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RESEARCH ARTICLE

In vivo gene delivery into ocular tissues by eye drops of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles

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The primary objective of this study was to investigate the feasibility of using PEO-PPO-PEO non-ionic copolymeric micelles as a carrier for eye-drop gene delivery of plasmid DNA with lacZ gene in vivo. Using pyrene fluorescence probe methods, zeta potential, and dynamic light scattering test (DLS), the ability of micelle formation of these block copolymers with plasmid was studied. Gene expressions were visualized by both the quality of enzymatic color reaction using X-gal staining and by the quantification of the substrate chlorophenol red galactopyranoside (CPRG) in enucleated eyes on day 2 after gene transfer. In addition, microscopy to identify the types of cell showing uptake and expression of the transferred gene was used. We found that the block polymeric micelles were formed above 0.1% (w/v) of block copolymer with a size of 160 nm and a zeta potential of −4.4 mV. After 2 days of topically delivery three times a day, the most intense gene expression was observed on days 2 and 3. Reporter expression was detected around the iris, sclera, conjunctiva, and lateral rectus muscle of rabbit eyes and also in the intraocular tissues of nude mice upon in vivo topical application for 48 h with a DNA/polymeric micelle formulation. Furthermore, after two enhancement treatments, the transport mechanisms of the block copolymeric micelles were found through endocytosis in tissues by enhancement through the tight junction pathway. Thus, efficient and stable transfer of the functional gene could be achieved with PEO-PPO-PEO polymeric micelles through topical delivery in mice and rabbits. These in vivo experiments indicate the possible potential use of block copolymers for DNA transfer. Gene Therapy (2001) **8,** 999–1004.

Keywords: *eye drops; gene delivery; polymeric micelles*

Introduction

Nonviral delivery systems have been developed with the hope of overcoming some of the problems associated with viral gene delivery.[1] In nonviral methods, some types of lipid vehicle, usually a cationic liposome,[2] chitosan,[3] or a cationic biopolymer,[4] etc are used as gene carriers. However, in developing nonviral gene carriers, those that are efficient *in vitro* often fail to show the same efficiency when applied *in vivo*.[5] The reasons for poor efficacy *in vivo* could be the sensitivity of the carrier to serum, the stability of complex formation between DNA and the carrier, and unknown mechanisms of cellular uptake and intracellular trafficking of the complex. Little is known concerning the individual parameters that influence complex formation and the characteristics of the complex that are relevant to transfection efficiencies in gene therapy.

Evaluation of transfection efficiency usually involves comparison of different stable formulations using transgene expression as the endpoint. Recently, a non-ionic PEO-PPO-PEO copolymer with an average molecular weight of 8400, a group of triblock copolymers derived from propylene oxide (20%) and ethylene oxide (80%),[6] have been widely used in medical, pharmaceutical, and cosmetic systems as a solubilizing, wetting, and emulsifying agent with relatively low toxicity.[7,8] In addition, there are many nano-polymeric micelle drug systems which contain hydrophilic PEO chains as palisade regions, can prohibit protein/serum absorption *in vitro*,[9] liver cellular interaction, and can increase stability in the blood stream.[10] This PEO-type carrier of block copolymer not only leads to enhanced passive transport but also avoids liver degradation. In our previous study,[11] we found through pharmacokinetic data, that using a PEO-PPO-PEO copolymer micelle-fentanyl patch *in vivo* prolonged the half-life ($t_{1/2}$) of fentanyl in the blood stream up to five times. Thus, this type of copolymer micelle carrier appears to have the potential to act as a topical drug delivery carrier since it prevents serum/protein interaction and the micelle carrier has good stability characteristics. Thus, the aim of the present study was to evaluate the ability of non-ionic ABA-type PEO-PPO-PEO block copolymeric micelles to improve gene transfer *in vivo*. The relatively simple preparation described here may

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have profound effects on the ability to transfect a wide variety of cell types and tissues with plasmid DNA. Finally, on the basis of these findings, we propose a mechanism to explain the effects of polymeric micelles upon the process of gene transfer.

