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Nonionic Polymeric Micelles for Oral Gene Delivery *In Vivo*

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ABSTRACT

The main aim of this study was to investigate the feasibility of using nonionic polymeric micelles of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO-PPO-PEO) as a carrier for oral DNA delivery *in vivo*. The size and appearance of DNA/PEO-PPO-PEO polymeric micelles were examined, respectively, by dynamic light scattering and atomic force microscopy, and their ζ potential was measured. Expression of the delivered *lacZ* gene in various tissues of nude mice was assessed qualitatively by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of sections and quantitatively by measuring enzyme activity in tissue extracts, using the substrate of β-galactosidase, chlorophenol red-β-D-galactopyranoside. In addition, the types of cells expressing the *lacZ* gene in the duodenum were identified by histological analysis. DNA/PEO-PPO-PEO polymeric micelles are a single population of rounded micelles with a mean diameter of 170 nm and a ζ potential of -4.3 mV. Duodenal penetration of DNA/PEO-PPO-PEO polymeric micelles was evaluated *in vitro* by calculating the apparent permeability coefficient. The results showed a dose-independent penetration rate of $(5.75 \pm 0.37) \times 10^{-5}$ cm/sec at low DNA concentrations (0.026–0.26 μg/μl), but a decrease to $(2.89 \pm 0.37) \times 10^{-5}$ cm/sec at a concentration of 1.3 μg/μl. Furthermore, when 10 m*M* RGD peptide or 10 m*M* EDTA was administered before and concurrent with the administration of DNA/PEO-PPO-PEO polymeric micelles, transport was inhibited ($[0.95 \pm 0.57] \times 10^{-5}$ cm/sec) by blocking endocytosis or enhanced ($[29.8 \pm 5.7] \times 10^{-5}$ cm/sec) by opening tight junctions, respectively. After oral administration of six doses at 8-hr intervals, the highest expression of transferred gene *lacZ* was seen 48 hr after administration of the first dose, with gene expression detected in the villi, crypts, and goblet cells of the duodenum and in the crypt cells of the stomach. Reporter gene activity was seen in the duodenum, stomach, and liver. Activity was also seen in the brain and testis when mice were administered 10 m*M* EDTA before and concurrent with DNA/PEO-PPO-PEO polymeric micelle administration. *lacZ* mRNA was detected in these five organs and in the blood by reverse transcription-polymerase chain reaction. Taken together, these results show efficient, stable gene transfer can be achieved in mice by oral delivery of PEO-PPO-PEO polymeric micelles.

OVERVIEW SUMMARY

Gene delivery through the gastrointestinal tract not only has many potential applications, but is also less invasive and more easily performed. PEO-PPO-PEO polymeric micelles were investigated to determine the feasibility of oral gene delivery and their usefulness as vectors for gene delivery *in vivo*. The stomach, duodenum, liver, and circulating blood are the primary targets for gene transfer by oral gene delivery using these nonionic polymeric micelles. Furthermore, gene transfer could be enhanced by administration of 10 m*M* EDTA before the administration of DNA/PEO-PPO-PEO polymeric micelles. Our results further advance the promising therapeutic usage of PEO-PPO-PEO polymeric micelles for gene transfer by oral delivery.

INTRODUCTION

GENE DELIVERY through the gastrointestinal (GI) tract not only has many potential applications, such as the comple-

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mentation of single gene disorders (e.g., cystic fibrosis), but also has the advantages of ease of administration, a large surface area for targeting, and the accessibility to stem cells located in the GI tract (Alton *et al.*, 1993). Nanotechnology and nanomaterials are being developed as advanced approaches to overcome some of the problems associated with oral viral gene delivery (Ledley, 1996; Croyle *et al.*, 1998a; Mao *et al.*, 2001; Cheng *et al.*, 2003). Limitations in the use of nanoparticles as oral gene delivery carriers are that they must be absorbed from the GI tract and they must overcome the physiological conditions in the GI tract, such as extreme pH, enzyme activity, and GI motility. However, factors affecting the absorption of polymeric nanoparticles in GI membranes are still not well understood. In particular, studies on the relationship between gene delivery and the physicochemical properties of the polymeric carrier, such as the stability of the complexes, their interaction with biomembranes, and the possible entry pathways of these polymeric complexes, are needed to reveal the application potential of these polymers (Florence, 1997; Kabanov *et al.*, 2002). In addition, biocompatibility and biodegradability of the polymer complexes are another two factors requiring more consideration. New biomaterials able to overcome all these problems would be of great interest for designing nanoparticulate carriers for mucosal oral gene delivery.

A nonionic PEO-PPO-PEO triblock copolymer has proved useful in the medical, pharmaceutical, and cosmetic fields (Saski, 1968; Guy and Hadgraft, 1987; Stolnik *et al.*, 1995; Liaw and Lin, 2000; Kabanov *et al.*, 2002). The PEO-PPO-PEO copolymer is resistant to adsorption by serum proteins, liver uptake, and enzyme degradation (Yalin *et al.*, 1997; Kabanov *et al.*, 2002). Drug formulations containing these copolymer complexes were found to be more stable in the blood stream (Schmolka, 1991; Kabanov *et al.*, 2002). Miyazaki *et al.* (1995) found that this type of nanopolymeric micelle showed good potential as a drug delivery system because of the simplicity of micelle preparation and the ease of drug incorporation into the micelles. Further advantages of the nonionic PEO-PPO-PEO triblock copolymer are its increased drug-loading capacity, sustained systemic release, and increased half-life (Liaw and Lin, 2000). We have reported on the efficacy of gene delivery of topical eye drops formulations using DNA/PEO-PPO-PEO polymeric micelles in the presence of interfering factors, such as tear mucin and degradation enzymes (Liaw *et al.*, 2001). Using these hydrophilic micelles, gene expression was increased by 28 and 38% in the eyes of nude mice and rabbits, respectively. Gene expression was further enhanced up to 70% by pretreatment with EDTA or cytochalasin B, which are thought to alter tight junctions in the cornea (Rojanasakul *et al.*, 1990; Liaw and Robinson, 1992; Liaw *et al.*, 2001). The aim of the present study was to explore the ability of nonionic PEO-PPO-PEO polymeric micelles to protect and deliver plasmid DNA orally *in vivo*. We assessed the expression of a reporter gene in GI tissues and examined gene distribution to other organs through the blood circulation. The *in vitro* duodenal penetration of nanosized DNA/PEO-PPO-PEO polymeric micelles was quantified with Hoechst H33258 DNA-staining dye. EDTA, a tight junction-opening enhancer (Rojanasakul *et al.*, 1990; Liaw *et al.*, 2001), and RGD peptide, a receptor-mediated endocytotic inhibitor (Croyle *et al.*, 1998b), were used to modify *in vitro* gene transfer in the duodenum.

MATERIALS AND METHODS

Materials

PEO-PPO-PEO copolymer, with an average molecular mass of 8400 Da, was obtained from BASF (Ludwigshafen, Germany). EDTA and RGD peptide (arginine-glycine-aspartic acid) were obtained from Sigma (St. Louis, MO). RGE peptide (arginine-glycine-glutamic acid) was obtained from GIBCO-BRL (Rockville, MD). All other chemicals were of analytical reagent grade and were used without further purification.

Animals

Male nude mice (BALB/c-nu), 6 to 8 weeks old, were used for *in vivo* and *in vitro* oral delivery studies and were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). They were maintained under specific pathogen-free conditions.

