] BALB/cAnN.Cg-*Foxn1nu*/CrlNarl nude mice were orally received six 40 μg doses of formulations at 8 h interval for 2 days. Organs were collected at 48 h after the first dosage delivered and the β-Gal activity was measured.
[b] The β-Gal activity was represented by mean with (SEM).
[c] Mice were orally received six 40 μg doses of P in water.
[d] 6% w/v of polymeric micelles (PM).
[e] Mice were orally received six doses of PM co-formulated with 10% or 40% ethanol (Et) following orally delivery with P/PM.
[f] Not determined (ND).
* Indicated a significant difference ($p<0.05$) compared with the same tissue of the control group.

administered with PM alone or pCMV-*Lac Z*/PM mixture, there was no significant difference on the β-Gal activities in all tissues tested, compared to that detected in mice orally administered with pCMV-*Lac Z* alone (Table 3). Yet, the β-Gal activities were significantly increased in the lung (39%), stomach (37%) and duodenum (71%) tissues from mice orally administered with pCMV-*Lac Z*/PM mixture co-formulated with 10% ethanol. Lower levels of elevated β-Gal activities were detected in

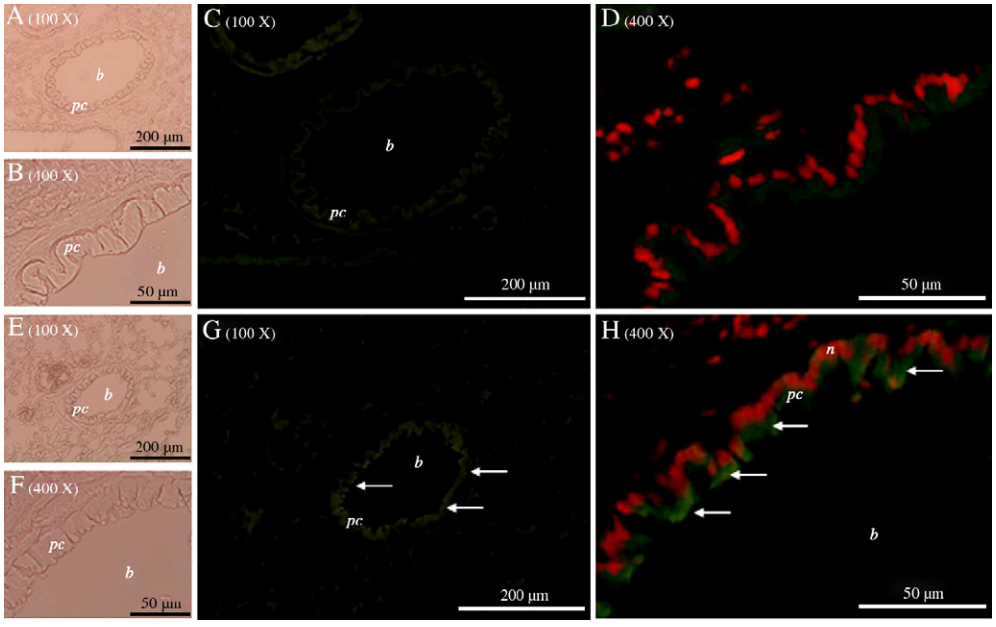

Fig. 5. *In vivo* inhaled delivery six 100 μg doses of pcDNA3-CFTR/polymeric micelles (PM) with co-administrated with 10% ethanol on nude mice. The lung cryosections were hybridized with goat anti-CFTR antibody (1:200) and donkey anti-goat IgG-FITC (1:200), and were co-stained with propidium iodide (PI) for localization of nucleus (*n*). Phase and immunofluorescence images of cryosections were taken from control (A–D) and delivered with pcDNA3-CFTR/PM co-formulated with 10% ethanol (E–H), respectively. The CFTR protein and cell nucleus were observed with FITC (green signal) and PI filters (red signal) under immunofluorescence microscope. The arrows indicated the expression of CFTR in pseudostratified columnar epithelium (pc) around bronchiole (b). Original magnification was indicated on each picture.

stomach (28%) and duodenum (29%) tissues from mice orally administered with pCMV-*Lac Z*/PM co-formulated with 20% ethanol, compared to the control groups (Table 3). No significant β-Gal activity was detected in mice administered with pCMV-*Lac Z*/PM co-formulated with 40% ethanol. These were similar to the results from inhalation delivery and no influences of β-Gal activity under oral delivery with PM alone, 10% ethanol alone and PM or plasmid co-formulated with 10% ethanol were detected (Table 3). Furthermore, in mice pretreated with PM co-formulated with 10% ethanol (PM in 10% Et) and followed by oral delivery with pCMV-*Lac Z*/PM (P/PM), the β-Gal activity was significantly increased in lung (34%), stomach (39%) and duodenum (44%) tissues, but no detectable β-Gal activity found in mice pretreated with PM co-formulated with 40% ethanol, compared to the control groups (Table 3). The increased *Lac Z* expression in lung through both inhalation and oral delivery with pCMV-*Lac Z*/PM mixture co-formulated with 10% ethanol indicated that modification of GI tissues could influence gene transfer to multiple tissues including lung, stomach and duodenum.