Results and discussion

Characterization of plasmid/PEO-PPO-PEO polymeric micelles formulation using a pyrene fluorescence probe, dynamic light scattering, and zeta potential

The critical micelle concentration (CMC) of PEO-PPO-PEO polymeric micelle formation was confirmed by a fluorescence probe technique using pyrene, and the partitioning of pyrene into the hydrophobic micellar phase was determined using the ratio of peak I_1/peak I_3 of the pyrene spectrum as previously reported.[11] By measuring the intensity ratios of the first to the third vibrational bands of pyrene, it was found that the I_1/I_3 peak height ratio decreased above 0.1% w/w of only block copolymers (Figure 1, triangles). The magnitude of I_1/I_3 in the high concentration range (here 1.55) is somewhat higher than that for pyrene in a toluene solution (1.04) but significantly lower than that in water (1.75). This ratio of fluorescence intensities has been correlated to the hydrophobicity of the molecular environment of the pyrene probe and it was indicated that above the CMC (0.1%) of PEO-PPO-PEO polymers polymeric micelles were formed.

When pyrene interacted in plasmid DNA with PEO-PPO-PEO polymeric micelles (Figure 1, circles), the magnitude of I_1/I_3 above 0.1% of PEO-PPO-PEO polymers has a similar pattern compared with that for polymeric micelles only. The lower I_1/I_3 ratio of fluorescence of pyrene interacting with DNA below 0.1% of PEO-PPO-PEO polymers may be explained by the plasmid DNA interacting more intensely with pyrene. Above 1% PEO-PPO-PEO polymers, the I_1/I_3 ratio of fluorescence of pyrene began to decrease. This indicates that the probe no longer fully interacted with the internal DNA and released the inside core of the PEO-PPO-PEO polymer micelles. Plasmid DNA/PEO-PPO-PEO polymeric micelle formation is likely to occur by weak interactions, such as hydrogen bonds between DNA and the poly(ethylene) oxide part of the polymers and decreased interfacial free energy as well as decreased entropy of core-forming segments. Similar results were found by Cherng *et al*[12] using acridine orange to intercalate in plasmid DNA. Taken together, at a concentration above 0.1% PEO-PPO-PEO copolymer (CMC) with plasmid DNA, it was indicated that DNA was incorporated inside of polymeric micelles and pyrene was transferred into the hydrophobic domain of the micelles.

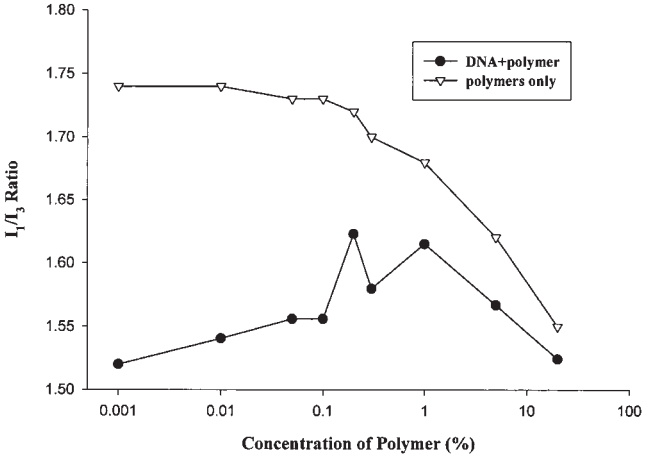

Figure 1 *Comparison of intensity of the I_1/I_3 ratio of vibrational bands in the pyrene fluorescence spectrum with 0.08 μl/μl plasmid DNA and 0.3% PEO-PPO-PEO polymeric micelles (●), and as a function of PEO-PPO-PEO copolymer concentration (∇) in pyrene solution.*