Plasmid DNA

The pCMV-*lacZ* plasmid, carrying the *lacZ* gene encoding β-galactosidase (β-Gal) under the control of the cytomegalovirus (CMV) promoter, was used as the transferred gene. Plasmid DNA was amplified in *Escherichia coli* host strain TG-1 and purified by equilibrium centrifugation on a CsCl–ethidium bromide gradient (Macgregor and Caskey, 1989). Purity was determined with a QIAprep Spin column under endotoxin-free conditions. Stability of the plasmid DNA prepared was determined by electrophoresis on an agarose gel followed by ethidium bromide staining. DNA concentration was measured by ultraviolet (UV) absorption at 260 nm. Stability of plasmid DNA in DNA/PEO-PPO-PEO polymeric micelle formulations was determined as described previously by electrophoresis immediately after preparation, after 2 days of storage at room temperature, and after 3 freeze–thaw cycles (Liaw *et al.*, 2001).

Preparation of DNA/PEO-PPO-PEO polymeric micelles

All DNA/PEO-PPO-PEO polymeric micelles were freshly prepared on a weight percentage basis as described previously (Liaw *et al.*, 2001). Polymeric micelles were formed with 6% (w/w) PEO-PPO-PEO in water. A pyrene fluorescence probe was used to determine the formation of micelles as previously reported (Liaw and Lin, 2000). Various concentrations of plasmid DNA were gently mixed with PEO-PPO-PEO polymeric micelles in a vial for 2 hr at 25°C.

Size and ζ potential of DNA/PEO-PPO-PEO polymeric micelles

The size and ζ potential of complexes in mixtures containing plasmid DNA (0.26 μg/μl) and a 6% PEO-PPO-PEO polymer solution were measured and compared with mixtures containing plasmid DNA (0.26 μg/μl) or 6% PEO-PPO-PEO polymer solution alone. The average particle size and ζ potential of the polymeric micelles were determined by quasielastic laser dynamic light scattering (DLS) (Zetasizer 3000; Malvern Instruments, Malvern, UK), using an assumed refractive index ratio of 1.33 and a viscosity of 0.88 (Liaw *et al.*, 2001). The

sampling time for each sample was 10 μsec and the experimental duration was 100 sec. All measurements were performed at 25°C at a measurement angle of 90°.

Atomic force microscopy

Ten microliters of DNA/PEO-PPO-PEO polymeric micelles was placed on a mica surface with no further treatment. The AFM (NanoScope III; Digital Instruments/Veeco Metrology Group, Santa Barbara, CA) was operated in constant tapping mode, as described in the previous study (Liaw *et al.*, 1999). The cantilevers were standard NanoProbe silicon single-crystal levers (125 μm); the constant force mode was used with a typical scan frequency of 5 Hz. A scanner with a 1-μm scanning range was used, and all images were collected within a 1×1 or 3×3 μm^2 area. Unless otherwise stated, all images shown were subjected only to the normal image processing of leveling.

In vitro *duodenal penetration studies*

For the *in vitro* studies, nude mice were killed by cervical dislocation and upper duodenal sections, from the pylorus to 1 cm distal to the ligament of Treitz, were retrieved. Duodenal tissues were gently rinsed three times in warm phosphate-buffered saline (PBS) and then placed in an *in vitro* vertical diffusion apparatus (Liaw and Lin, 2000). A tissue surface area of 0.09 cm^2 was exposed to the donor and receiver compartments of the diffusion apparatus, containing 0.5 and 6 ml of PBS, respectively. DNA/PEO-PPO-PEO polymeric micelles and other reagents were added to the donor compartment, and 0.5-ml samples were taken from the receiver compartment at various sampling times; the volume in the receiver compartment was maintained by the addition of 0.5 ml of prewarmed PBS. The concentration of DNA penetrating through the duodenum in the presence or absence of 10 m*M* RGD, 10 m*M* RGE, or 10 m*M* EDTA was assayed with Hoechst H33258 dye (Molecular Probes, Eugene, OR). A F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) was set at a fixed excitation wavelength of 352 nm and fluorescence was determined at an emission wavelength of 460 nm. The fluorescence of penetrating plasmids in the tissues was compared with a standard curve generated using plasmid DNA at 0.1 to 500 μg/ml with H33258 dye (Jong *et al.*, 1997). The apparent permeability coefficient (P) was calculated according to the following equation as described previously (Liaw *et al.*, 1999): $P = (dC/dt)V/A \times C_0$, where $V(dC/dt)$ is the steady state rate of DNA appearing in the receiver chamber after the initial lag time, C_0 is the initial plasmid concentration in the donor chamber, and A is the area of duodenal tissue exposed (0.09 cm^2). Data from all experiments were pooled to determine the mean and standard error of the mean. Analysis of variance (ANOVA) using Dunnett's multiple comparison tests with a 95% confidence level determined the significance of differences between each group of experiments.

Oral gene transfer in vivo

For the *in vivo* studies, nude mice were fasted for 24 hr before the experiments, but were allowed free access to water. Formulations were administered with a stomach feeding needle for mice (KN-342; Natume Seisakusho,). Six doses of each polymeric micelle formulation (150 μl), formed from plasmid (0.26 μg/μl) and 6% (w/v) PEO-PPO-PEO, were administered at 8-hr intervals (6 A.M., 2 P.M., and 10 P.M.). Mice receiving only plasmid DNA or polymeric micelles served as control groups. To evaluate gene transfer *in vivo*, mice were killed by cervical dislocation at set time points and all major organs and tissues were removed and processed immediately for individual analysis. The duodenum was dissected from the pylorus to 1 cm distal to the ligament of Treitz. All tissues were gently rinsed three times in warm PBS, pH 7.4. In transfection enhancer studies, 150 μl of enhancer (10 m*M* EDTA) was administered orally 10 min before and concurrent with the administration of each dose of DNA/PEO-PPO-PEO polymeric micelles.

β-Galactosidase enzyme histochemistry

Organs and tissues were immersed for 5 min in ice-cold fixation solution (2% paraformaldehyde, 0.2% glutaraldehyde, and 5 m*M* EGTA in 100 m*M* sodium phosphate buffer, pH 7.6) and then rinsed in ice-cold PBS containing 5 m*M* EGTA. EGTA was added to these solutions to abolish endogenous β-Gal activity (Rosenberg *et al.*, 1992; Sferra *et al.*, 1997; Weiss *et al.*, 1999). Two adjacent 1-cm sections were taken from the entire length of the duodenum and separated in paired series. One series of sections was frozen in cryoprotective medium (O.C.T.; Sakura Finetek U.S.A., Torrance, CA) and the other series of sections was processed for *en bloc* β-Gal staining. Sections from brain, liver, stomach, and testis were paired and processed in the same way.

Cryosections (10 μm) of the O.C.T.-embedded organs were fixed for 5 min at 4°C in 4% paraformaldehyde and 0.2% glutaraldehyde in 100 m*M* phosphate buffer, pH 7.4, washed twice in PBS, and stained for 24 hr at 37°C in 100 m*M* sodium phosphate buffer, pH 7.4, containing 1.3 m*M* $MgCl_2$, 3 m*M* $K_3Fe(CN)_6$, 3 m*M* $K_4(CN)_6$, and a 1-mg/ml concentration of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; GIBCO-BRL, Grand Island, NY), according to the method of Oshima *et al.* (1998). Tissues to be stained *en bloc* were fixed for 90 min at 4°C in 4% paraformaldehyde and then incubated for 24 hr at 37°C with PBS containing 10 m*M* $K_4Fe(CN)_6$, 10 m*M* $K_3Fe(CN)_6$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 (NP-40), 2 m*M* $MgCl_2$, and X-Gal (1 mg/ml). The *lacZ* gene was considered to be expressed when the tissue was blue-green in color under an operating microscope at magnifications of $\times$40 and $\times$100, unless specifically mentioned otherwise. In addition, cryosections (10 μm) were stained with hematoxylin–eosin for pathological assessment.