3.8. Immunofluorescence detection of delivered CFTR in lung

To evaluate the feasibility of using 10% ethanol to enhance the functional gene delivery to lung area, six doses of PM co-formulated with 10% ethanol containing 100 μg of pcDNA3-CFTR were delivered to mice. Fig. 5A–D and E–H were the phase and immunofluorescence images of cryosections of lung tissues from control mice and mice delivered with pcDNA3-CFTR/PM co-formulated with 10% ethanol, respectively. The CFTR protein and cell nucleus were observed with FITC (green signal) and PI (red signal) filters under the immunofluorescence microscope. The 40% higher intensity of green signal was observed at lung sections from mice delivered with pcDNA3-CFTR/PM co-formulated with 10% ethanol by *SimplePCI* image software system, and the expression of CFTR was increased in pseudostratifled columnar (*pc*) epithelium around bronchiole (Fig. 5H compared with Fig. 5D).

4. Discussion

The non-ionic PEO-PPO-PEO polymers consisting of ethylene oxide (EO) and propylene oxide (PO) chains are arranged in a triblock structure and at concentration above the CMC, the unimer molecules tend to aggregate and form micelles which have hydrophobic core formed by PPO chains and hydrophilic shell formed by PEO chains [6,35,36]. It has been suggested that aggregation may enclose the biologic molecules (such as DNA or protein) into the micelles, leading to a decrease in the amount of free molecules available [5,18]. Thus, the plasmid may be enclosed to the aggregates during the micelle formation, reducing the total negative charge of the mixture and decreasing the amount of DNA exposed to DNase I.

The airway epithelium lining the alveoli establishes the dominant airway barrier and mostly prevents the uptake of foreign materials, including gene transfer targeted to airway region [37]. In addition, extracellular barriers such as mucus and glycocalyx may need to be conquered for airway gene transfer. It has been shown that mucus reduces the transfection efficiency of most viral- and non-viral driven gene transfer. However, the transfection efficiency could be increased through pre-treatment with mucolytics or the antichiolinergic drug, glycopyrolate *in vitro* and *in vivo* [38]. Ethanol was also shown to be one of the modifiers to enhance penetration of particles to airway epithelium with prolonged retention in trachea and bronchial area [39,40]. It has been demonstrated that ethanol was able to increase the absorption of ^{131}I-albumin from the tracheobronchial face into the circulation system [40]. We observed that β-Gal activities were enhanced by 32% at tracheal and 38% at lung tissues as well as 22% and 26% at stomach and duodenum areas by inhalation co-formulated of 10% ethanol with pCMV-*Lac Z*/PM (six doses of 100 μg). Desigaux et al. [19] also showed a similar β-Gal expression (0.08 mol/lung vs. ours at 0.05 mol/lung) after direct intratracheal administration of Lutrol-DNA and their formulation gave 5-fold higher gene expression level compared with PEI-DNA polyplexes administered via the same delivery route. Both their and our studies found that the around bronchioles of lung showed positive staining that reflects the β-Gal activity, where stem cells are located [41,42]. There was a similar β-Gal activity detected in all tissues between mice pre-treated with 10% ethanol and inhaled with pCMV-*Lac Z*/PM co-formulated with 10% ethanol (Table 2). Yang et al. [36] have showed that PEO-PPO-PEO polymer enhanced reporter gene expression in a mouse strain- and promoter-type dependent manner. The transcriptional activation of gene expression by this polymer was reported through the activation of NF-êB signaling pathway [35]. However, no enhanced reporter gene expression was detected in athymic Balb/c nu/nu mice [36]. In our study, the results showed that inhalation of 10% ethanol alone, PM alone, PM or plasmid co-formulated with 10% ethanol in nude mouse did not enhance β-Gal activity (Table 2), suggesting that the detected enhanced β-Gal activity may not through PM or 10% ethanol.

We also found that β-Gal activities were significantly increased by 22% and 26% at stomach and duodenum areas after inhalation delivery with pCMV-*Lac Z*/PM co-formulated with 10% ethanol. In general, it has been known that absorption takes place from multiple sites of inhalation deposition. In addition, the delivered particles or drugs released from particles either (1) deposited in superficial lipid layer of the mucus, (2) redistributed over the epithelium, (3) or even could be cleared in the GI tract [39]. Sakagami et al. [34] used nose-only inhalation with fluorescein in rodent had demonstrated that 63.3% of delivered fluorescein was distributed to GI tact, and only 24.2% and 12.5% to lung and nasal, respectively. From our *in vitro* diffusion study with either inhalation or oral formulation delivery of 10% ethanol at 8 h interval for 2 days, our data suggested that the apparent permeability coefficients of stomach and intestine were all increased in both inter- and intracellular pathways through GI and airway. These indicated that GI would be the major absorption area after either inhaled or oral delivery. In addition, Brown et al. [43] showed that chronic oral ingestion of ethanol can increase alveolar barrier permeability in alveolar type II cells. Guidot et al. [44] also showed that ethanol could modify rat alveolar epithelial barrier function by orally feeding

with liquid diet containing 36% of ethanol. In our studies, similar results showed that co-administration of 10% ethanol with pCMV-*Lac Z*/PM by oral delivery could enhance the permeation in GI tissue, subsequently enhance the *Lac Z* expression in lung by 34% (Table 3).