By dynamic light scattering for particle size measurement, the PEO-PPO-PEO copolymers at a concentration above 0.1% (w/w) also exhibited a single modular population of particle distribution within the 160 nm range (Table 1). In the presence of sufficient PEO-PPO-PEO polymers, plasmid DNA condensed. The size of PEO-PPO-PEO/DNA polymeric micellar complexes decreased from 394 ± 38 nm to 155 ± 44 nm, and this was similar to that of polymeric micelles alone (163 ± 26 nm) (Table 1). Using poly(ethyleneimine) cationic polymer/DNA, Tang and Szoka[13] also showed the diameter range of complexes in solution to be around 90–130 nm. In addition, in our previous study,[14] we found that using cytochalasin B or EDTA could open the tight junction paracellular pathway and could only increase permeation up to PEG 1000 a linear, hydrophilic molecule. Thus, induction of DNA condensation from 400 to 155 nm could influence junctional penetration ability as well as providing compact carrier for endocytosis and affording enzyme digestion/interaction with serum in tears.

The surface charges of plasmid DNA only, PEO-PPO-PEO polymeric solution, and DNA/PEO-PPO-PEO polymeric micellar complexes fell to –17.6 ± 2.3, –4.8 ± 1.7, and –4.4 ± 2.0 mV, respectively. Although the positive surface charge of the transfecting carriers is thought to be an important factor in the complexes' association with the plasma membrane, our polymeric micelles, with almost no zeta potential (–4 mV), could still enhance the transfection through both *in vivo* systems. The microscopic pictures with complexes showed that the carrier enhanced DNA distribution through the tissue. There is an idea that hydrophilic poly(ethylene) oxide polymers can prevent protein or serum binding or enzyme degradation and that they are carried to different liposome or cationic block biopolymers. Indeed, these types of carriers found outside of the poly(ethylene) oxide polymer chain show promise for *in vivo* gene delivery.[15] Thus, at less than the 155-nm size of the non-ionic plasmid/PEO-PPO-PEO polymeric micelle carrier, the transport pathway of these carriers could be involved in endocytosis through tissue. However, the mechanisms of action of PEO-PPO-PEO polymeric micelles on gene transfer remain to be further explored.

In vivo gene transfer to ocular tissues

Forty-eight hours after topical eye-drop delivery, whole-mount preparations of mouse and rabbit eyes with CMV LacZ plasmid/PEO-PPO-PEO polymeric micelles reacted with the β-Gal substrate, X-gal, revealing dark blue spots corresponding to speckled staining of the choroid sclera, conjunctiva, iris, tendon of the lateral rectus muscles, etc (Figure 2). Expression of the reporter *lacZ* gene was detected at low levels in other ocular tissues such as the anterior chamber, cornea, retinal pigment epithelium,

Table 1 Size and zeta potential of plasmid DNA/PEO-PPO-PEO polymeric micelles[a]

Formulation	*Size (nm)*	*Poly-dispersity*	*Zeta potential (mV)*
Plasmid DNA only[b]	394 ± 38	0.19	−17.6 ± 2.3
PEO-PPO-PEO polymeric micelles[c]	163 ± 26	0.20	−4.8 ± 1.7
Plasmid DNA/PEO-PPO-PEO polymeric micelles[d]	155 ± 44	0.22	−4.4 ± 2.0

[a]Results are expressed as mean values with standard deviation of five experiments.
[b]0.08 μg/μl plasmid DNA.
[c]0.3% PEO-PPO-PEO polymer.
[d]0.08 μg/μl plasmid DNA + 0.3% PEO-PPO-PEO polymers.