Preparation of tissue extracts and determination of transgene expression

β-Gal expression 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 previously described (Liaw *et al.*, 2001). Total tissue protein was measured with a DC protein assay reagent kit (Bio-Rad, Hercules, CA) and used to normalize the β-Gal activity for each sample. Statistical comparisons were determined by ANOVA (Dunnett's multiple comparison tests) with a 95% confidence level.

TABLE 1. SIZE AND ζ POTENTIAL OF DNA/PEO-PPO-PEO POLYMERIC MICELLES[a]

Formulation	*Size (nm)*	*AFM (nm)*	*ζ potential (mV)*
Plasmid DNA[b]	403 ± 27	—	−20 ± 1.3
PEO-PPO-PEO polymeric micelles[c]	165 ± 19	160 (100–200)[d]	−4.5 ± 2.3
DNA/PEO-PPO-PEO polymeric micelles[e]	170 ± 20	170 (88–236)[d]	−4.3 ± 1.7

[a]Results are expressed as the mean and standard deviation for five experiments.
[b]Plasmid DNA (0.26 μg/μl).
[c]PEO-PPO-PEO polymers (6%).
[d]Values in parentheses represent the range of particle sizes measured by AFM.
[e]Plasmid DNA (0.26 μg/μl) plus 6% PEO-PPO-PEO polymeric micelles.

Polymerase chain reaction analysis of lacZ *gene mRNA*

Forty-eight hours after the first oral dosing of DNA/PEO-PPO-PEO polymeric micelles, total RNA was extracted from the duodenum, stomach, blood, brain, liver, and testis with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. cDNA was prepared with SuperScript II reverse transcriptase (Invitrogen Life Technologies) and stored at −20°C. A 249-bp segment of the *lacZ* gene was amplified by polymerase chain reaction (PCR) as described below. Sequences of the primer pair for amplification of the transferred β-Gal-encoding gene were 5′-CTA CAC CAA CGT AAC CTA TCC C-3′ and 5′-TCC TCC GGC GCG TAA AAA TGC G-3′ (Hanazono *et al*., 1999). The level of housekeeping gene β-actin expression was analyzed and used to demonstrate the presence of the same amount of total cDNA in each RNA sample. Sequences of the primer pair for amplification of a 659-bp segment of the β-actin gene were 5′-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3′ and 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ (Hanazono *et al*., 1999). PCRs included 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C, and extension for 2 min at 72°C. PCR products were loaded onto a 0.8% acrylamide gel for electrophoresis and then the DNA bands were visualized under UV light. Quantification of band intensities was performed with a Kodak EDAS290 Analysis system (Kodak Scientific Imaging Systems, New Haven, CT).

RESULTS

Characterization of DNA/PEO-PPO-PEO polymeric micelles

Using DLS for particle size measurement, PEO-PPO-PEO polymers at a concentration of 6% (w/w) were found to exist as a single population of particles with a mean diameter of 165 ± 19 nm (Table 1). In the presence of PEO-PPO-PEO polymers, plasmid DNA carrying the *lacZ* gene condensed from a size of 403 ± 27 to 170 ± 20 nm; the condensed DNA size is similar to that of polymeric micelles alone (Table 1). AFM was used to vi-

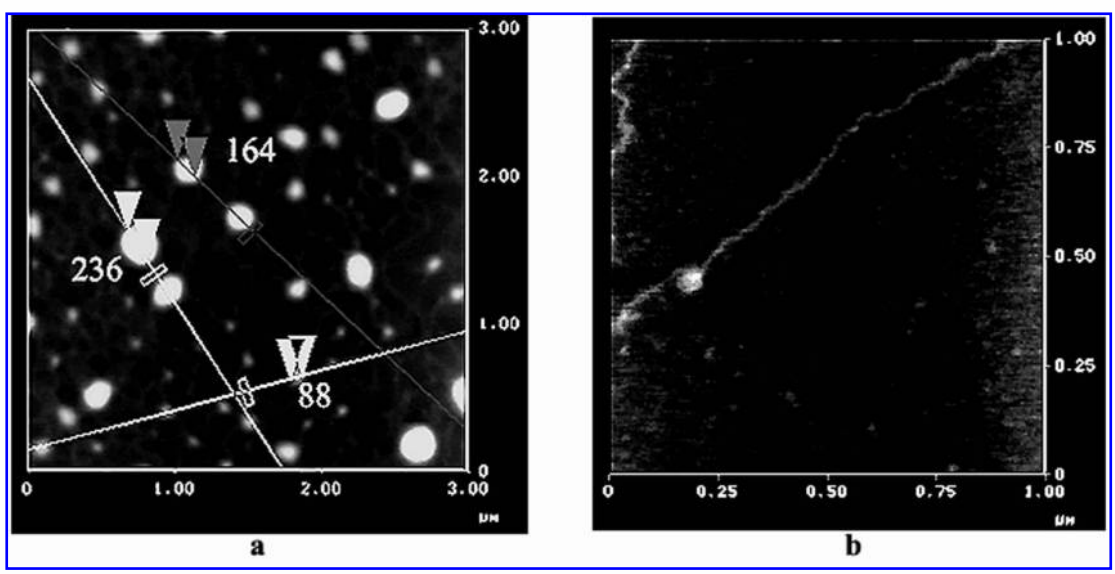


FIG. 1. Size distribution of DNA/PEO-PPO-PEO polymeric micelles. (**a**) AFM images of 10 μl of PEO-PPO-PEO polymeric micelles (6%) with plasmid solution (0.26 μg/μl) on a mica surface (the size section analysis of particles 1, 2, and 3 are 164, 236, and 88 nm, respectively); (**b**) AFM images of plasmid alone.

TABLE 2. EFFECT OF DNA CONCENTRATION WITH 6% PEO-PPO-PEO POLYMERIC MICELLES ON *In Vitro* DUODENAL PERMEABILITY IN NUDE MICE[a]

Treatment	*Apparent permeability coefficient* (P) *($\times 10^{-5}$ cm/sec)*[b]
DNA concentration with PEO-PPO-PEO	
0.026 μg/μl	5.12 ± 0.98
0.13 μg/μl	5.52 ± 1.31
0.26 μg/μl	5.75 ± 0.37
1.30 μg/μl	2.89 ± 0.37[c]
10 m*M* RGD + DNA (0.26 μg/μl) with PEO-PPO-PEO[d]	0.95 ± 0.57[c]
10 m*M* RGE + DNA (0.26 μg/μl) with PEO-PPO-PEO[d]	5.60 ± 0.62
10 m*M* EDTA + DNA (0.26 μg/μl) with PEO-PPO-PEO[e]	29.81 ± 5.73[f]