Our previous study using arginine-glycine-aspartic acid (RGD peptide) and EDTA indicated that the internalization of PM mainly involved intracellular endocytosis pathway and could be enhanced by the opening of tight junction in the duodenunal tissues [5]. In this study, our results further showed that the intracellular permeability was increased by co-formulated with 10% ethanol, but decreased along with higher concentrations (20% and 40%) of ethanol used in the formulation, meanwhile, the *Lac Z* expression in tissues was decreased when mice inhaled or oral delivery with the pCMV-*Lac Z*/PM formulations co-administrated with 20% or 40% of ethanol (Tables 2 and 3). Similar results from other studies have showed that ethanol at concentration greater than 30% could decrease adenovirus-mediated gene expression to the bladder epithelium of rodents [22]. Also, an ethanol provoked decrease in the fluidity of synaptic plasma membrane, which subsequently affected the membrane transport, might be through the intracellular pathway [45]. Wilson and Hoyumpa [46] showed that ethanol affected the lipid integrity of cell membrane and increase the intramembrane fluidity for the intestinal transport. The increasing intramembrane fluidity could directly enhance the intracellular internalization of vesicle by inducing perturbation of the lipid bilayer [47,48]. However, it is also known that high concentration, long-term or chronic administration of ethanol was able to rigidify cell membrane by decreasing its fluidity [49,50]. The rigidifying effect of ethanol was resulted from an increase of phosphatidylethanolamine (PE) in the membrane composition. The PE has been described as rigidifying phospholipids because of the hydrogen bond formations between its primary amine and a neighboring phospholipids head group [49]. In our inhaled/oral formulation co-formulated with 20% or 40% ethanol, the intracellular pathway was decreased in GI membrane, indicated that PM internalization would be reduced and following decrement of the transgene expression in GI and even the lung area.

On the other hand, it has been reported that ethanol could open an effective pore on intact human epidermal membrane to be an effective radius of 15–20 Å [51] and disrupt tight junction by activating MLCK (myosin light chain kinase) in Caco-2 cell [26]. In our studies, the resistance values were at a similar range (1047–4573 Ω cm^2) in gastric mucosa reported by other researchers [52] and 10% ethanol decreased the resistance values of GI tissues (Fig. 4C and D). This indicated that the membrane integrity was impaired. Although co-administration of ethanol (40%) could significantly increase GI intercellular permeability, we did not observe the enhancement of gene expression in all tissues. In general, the average range of pore radius of human small intestine was reported to be 8–15 Å [53,54] and the junctional pore size was opened only up to 12–23 Å by EGTA. In previous studies, using a series of polyethylene glycol (PEG) which is a linear and hydrophilic molecules with different molecular weights, the maximum pore opened by those tight junction openers (sodium lauryl sulfate, cytochalasin B and EDTA) could only increase the permeation of PEG on mucus membrane up to molecular weight of 2000 [55–57]. However, using DLS measurement the particle size of pCMV-*Lac Z*/PM co-formulated with ethanol (10%–40%), our data showed that the particle size increased up to 480 nm above, indicating that it must be larger than the opened pore located at intercellular tight junction by ethanol. Therefore, the transport pathway could be predominantly involved in the intracellular endocytosis with less permeability through intercellular pathway.

With inhalation delivery of plasmid/PM co-formulated with 10% ethanol in our system, this formulation was able to deliver foreign genes (including *Lac* Z and CFTR genes) into airway and GI tract. We were able to demonstrate that an increased CFTR expression (about 40%) around the bronchioles after the inhaled delivery. This enhanced effect was similar to the increased *Lac* Z (38%) after inhalation with the pCMV-*Lac Z*/PM co-formulated with 10% ethanol in lung area. Alton et al. [58] also delivered CFTR cDNA−liposome complexes into the airways of mutant mouse (*cf/cf)* by nebulization, and observed an about 50% correction in cAMP related chloride responses restoration of the deficit between wild type mice and untreated (*cf/cf)* controls. Since the cystic fibrosis occurs at the respiratory, GI and reproductive systems as well as sweat glands, our approach would allow the delivered gene distributing to trachea, lung, stomach and duodenum. Further investigations on gene therapy applied for cystic fibrosis will be examined in our laboratory.

5. Conclusions

The present study provides a promising system to deliver functional plasmid into lung tissues. The plasmid/PM co-formulated with 10% ethanol was able to achieve promising transfection efficiency *in vivo*. The possibility of enhanced PM-mediated transfecting efficiency is through the mechanism involving the increasing intracellular pathway in stomach and intestine.

Acknowledgements

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