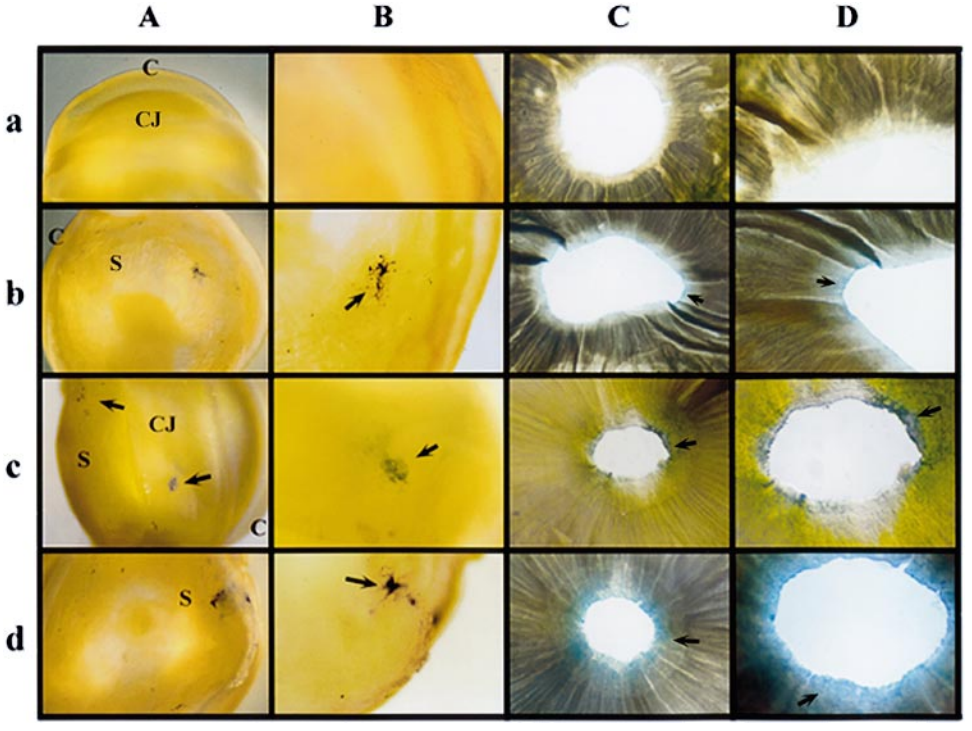

Figure 2 *Whole-mount of eye 2 days after topical eye-drop administration in nude mice (columns A and B) and irises of rabbit (columns C and D) with the 0.08 μl/μl plasmid/PEO-PPO-PEO polymeric micelles. (a, plasmid only; b, plasmid/PEO-PPO-PEO polymeric micelles; c, addition of 5 mM EDTA before plasmid/PEO-PPO-PEO polymeric micelles delivery; d, addition of 3 mM cytochalasin B before plasmid/PEO-PPO-PEO polymeric micelles delivery.) Note the speckled staining of blue color (arrow) corresponding to X-gal activity in the ocular tissues (C, cornea; CJ, conjunctiva; S, sclera). There is no ocular cloudiness or edema in areas with polymeric micelle delivery. Original magnification ×12 in columns A and C, and ×25 in columns B and D.*

and vitreous body (data not shown). No β-Gal activity was detected in cells in the ocular tissues of control eyes or those with delivery of DNA plasmid only (Figure 2, column a). There was no evidence of any cellular inflammatory reaction as judged by an absence of polymorphonuclear or round cell infiltration with hematoxylin and eosin staining of the eyes (data not shown). In addition, for the entire study period, there was no light microscopic evidence of toxic or cytological disruptive effects caused by the polymeric micelles in any eyes examined or alterations in the food/water consumption for animals. Furthermore, from 1 to 5 days after topical delivery, β-Gal activity could also still be observed in some parts of ocular tissues in all eyes, eg sclera, conjunctiva, iris (data not shown). The most intense gene expression was observed on days 2 and 3 after delivery. The quantitative expression levels of β-Gal by plasmid DNA/0.3% PEO-PPO-PEO polymeric micelles in nude mice and rabbits were therefore, determined at 2 days after delivery (Table 2). The enhanced ability statistically increased the expression of reporter activity to 28% (from 4.9 ± 0.2 to 6.3 ± 0.1 mU/mg protein) and 38% (from 3.1 ± 0.4 to 4.3 ± 0.7 mU/mg protein) for nude mice and rabbits, respectively. Shiraishi *et al*[16] also observed similar results using gene gun delivery of keratin 12 promoter-β-gal gold particles (0.6 μm) in the conjunctival area (2.4 ± 1.1 mU/mg protein). However, their transfection in the cornea area (23 ± 10 mU/mg protein) was higher than our results and this could be partially due to using invasive delivery through the cornea.