[a] $n = 9$.
[b] Values are expressed as means ± SEM.
[c] Denotes a statistically significant decrease ($p < 0.01$) compared with DNA (0.26 μg/μl) with polymeric micelles.
[d] RGD or RGE (10 m*M*) was added to the donor compartment 10 min before addition of DNA (0.26 μg/μl) with polymeric micelles.
[e] EDTA (10 m*M*) was added to the donor compartment 10 min before addition of DNA (0.26 μg/μl) with polymeric micelles.
[f] Denotes a statistically significant increase ($p < 0.01$) compared with a DNA concentration of 0.26 μg/μl.

sualize the morphology of DNA/PEO-PPO-PEO polymeric micelles, and the results showed that the complexes readily attached and remained bounded to mica and could be imaged with an AFM tip (Fig. 1a). DNA/PEO-PPO-PEO polymeric micelles appear to exist as a single population of round micelles with diameters ranging from 88 to 236 nm and an average diameter of 170 nm (Table 1). Images of plasmid DNA alone on mica are shown in Fig. 1b. The measured surface charge was −20 ± 1.3 mV for plasmid DNA alone, −4.5 ± 2.3 mV for PEO-PPO-PEO polymeric micelles alone, and −4.3 ± 1.7 mV for DNA/PEO-PPO-PEO polymeric micelles (Table 1).

In vitro *nude mouse duodenal penetration of DNA/PEO-PPO-PEO polymeric micelles*

To evaluate mechanisms involved in the penetration of DNA/PEO-PPO-PEO polymeric micelles through duodenal tissues, *in vitro* penetration studies were performed. Apparent permeability coefficients (*P*) for DNA/PEO-PPO-PEO polymeric micelles formulated with various concentrations of plasmid DNA are shown in Table 2. No significant differences were seen for formulated micelles with plasmid DNA at 0.026, 0.13, or 0.26 μg/μl. However, the *P* value decreased when the DNA

TABLE 3. β-GALACTOSIDASE ACTIVITY AFTER ORAL ADMINISTRATION OF REPORTER VECTOR WITH PEO-PPO-PEO POLYMERIC MICELLES IN NUDE MICE[a]

Treatment	*β-Gal activity*[b] *(mU/mg protein)*
Polymeric micelles alone	5.05 ± 0.26 ($n = 12$)[c]
DNA alone	5.89 ± 0.55 ($n = 12$)[c]
DNA/polymeric micelles[a]	
One dose (48 hr)	5.89 ± 0.35 ($n = 12$)
Three doses (three times per day for 1 day, 12 hr)	6.59 ± 0.18 ($n = 12$)[d]
Three doses (three times per day for 1 day, 48 hr)	7.10 ± 0.35 ($n = 12$)[d]
Six doses (three times per day for 2 days, 48 hr)	7.90 ± 0.50 ($n = 12$)[d]
Six doses (three times per day for 2 days, 72 hr)	6.20 ± 0.48 ($n = 12$)[d,e]
Six doses (three times per day for 2 days, 96 hr)	5.78 ± 0.18 ($n = 12$)
10 m*M* EDTA + DNA/polymeric micelles	9.00 ± 1.15 ($n = 10$)[d]

[a] Six- to eight-week-old BALB/c nude mice were administered one, three, or six doses of plasmid (0.26 μg/μl) plus 6% polymeric micelles over the indicated time period (days); then, at the indicated time (hr) after the start of treatment, the duodenum was removed and homogenized, and its β-Gal activity was measured.
[b] Values are expressed as means ± SEM. Numbers in parentheses are the numbers of specimens examined.
[c] No significant difference between polymeric micelles alone and DNA alone ($p > 0.1$).
[d] Significant ($p < 0.05$) increase compared with DNA-only mice.
[e] Significant ($p < 0.05$) decrease between 48 and 72 hr of observation.

concentration was 1.30 μg/μl, indicating mediated and saturated penetration processes for the DNA/PEO-PPO-PEO polymeric micelles formulation. P values of highly penetrating compounds, such as phenol red, in the GI membrane are on the order of 10^{-5} cm/sec, whereas the P value of insulin is lower, on the order of 10^{-7} cm/sec (Schilling and Mitra, 1990). These data suggest that the penetration of DNA/PEO-PPO-PEO polymeric micelles across the GI membrane was relatively high.

To investigate the subsequent internalization of DNA/PEO-PPO-PEO polymeric micelles after entry into the duodenal membrane, RGD peptide was used to block the clathrin-coated vesicle pathway (Croyle *et al.*, 1998b). As shown in Table 2, pretreatment with 10 m*M* RGD peptide for 10 min before and concurrent with polymeric micelle administration resulted in a significant 6-fold decrease in the P value of DNA/PEO-PPO-PEO polymeric micelles. However, administration of a 10 m*M* concentration of the analog RGE, in which aspartic acid was replaced by glutamic acid, in place of RGD, did not affect transport in the duodenum. In contrast, the P value increased by about 5- to 6-fold when the tight junction-opening reagent EDTA was used before and concurrent with polymeric micelle administration.

In vivo *oral gene transfer*

An intubation procedure was used to administer DNA/PEO-PPO-PEO polymeric micelles directly to the stomach of nude mice and to assess quantitatively the efficiency of gene transfer. β-Gal activity in the duodenum with various numbers of doses (one, three, or six) after administration of DNA (0.26 μg/μl) with PEO-PPO-PEO polymeric micelles was measured 48 hr after the first dose. In mice administered three doses at 8-hr intervals, we detected significant enhancement of transgene expression in duodenal tissue both 12 and 48 hr after initial dosing (Table 3). However, in mice administered only a single dose, no significant transgene expression in the duodenum was seen 48 hr postadministration (Table 3). Results showed that transfer of the *lacZ* gene could be achieved in the duodenum, and that β-Gal activity in the duodenum was most significantly increased (by 32%) when six doses were administered, compared with the control groups. There was no significant difference between the two control groups receiving six doses of either plasmid DNA alone or polymeric micelles alone ($p > 0.1$), further indicating that the detected β-Gal activity stated above was from plasmid DNA transferred by the polymeric carrier. In addition, we investigated the duration of *lacZ* expression in the duodenum after administration of six doses of DNA/PEO-PPO-PEO polymeric micelles by measuring β-Gal activity 48, 72, and 96 hr postadministration of the first dose. Transgene expression at 72 hr was lower than that detected at 48 hr and returned to background levels by 96 hr. To examine the effect of EDTA on copolymer complex-mediated gene transfer, 150-μl doses of 10 m*M* EDTA were administered 10 min before and concurrent with administration of the micelle formulation for each of the six doses of DNA/PEO-PPO-PEO polymeric micelles. Transgene expression at 48 hr was significantly increased (by 52%) compared with that in mice administered DNA alone, as shown in Table 3.

Six doses were administered at 8-hr intervals (at 6 A.M., 2 P.M., and 10 P.M.), and expression of the delivered *lacZ* gene was evaluated 48 hr after administration of the first dose, using histological tissue sections with β-Gal substrate or whole-mount staining with X-Gal. In mice administered DNA/PEO-PPO-PEO polymeric micelles orally, a blue–green color, indicating expression of exogenous β-Gal, was detected mainly in the epithelium of the duodenal villi (Fig. 2D–F). Most of the transgene expression was localized in the duodenal epithelium (Fig. 2G–K); the intensity of staining was much stronger in the villi and the crypts (Fig. 2J and K). EGTA treatment was used to block endogenous β-Gal activity (Rosenberg *et al.*, 1992; Sferra *et al.*, 1997; Weiss *et al.*, 1999) (see β-Galactosidase Enzyme Histochemistry, above), and no background staining was seen in control tissues (Fig. 2A–C and L–O). In addition, no signs of toxicity or inflammatory response to the delivered polymeric micelles were evident by microscopy in any duodenal specimens examined during the entire study period; this observation was supported by analysis of hematoxylin and eosin-stained tissue sections (Fig. 2M and N).

Biodistribution of β-Gal in other tissues

In this study, a delivery regimen of six doses administered at 8-hr intervals was used, because transgene expression in duodenal tissues was highest with this dosing regimen. Representative examples of X-Gal staining of other major tissues of the nude mouse after oral gene delivery using DNA/PEO-PPO-PEO polymeric micelles are presented in Fig. 3, and a summary of the quantitative data for the amount of gene transfection is given in Table 4. β-Gal activity in the stomach extract showed a significant increase from 0.46 $\pm$ 0.09 mU/mg protein in the control group to 0.53 $\pm$ 0.06 mU/mg protein in the group receiving DNA/PEO-PPO-PEO polymeric micelles ($p < 0.05$), and a further increase to 0.66 $\pm$ 0.05 mU/mg protein when using EDTA as a penetration enhancer. A similar pattern of increased β-Gal activity was seen in the liver. Significant increases in transgene expression were seen in the brain and the testis (95 and 21%, respectively) only after EDTA treatment. Compared with the evenly distributed pattern of β-Gal expression in the duodenum, the small, localized areas of blue–green color in the stomach, brain, and testis indicated only low amounts of β-galactosidase activity in these tissues. No increases in β-Gal activity were observed

FIG. 2. Whole-mount mucosa of duodenal tissue analysis (**A–F**) and histological analysis of the duodenum (**G–O**) of nude mice 48 hr after the start of oral delivery of six 0.26-μg/μl doses of plasmid/PEO-PPO-PEO polymeric micelles (PPM) administered at 8-hr intervals [(**A–C**) delivery plasmid alone; (**D–K**) plasmid/PPM; (**L**) delivery plasmid alone; (**O**) delivery polymeric micelles (PM) alone]. Note the speckled blue–green staining corresponding to exogenous β-Gal activity in the duodenal tissues (villi [V] and crypt cells [cr] are indicated by asterisks and arrows, respectively). Light micrographs of 20-μm O.C.T. cryosections of duodenum counterstained with eosin (E) (**I–K**) or hematoxylin–eosin (H/E) (**M** and **N**). No inflammatory reaction was noted after DNA/PPM delivery. Original magnification is indicated in parentheses in each panel.

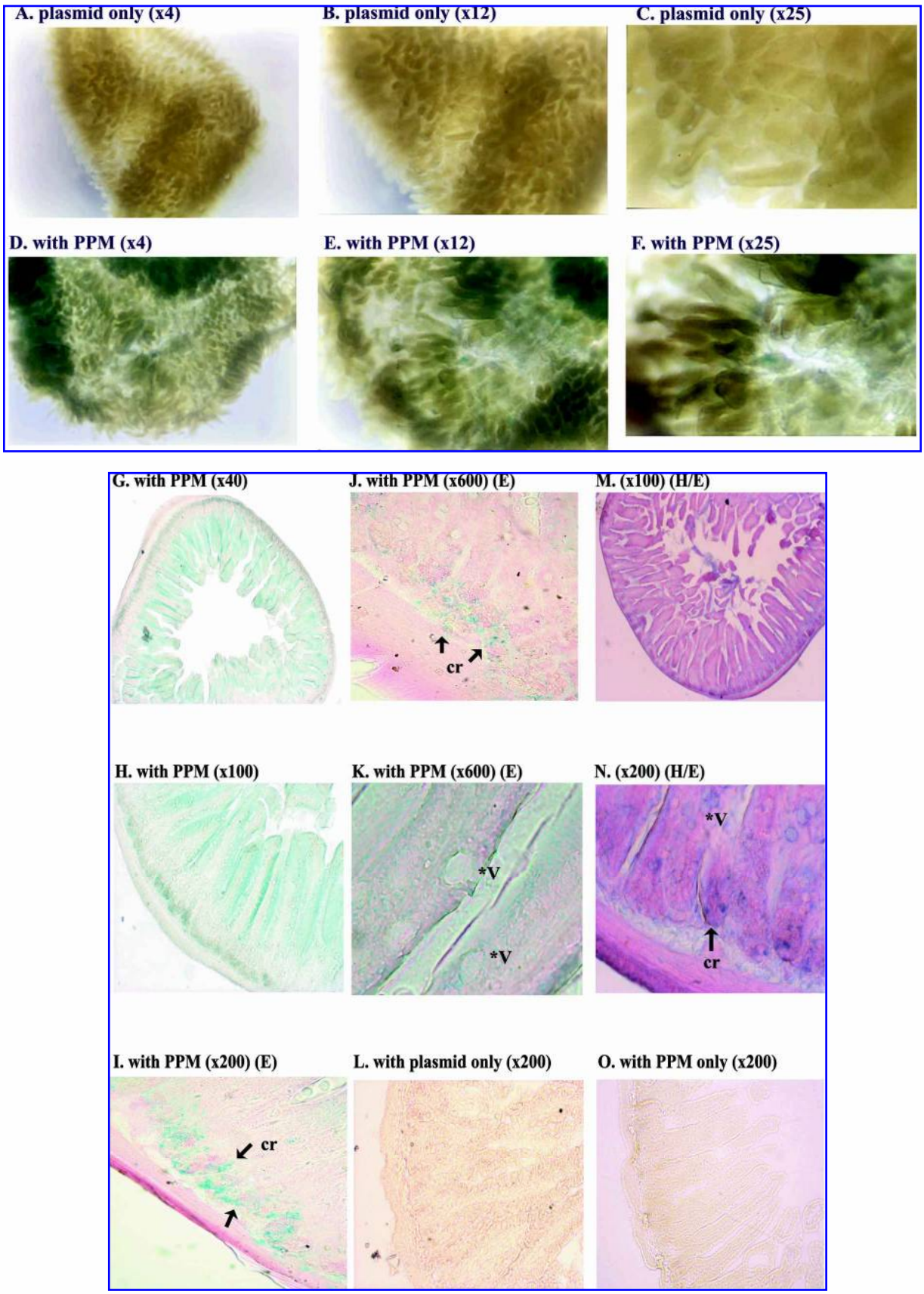

A. plasmid only (x4)
B. plasmid only (x12)
C. plasmid only (x25)
D. with PPM (x4)
E. with PPM (x12)
F. with PPM (x25)
G. with PPM (x40)
J. with PPM (x600) (E)
M. (x100) (H/E)
cr
H. with PPM (x100)
K. with PPM (x600) (E)
N. (x200) (H/E)
*V
*V
*V
cr
I. with PPM (x200) (E)
L. with plasmid only (x200)
O. with PPM only (x200)
cr

FIG. 2.

with or without EDTA treatments in other tissues, such as the heart, lung, spleen, and kidney.