In addition, two different concentrations of polymeric micelles, 0.01% (below CMC) and 2.5% (above CMC), influenced the expression of reporter activity in mice by 14% (5.7 ± 0.2 mU/mg protein) and 24% (6.2 ± 0.2

Table 2 Effect of two enhancers on plasmid DNA with PEO-PPO-PEO polymeric micelle delivery in nude mice and rabbits[a]

Treatment	*Nude mice*[b] *(mU/mg protein)*		*Rabbits*[c] *(mU/mg protein)*
DNA only	4.9 ± 0.2 ($n = 13$)		3.1 ± 0.4 ($n = 8$)
DNA + 0.01% polymer	5.7 ± 0.2^{d} ($n = 5$)		ND
DNA + 0.3% polymer	6.3 ± 0.1^{d} ($n = 13$)		4.3 ± 0.7^{e} ($n = 5$)
DNA + 2.5% polymer	6.2 ± 0.2^{d} ($n = 5$)		ND
DNA + 0.3% polymer + 5 mM EDTA	6.5 ± 0.3^{d} ($n = 10$)		5.1 ± 0.4^{d} ($n = 5$)
DNA + 0.3% polymer + 3 mM cytochalasin B	7.3 ± 0.2^{d} ($n = 9$)	Cornea	5.3 ± 0.2^{d} ($n = 5$)
		Iris	5.2 ± 0.6^{d} ($n = 5$)
		Others[f]	4.9 ± 0.3^{d} ($n = 5$)

[a]Values are averages of the β-Gal activity ± standard error of the mean per specimens (mU/mg proteins). The numbers in parentheses are numbers of specimens examined.
[b]Represents the expression of β-Gal activity in whole-enucleated eyes.
[c]Represents the expression of β-Gal activity in irises of rabbits.
[d]Denotes a statistically significant difference at $P < 0.01$ as compared with DNA only.
[e]Denotes a statistically significant difference at $P < 0.05$ as compared with DNA only.
[f]Represents whole-enucleated eyes, except cornea and iris tissues, quantitative expression in β-Gal activity.
ND, not determined for the tests in rabbits.

mU/mg protein), respectively. Although there was no statistically significant difference of transfection between 0.01 and 0.3% polymers, it could be suspected due to a different transfection mechanism which influenced DNA interaction with the extracellular matrix and its cellular uptake. Alakhov *et al*[17] demonstrated that ABA types of poly(ethylene) oxide polymers dramatically increase membrane permeability by surfactant interaction on tissue membranes. However, it is not yet clear whether each polymeric micelle particle of PEO-PPO-PEO was directly endocytosis permeation to tissues/membrane or release some monomers of PEO-PPO-PEO to perturb the membrane structure and enhance DNA diffuse into the cells. On the other hand, Masuda *et al*[18] reported that using topical gene delivery by liposome could also transfer the gene to retinal ganglion cells. They suggested that liposome with DNA might penetrate the cornea, diffuse in the intraocular fluid, and then reach the retina. It remains unknown how gene-carrying PEO-PPO-PEO polymer and polymeric micelles applied topically as eye drops to the ocular surface can reach the retina and transfer the gene. More detailed studies on this subject are in progress in this laboratory.