Reverse transcription-polymerase chain reaction for RNA analysis

Transcription of the *lacZ* transgene was further analyzed by reverse transcription-polymerase chain reaction (RT-PCR) of total duodenal RNA extracts from mice administered six doses of DNA/PEO-PPO-PEO polymeric micelles; β-actin expression served as internal control. Total RNA was extracted from duodenal samples 48 hr after administration of the first dose and a 249-bp DNA fragment of the *lacZ* gene amplified by RT-PCR is shown in Fig. 4. Interestingly, *lacZ* mRNA was detectable in samples from blood, brain, stomach, liver, and testis tissues at 48 hr with or without EDTA pretreatment (Fig. 4). No PCR product was detected when using cDNA from tissues of the plasmid DNA-treated control group. Direct PCR analysis of the RNA samples did not produce the 249-bp band, showing that DNA contamination of RNA extracts was not responsible for the appearance of the 249-bp band (data not shown).

DISCUSSION

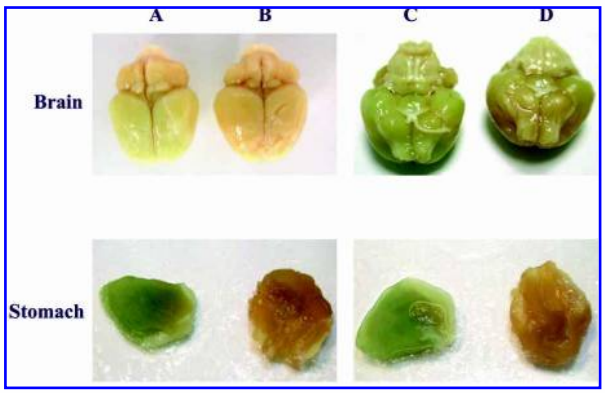
It is generally believed that certain features of the polymeric carrier system, including size, surface charge, and hydrophilicity of the carriers, affect intestinal absorption characteristics (Jong *et al*., 1997). Jani *et al.* (1990) observed that particles with mean diameters of 50–100 nm showed a higher uptake in rat intestine than did larger particles. Rejman *et al.* (2004) also revealed that the mechanism by which the beads were internalized, and their subsequent intracellular routing, were strongly dependent on the particle size of the carrier system. Internalization of microspheres with a diameter less than 200 nm involved clathrin-coated pits. With increasing size, a shift to a mechanism that relied on caveola-mediated internalization became apparent; caveola-mediated internalization is the predominant pathway of entry for particles with a size of 500 nm. In our study, the diameter of DNA/PEO-PPO-PEO polymeric micelles ranged from 88 to 236 nm, which is comparable to that reported by Jeong and Park (2001) for DNA/poly(D,L-lactic-co-glycolic acid) copolymer complexes, as determined by AFM. The condensation of DNA from 400 to 170 nm on interaction with the polymeric micelles indicates that this formulation may generate more compact particles for endocytosis, thus making it more resistant to enzymatic digestion and to the pH of the GI tract, as well as more sensitive to influence by junctional penetration enhancer.

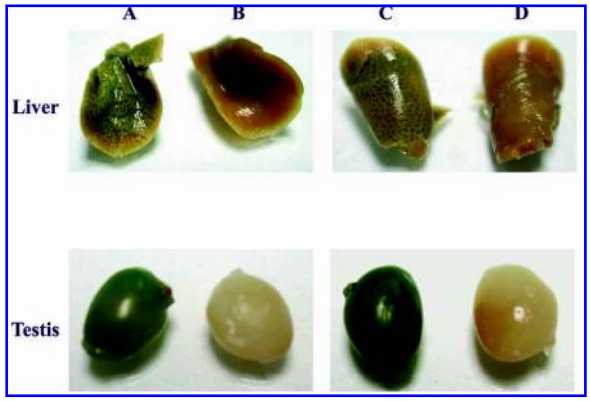
Particles with less hydrophobic surfaces show higher uptake in the GI tract than do particles with more hydrophobic surfaces (Norris and Sinko, 1997). These authors found that, in contrast to a more hydrophilic polymeric carrier system, hydrophobic poly(styrene) beads showed poor mucus penetration. The hydrophilic polymeric micelles we used in this study, which had almost no ζ potential (-4 mV), enhanced gene transfer *in vivo*. These surface properties may partially explain the ability of EDTA to increase the ability of these hydrophilic, nanosized particles to penetrate the duodenal epithelium and distribute to other tissues (Tables 2–4), because such particles would be distributed into the extracellular space and be able to pass around cells. This process would be aided by the opening of paracellular junctions. Furthermore, it has been hypothesized that the addition of hydrophilic PEO polymers to liposome or polymers can prevent complexes from binding to protein/serum or from being degraded by digestive enzymes (Kwon *et al*., 1994; Torchilin, 2001). Mathiowitz *et al.* (1997) and Luessen *et al.* (1996) showed that surface charge seems to be a prerequisite structural factor for colloidal carriers, leading to significant bioadhesion to the mucosa of the small intestine. However, the mechanisms of gene transfer by PEO-PPO-PEO polymeric micelles under physiological conditions seen in the GI tract are still under examination in our laboratory.

β-Gal activity in duodenal extracts increased 32% over controls when using the PEO-PPO-PEO polymeric micelles transfer system. Foreman *et al.* (1998) obtained a similar enhancement of 20% after direct injection of the β-Gal gene into the duodenum. However, Cheng *et al.* (1997) demonstrated an approximately 9-fold increase in transfection when using an adenoviral vector to deliver the β-Gal gene into the duodenum; this higher transfection efficiency could be partially due to their combination use of gentamicin, ranitidine, prednisolone, and glucagons before inserting the oral feeding tube, which reduced the bacterial burden, reduced secretion of acid fluid from stomach, lowered the intestinal mucous barrier, and slowed bowel motility in the duodenum. We found unacceptable levels of β-Gal background activities in the jejunum, the ileum, and the colon of our experimental mice even after EGTA incubation. This endogenous β-Gal activity in the intestine has been observed in other studies (Sferra *et al*., 1997; Foreman *et al*., 1998; Weiss *et al*., 1999). A study by Foreman *et al.* (1998) indicated that potential sources of this background activity included mammalian lysosomal enzymes, the digestive enzyme lactase, and β-Gal produced by enteric bacteria. We therefore focused our study on duodenal sections of the GI tract.

PEO-PPO-PEO copolymers have long been used as components of various pharmaceutical formulations to enhance the oral bioavailability of drugs (Saski, 1968; Schubert and Wretlin,

FIG. 3. (**A**–**D**) Whole-mount and (**E**–**G**) histological analysis of brain, stomach, liver, and testis 48 hr after the start of oral delivery of six 0.26-μg/μl doses of plasmid/PEO-PPO-PEO polymeric micelles administered at 8-hr intervals. [(**B** and **D**) plasmid alone or polymeric micelles alone; (**E**) stained with hematoxylin/eosin (H/E); (**G**) counterstained with eosin (E); (**A**, **C**, **F**, and **G**) treatment with 10 m*M* EDTA before and during DNA/PEO-PPO-PEO polymeric micelle delivery]. Note the speckled blue–green staining corresponding to X-Gal activity (brain in the cortex area [co]; stomach in the chief cells of the gastric mucosa [m]; liver in hepatocytes [h]; testis in Leydig's cells [l] are indicated by arrows). No inflammatory reaction was noted after DNA/PEO-PPO-PEO polymeric micelle delivery. Original magnification is indicated in parentheses in each panel. Whole mount original magnification in (**A**–**D**), $\times$10.


A
B
C
D
Brain
Stomach


A
B
C
D
Liver
Testis

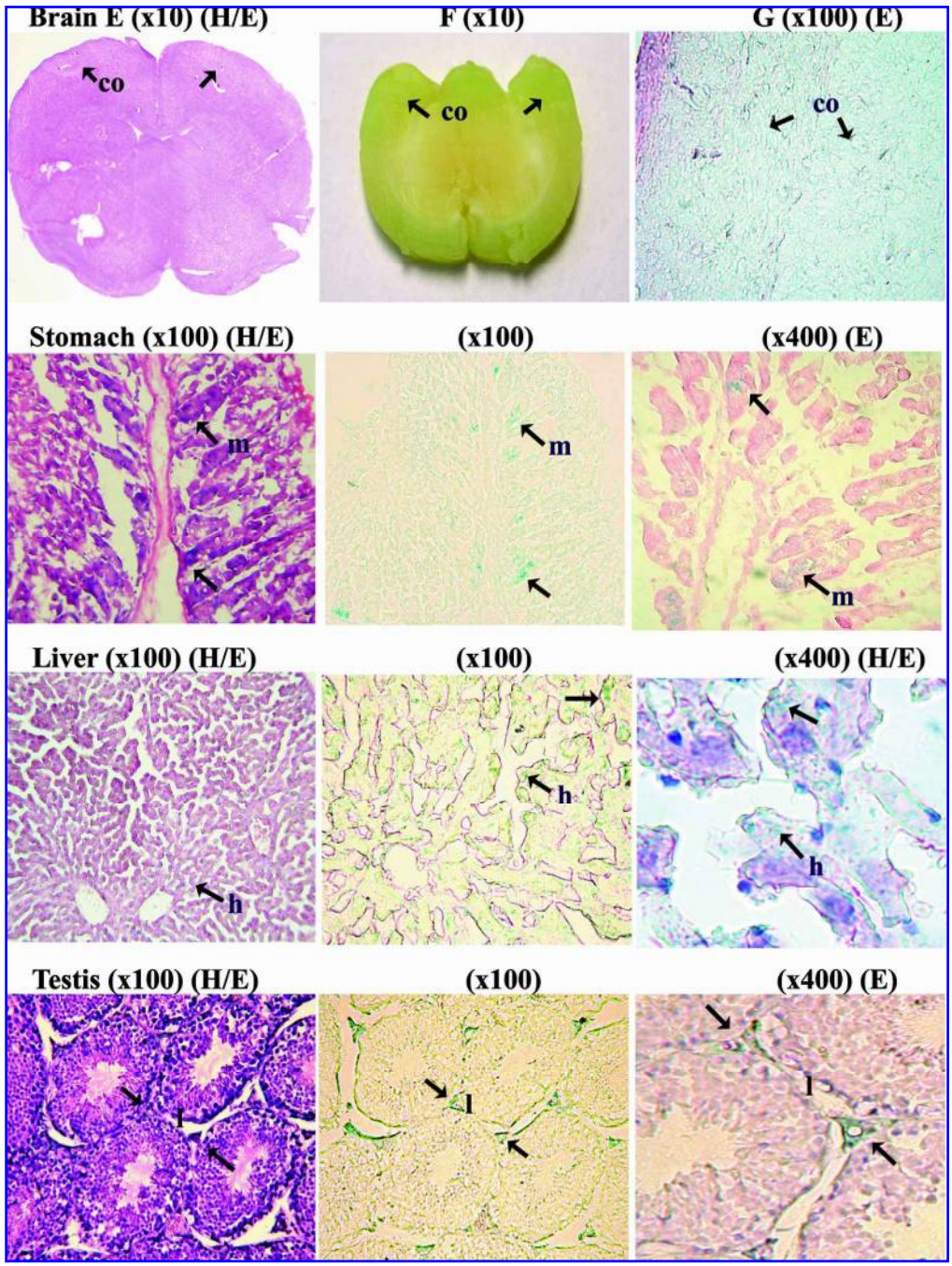

Brain E (x10) (H/E)
F (x10)
G (x100) (E)
co
co
co
Stomach (x100) (H/E)
(x100)
(x400) (E)
m
m
m
Liver (x100) (H/E)
(x100)
(x400) (H/E)
h
h
h
Testis (x100) (H/E)
(x100)
(x400) (E)
l
l
l

FIG. 3.

TABLE 4. β-GALACTOSIDSE ACTIVITY IN OTHER MAJOR TISSUES OF NUDE MICE AFTER ORAL ADMINISTRATION OF REPORTER VECTOR WITH PEO-PPO-PEO POLYMERIC MICELLES

	β-Gal activity[a] *(mU/mg protein)*		
Organ	*Plasmid control*	*Oral delivery*[b]	*EDTA + oral delivery*[c]
Stomach	0.46 ± 0.09 ($n = 7$)	0.53 ± 0.06[d] ($n = 7$)	0.66 ± 0.05[d] ($n = 7$)
Liver	0.37 ± 0.03 ($n = 6$)	0.45 ± 0.04[d] ($n = 6$)	0.42 ± 0.04[d] ($n = 6$)
Brain	0.27 ± 0.07 ($n = 8$)	0.33 ± 0.01 ($n = 8$)	0.52 ± 0.11[d] ($n = 8$)
Testis	0.63 ± 0.03 ($n = 7$)	0.66 ± 0.05 ($n = 8$)	0.76 ± 0.06[d] ($n = 8$)
Heart	0.15 ± 0.03 ($n = 8$)	0.15 ± 0.02 ($n = 8$)	0.16 ± 0.02 ($n = 8$)
Lung	0.40 ± 0.04 ($n = 8$)	0.38 ± 0.03 ($n = 8$)	0.44 ± 0.02 ($n = 6$)
Spleen	1.33 ± 0.18 ($n = 8$)	1.33 ± 0.18 ($n = 8$)	1.39 ± 0.12 ($n = 8$)
Kidney	1.23 ± 0.16 ($n = 7$)	1.19 ± 0.12 ($n = 7$)	1.20 ± 0.10 ($n = 7$)

[a]Values are expressed as means ± SEM. Numbers in parentheses are the numbers of specimens examined.
[b]Six- to eight-week-old BALB/c nude mice received six doses of plasmid (0.26 μg/μl) with micelles and then, 48 hr after the start of treatment, organs were removed and homogenized, and β-gal activity was measured.
[c]Mice were treated with 10 m*M* EDTA before and during treatment, as described in footnote *b*.
[d]Significant ($p < 0.05$) increase in activity compared with that in the same organ in plasmid-only control mice.

1983; Damage *et al.*, 1997; Yang *et al.*, 1999). However, their effects varied when different concentrations of the copolymer were used. First, at concentrations below the critical micelle concentration (CMC), the copolymers perturbed the plasma membrane and inhibited the P-glycoprotein efflux system in Caco-2 cell monolayers, resulting in a significant enhancement of absorption and permeability of drugs (Batrakova *et al.*, 1999). Second, at concentrations above the CMC, block copolymers self-assembled into micelles and increased the *P* value of various compounds in Caco-2 cell monolayers to $0.2–3.5 \times 10^{-5}$ cm/sec (Batrakova *et al.*, 1999). This range of *P* values is similar to that seen in our study ($\sim 10^{-5}$ cm/sec). Taken together, these results suggest that this copolymer system should be useful in increasing the oral bioavailability of plasmid DNA in the duodenum.