Enhanced in vivo gene transfer to ocular tissues by EDTA and cytochalasin B

In order to examine the effect of EDTA and cytochalasin B on complex-mediated gene transfer, the ocular tissues were treated with plasmid-PEO-PPO-PEO polymeric micelles after 20 min of 5 mM EDTA or 3 mM cytochalasin B treatment. In Table 2, transgene expression, which compared with DNA only, significantly increased to 32–64% (6.5 ± 0.3–7.3 ± 0.2 mU/mg protein) and 48–70% (4.9 ± 0.3–5.3 ± 0.2 mU/mg protein) for EDTA and cytochalasin B treatment in two intraocular tissues, respectively. β-Gal activity was observed to increase enhancement in both the ocular tissue area and layers such as sclera (S), conjunctiva (CJ) for nude mouse eye and iris for rabbit eye (Figure 2 columns c and d), which were transfected with complexes incubated in the presence of two enhancers. Improved transfection efficiency was achieved by the use of EDTA and cytochalasin B, which are known to open tight junctions of the cornea and increase paracellular transport,[19] for further distribution of DNA to tissue and depth of levels. However, it is also known that increased intracellular calcium levels can lead to increased rates of endocytosis[20] and thus can promote the uptake of complexes. Although the mechanism of EDTA, whereby calcium can stimulate uptake, is not yet clear, it probably arises from opening the tight junction to penetrate into different areas and depths of tissue levels following increased intracellular levels of calcium with enhancement of endocytosis. Thus, the transport mechanisms of hydrophilic block copolymeric micelles could be through endocytosis in tissues by enhancement through the tight junction pathway.

In conclusion, the current results provide a promising initial step in the development of simple topical techniques to introduce therapeutic genes into ocular tissues by PEO-PPO-PEO polymeric micelles. The ABA-type of non-ionic PEO-PPO-PEO polymeric micelles has low toxicity/high stability and appears to be useful in a variety of cell lines and animals. Using EDTA and cytochalasin B to enhance transfection properties of plasmid/PEO-PPO-PEO polymeric micelles, the enhanced mechanism appears to involve the plasmid/PEO-PPO-PEO polymeric micelles opening the tight junction of ocular tissues and increasing the paracellular pathway for further distribution (endocytosis) to different areas and depths of tissues.

Materials and methods

Materials

PEO-PPO-PEO copolymer with an average molecular weight of 8400 was obtained from BASF Corporation (Ludwigshafen, Germany). Cytochalasin B and EDTA were obtained from Sigma Chemical (St Louis, MO, USA). All other chemicals used in the study were of analytical reagent grade and were used with no further purification.

Animals

The nude mice (BALB/c-nu) used (under specific pathogen-free condition) in the *in vivo* eye-drop study were

aged 6 to 8 weeks and were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). A second animal model, male albino New Zealand rabbits (Animal Center of National Taiwan University) weighing between 2 and 3 kg were also used in the *in vivo* eye-drop studies.

Plasmid

The pCMVβ plasmid, which encodes the *lacZ* gene for the β-Gal protein, was driven by a cytomegalovirus (CMV) promoter to assess expression. Plasmid DNA was amplified in the *Escherichia coli* host strain, TG-1, and was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient.[21] The purity and stability of plasmid preparations were investigated by electrophoresis on agarose gel followed by ethidium bromide staining, and the DNA concentration was measured by UV absorption at 260 nm. Briefly, plasmid preparations were applied in slots of a 0.8% agarose gel containing 40 mM Tris/TBE buffer solution (pH 7.6), 1 mM EDTA, and 0.6 μg/ml ethidium bromide. Plasmids were dissolved in water before mixing with pyrene and polymeric micelles. Agarose gels demonstrate that the mainly circular plasmid is resolved as several bands with one dominating in the gels, while only the linear form of plasmid DNA shows on the gel after *Eco*RI or *Hin*dIII restriction enzyme digestion. A formulation of PEO-PPO-PEO polymeric micelles formed with DNA illustrates the circular DNA electrophoresis pattern, which is similar to that shown by the plasmid alone. The stability of plasmid DNA with PEO-PPO-PEO copolymeric micelles was confirmed by the integrity of the plasmid DNA electrophoresis pattern after preparation or 2 days at room temperature, or after three cycles of freezing and thawing.

Preparation of PEO-PPO-PEO copolymer formulation with plasmid

All PEO-PPO-PEO copolymer formulations of plasmid used in these studies were freshly prepared on a weight percentage basis as in a previous method.[11] The different weighed amounts of copolymer were added to water with gentle mixing. Different concentrations of plasmid were gently mixed with PEO-PPO-PEO polymeric micelles in a vial for 2 h.