Improved transfection efficiency was achieved both *in vivo* and *in vitro* by the use of EDTA, which is known to open tight junctions of the duodenum and increase paracellular transport, allowing hydrophilic polymeric micelles containing plasmid DNA to be distributed to deeper levels of tissues (Zhang *et al.*, 2003). This is similar to our previous results in which we enhanced transfection of ocular tissues by using EDTA and cytochalasin B (Rojanasakul *et al.*, 1990; Liaw and Robinson, 1992; Liaw *et al.*, 2001). However, it is also known that increased intracellular calcium levels lead to an increased rate of uptake of complexes (Yuan *et al.*, 2001), so the mechanism of enhancement by EDTA is not totally clear. We hypothesize that EDTA causes an increase in intracellular calcium levels, triggering endocytosis and the opening of tight junctions, thus allowing the complexes to penetrate into deeper tissues and distribute to other organs. However, our data also showed that, after treatment of the duodenum *in vitro* with RGD peptide before and concurrent with polymeric micelle administration, gene transfer efficiency using DNA/PEO-PPO-PEO polymeric micelles was reduced by 84%, implying the entry of particles into cells via receptor-mediated endocytosis. Croyle *et al.* (1998b)

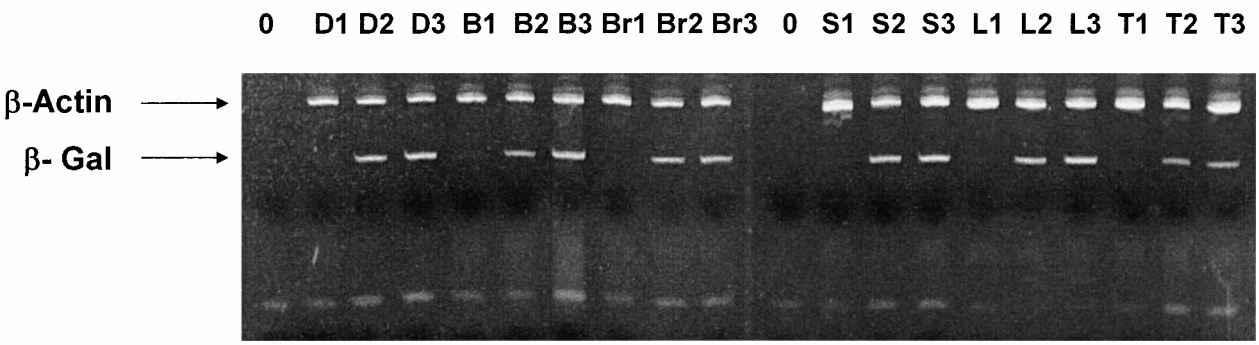


FIG. 4. RT-PCR analysis of *lacZ* gene transcription. Total RNA was prepared from duodenum (D), blood (B), brain (Br), stomach (S), liver (L), and testis (T) of animals administered plasmid alone (1) or DNA/polymeric micelles (2), or treated with 10 m*M* EDTA before and during DNA/polymeric micelle administration (3). The samples were amplified using a 249-bp sequence of the *lacZ* gene and a 659-bp sequence of the β-actin gene. A typical direct PCR analysis of the RNA samples (lane 0) did not result in production of the 249-bp band.

reported that pretreatment of Caco-2 cells with 3.5 m*M* RGD peptide reduced adenovirus-mediated gene transfer by 80%. Rejman *et al.* (2004) also demonstrated that internalization of microspheres with a diameter less than 200 nm involves mainly a clathrin-coated pit-mediated mechanism. It has been demonstrated that PEO polymeric micelles were taken up into cells via a receptor-mediated endocytotic mechanism that can be abolished by low temperatures (Batrakova *et al.*, 1999; Zhang *et al.*, 2003). Thus, taken together, our data suggest that the transport of PEO-PPO-PEO copolymer micelles into tissues may be through endocytosis and could be enhanced by the opening of tight junctions.

We found that X-Gal staining of epithelial cells was localized to the cytoplasm of intestinal enterocytes. Under higher magnification, an apical duodenal localization of exogenous β-Gal activity was observed in the region of the villi. The finding that the majority of goblet cells showed positive staining, also seen in the crypts, where stem cells are located, is similar to other reports (Lau *et al.*, 1995; Cheng *et al.*, 2003) using a viral delivery system. In contrast to retroviral vectors (Lau *et al.*, 1995), plasmid-containing polymeric micelles cannot insert their DNA into host chromosomal DNA; hence the crypt stem cells of the villi may not be able to express the delivered β-Gal reporter gene because of loss of episomal genetic material after continuous cell division. Almost all enterocytes in the crypt–villus axis were β-Gal positive, which is consistent with the migration of crypt cells to the villi by 48 hr (Clatworthy and Subramanian, 2001), suggesting that crypt cells are the possible target cells for plasmid DNA delivered by polymeric micelles. However, we cannot rule out the possibility that the X-Gal-stained epithelial cells in the villi were cells derived from crypt stem cells harboring the transferred gene delivered by polymeric micelles.

After oral administration of DNA/PEO-PPO-PEO polymeric micelles, β-Gal activity in the duodenum was maximal at 48 hr and returned to background levels by 96 hr. This finding further suggests that rapid turnover of intestinal epithelial cells limits the time that the micelles remain in the tissue and thus the expression of the delivered gene. However, it is unclear whether the time when reporter gene expression started to decrease was affected by mucus, enzymes, pH, and bacterial components in the duodenum, resulting in a decrease in transfection efficiency. Furthermore, because of the extremely low gene transfer efficiency at time points beyond 96 hr, it was difficult to tell whether the trace signal observed was indeed due to the gene product. Possible explanations for this transient gene expression could be degradation of the delivered gene, shutdown of gene expression, or loss of transfected cells.

It is interesting that transfection was detected in the circulating blood 48 hr after oral delivery of polymeric micelles. Furthermore, we detected positive RT-PCR signals and protein expression in the liver, stomach, brain, and testis. These results are similar to those obtained in studies on the tissue biodistribution of genes transferred by adenoviral vectors after local *in vivo* gene transfer to the arterial wall, which showed that, after transfection, X-Gal staining of blood cells was increased by 1.8%, whereas that of the liver and testis was increased by 0.7 and 0.06%, respectively (Hiltunen *et al.*, 2000; Zhang *et al.*, 2003). In addition, after a single intravenous injection of plasmid packaged in the interior of PEO liposomes, Shi *et al.* (2001) detected expression of the exogenous gene in the brain, liver, and spleen that lasted for at least 6 days. Kabanov *et al.* (2002) reported that these PEO-PPO-PEO formulations not only remained stable at concentrations above the CMC in the blood stream, but also showed 3-fold higher stability in the brain and better oral bioavailability for various drugs. This further emphasizes that it should be possible to locally transfect the stomach and duodenum and other major organs through the blood circulation, using oral gene delivery with PEO-PPO-PEO polymeric micelles.

In summary, the present study demonstrates the feasibility of gene transfer into duodenal epithelial cells, using orally administered PEO-PPO-PEO polymeric micelles containing the *lacZ* gene. Expression of the β-Gal gene in the duodenal epithelium peaked at 48 hr and fell to background levels by 96 hr after polymeric micelle administration. Use of EDTA and RGD peptide to modify the transfection efficiency of DNA/PEO-PPO-PEO polymeric micelles suggested that the transfection mechanism involved endocytosis and was enhanced by the opening of tight junctions in the duodenal tissues and by augmenting the paracellular pathway, allowing distribution to deeper tissues and more distant organs.

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