Characterization of plasmid/PEO-PPO-PEO copolymer micelle formation using a pyrene fluorescence probe

The formation of PEO-PPO-PEO copolymer micelles with or without plasmid was confirmed by a fluorescence probe technique using pyrene, and the partitioning of pyrene into the micellar phase could be determined using the ratio of peak I_1/peak I_3 of the pyrene spectrum as previously reported.[11] The fluorescence emission spectrum of pyrene in the PEO-PPO-PEO copolymer micelle solutions was measured from 350 to 500 nm using a fixed excitation wavelength of 339 nm with a constant pyrene concentration of 6×10^{-7} M. The PEO-PPO-PEO block copolymer varied from 0.001% to 20% (w/w) with or without 0.08 μg/μl of plasmid. The spectral data were acquired using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). All fluorescence experiments were carried out at 25°C.

Size and zeta potential of the PEO-PPO-PEO/plasmid complex

Mixtures containing 0.08 μg/μl of plasmid DNA and 0.3% of PEO-PPO-PEO polymer were used to measure the size and zeta potential of complexes. A comparison was made with 0.08 μg/μl plasmid DNA or 0.3% PEO-PPO-PEO polymer solutions. The average particle size and the zeta potential were determined by quasi-elastic laser light scattering (DLS) (Zetasizer 3000, Malvern, UK) with an assumed refractive index ratio of 1.33 and viscosity of 0.88. As a measure of the homogeneity of particle size, the system reports a polydispersity index (PI). Perfectly monodispersed systems have a PI of 0, while completely heterodispersed systems will have a PI close to 1. The sample time for each sample was 10 μs, and the experimental duration was 100 s. All measurements were performed at 25°C at a measurement angle of 90°.

Gene transfer to ocular tissues in vivo

For the *in vivo* study, the eyes of nude mice and rabbits were treated with 0.08 μg/μl of plasmid and 0.3% (w/v) PEO-PPO-PEO polymeric micelles by simple instillation of drops (10 μl for mouse and 50 μl for rabbit, three times per day). The right eyes received drops and the left eyes received plasmid only or DNA/polymeric micelles as control/comparison. To evaluate PEO-PPO-PEO polymeric micelle gene transfer *in vivo*, the animals were killed either by cervical dislocation of the neck for nude mice or i.v. injection with 3% pentobarbital for rabbits. The enucleated eyes were immediately removed after 48 h of topical administration of formulation. For transfection enhancer studies, 2 μl of each enhancer (5 mM of EDTA or 3 mM of cytochalasin B) were applied as topical eye-drops to the cornea for 20 min before the PEO-PPO-PEO/plasmid carrier was applied to the animals.

β-Gal enzyme histochemistry

The presence of β-Gal activity was determined by histochemical analysis using X-gal substrate (Gibco BRL, Grand Island, NY, USA) following the method of Oshima *et al*.[22] Enucleated eyes were fixed with 4% paraformaldehyde at 4°C for 90 min. Eyes were next exposed to 10 mM $K_4Fe(CN)_6$, 10 mM $K_3Fe(CN)_6$, 0.01% sodium deoxycholate, 0.02% NP40, and 2 mM $MgCl_2$ in PBS solution containing 1 mg/ml of the X-gal substrate. The *lacZ* gene was considered to be expressed when the tissue area was a green-blue color under an operating microscope at two constant magnifications.

Determination of transgene expression

Quantification of β-Gal expression was assayed by using the substrate chlorophenol red galactopyranoside (CPRG) (Gene Therapy System, San Diego, CA, USA) at 1 mg/ml to induce color development, which was measured at 580 nm with a microtube. Total tissue protein was determined using the DC protein assay reagent kit (BioRad, Hercules, CA, USA), which was subsequently used to normalize β-Gal activities of each transfection. Statistical comparisons were made with ANOVA test with Dunnett's multiple comparison test at 99% confidence levels.

Acknowledgements

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